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Veterinary Immunology

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Ian Tizard BVMS, PhD, ACVM (Hons), DSc (Hons)

University Distinguished Professor of Immunology
Richard M. Schubot Professor of Exotic Bird Health
Department of Veterinary Pathobiology
Texas A&M University
College Station, Texas

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Table of Contents

Cover image

Title Page

Copyright

Dedication

Preface

Acknowledgments

1 Surviving in a Microbial World

- The Microbial World
- The Defenders
- Where to Go for Additional Information

2 Innate Immunity

- How Invaders Are Recognized
- Pathogen-Associated Molecular Patterns
- Damage-Associated Molecular Patterns
- Soluble Pattern-Recognition Receptors

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Sentinel Cells

3 Humoral Innate Immunity

Products of Sentinel Cells

Inflammatory Mediators

The Coagulation System

Antimicrobial Molecules

4 Humoral Innate Immunity

Complement Proteins

Activation Pathways

Regulation of Complement Activation

Other Consequences of Complement Activation

Complement Genes

Complement Deficiencies

5 Cellular Innate Immunity

Leukocyte Classification

Neutrophils

Emigration From the Bloodstream

Phagocytosis

Surface Receptors

Fate

6 Cellular Innate Immunity

Macrophages

Functions

Fate of Foreign Material

VetBooks.ir

Resolution of Inflammation

7 Sickness

Sickness Behavior

Systemic Innate Responses

Systemic Inflammatory Response Syndrome

Protein Misfolding Diseases

8 How Immune Cells Communicate

Cytokine Nomenclature

Cytokine Functions

Cytokine Structure

Cytokine Receptors

Cytokine Regulation

Signal Transduction

Gene Transcription

9 Antigens

Microbial Antigens

Nonmicrobial Antigens

What Makes a Good Antigen?

Epitopes

Cross-Reactions

10 Dendritic Cells and Antigen Processing

Dendritic Cells

Dendritic Cells in Domestic Animals

Other Antigen-Processing Cells

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Antigen Processing

Histiocytosis and Histiocytomas

11 The Major Histocompatibility Complex

Major Histocompatibility Complex

MHC Class Ia Molecules

MHC Class II Molecules

MHC Class III Molecules

MHC of Domestic Animals

MHC Molecules and Disease

MHC and Body Odors

12 Organs of the Immune System

Sources of Lymphocytes

Primary Lymphoid Organs

Secondary Lymphoid Organs

Tertiary Lymphoid Organs

13 Lymphocytes

Lymphocyte Structure

Lymphocyte Populations

Lymphocyte Surface Molecules

Species Differences

Lymphocyte Mitogens

14 Helper T Cells and Their Response to Antigens

Immunoglobulin Superfamily

T Cell Antigen Receptors

VetBooks.ir

Co-Stimulators

Immunological Synapse Formation

Signal Transduction

Overall Considerations

Superantigens

Helper T Cell Subpopulations

Species Differences

Memory T Cells

15 B Cells and Their Response to Antigens

B Cell Antigen Receptors

Antigen Presentation by B Cells

Co-stimulation of B Cells

B Cell Response

Cellular Responses

Plasma Cells

Memory B Cells

Germinal Centers

Myelomas

Hybridomas

16 Antibodies

Immunoglobulins

Immunoglobulin Classes

Three-Dimensional Structure of Immunoglobulins

Immunoglobulin Variants

Production of Immunoglobulin Heavy Chains

Immunoglobulins of Domestic Mammals

VetBooks.ir

17 How Antigen-Binding Receptors Are Made

Receptor-Antigen Binding

Antigen Receptor Genes

Immunoglobulin Diversity

Gene Recombination

Generation of Junctional Diversity

Somatic Mutation

Gene Conversion

Species Differences

T Cell Receptor Diversity

γ/δ T Cell Diversity

Epigenetic Regulation

18 T Cells and the Destruction of Cell-Associated Invaders

Endogenous Antigens

Apoptosis

Cell Cooperation

Cytotoxic T Cell Responses

Cytotoxic T Cell Subsets

Other Mechanisms of Cellular Cytotoxicity

Macrophage Activation

Effector T Cell Memory

19 Innate Lymphoid Cells

Innate Helper Cells

Innate Cytotoxic Cells

NKT Cells

VetBooks.ir

20 Regulation of Adaptive Immunity

Tolerance

T Cell Tolerance

B Cell Tolerance

Duration of Tolerance

Control of Immune Responses

Antigen Regulation of Immune Responses

Antibody Regulation of Immune Responses

Inhibitory Receptors

Regulatory Cells

Regulation by the Innate Immune System

Regulation of Apoptosis

Neural Regulation of Immunity

21 The Microbiota and the Immune System

The Locations of the Microbiota

The Functions of the Microbiota

Immune Responses to the Microbiota

Dysbiosis

Odors

The Virome

Inflammatory Bowel Diseases

22 Immunity at Body Surfaces

Immunity on Body Surfaces

Mucosal Lymphoid Tissues

Adaptive Protective Mechanisms

Immunity to Food

VetBooks.ir

Vaccination on Body Surfaces

23 Immunity in the Fetus and Newborn

Development of the Immune System

Immune Response of Newborn Mammals

Transfer of Immunity From Mother to Offspring

Failure of Passive Transfer

Cell-Mediated Immunity and Colostrum

Development of Adaptive Immunity in Neonatal Mammals

Vaccination of Young Animals

24 Vaccines and Their Production

Types of Immunization Procedures

Passive Immunization

Active Immunization

Modern Vaccine Technology

Adjuvants

25 The Use of Vaccines

Administration of Vaccines

Vaccination Strategies

Vaccine Assessment

Failures in Vaccination

Adverse Consequences of Vaccination

Adverse Effect Principles

Production, Presentation, and Control of Vaccines

26 Immunity to Bacteria and Fungi

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Innate Immunity

Adaptive Immunity

Evasion of the Immune Response

Some Antibacterial Vaccines

Adverse Consequences of the Immune Responses

Serology of Bacterial Infections

Immunity to Fungal Infections

27 Immunity to Viruses

Virus Structure and Antigens

Pathogenesis of Virus Infections

Innate Immunity

Adaptive Immunity

Evasion of the Immune Response by Viruses

Adverse Consequences of Immunity to Viruses

Some Selected Virus Diseases

Some Antiviral Vaccines

Serology of Viral Diseases

28 Immunity to Parasites

Immunity to Protozoa

Immunity to Helminths

Immunity to Arthropods

29 Mast Cell and Eosinophil-Mediated Hypersensitivity

Induction of Type I Hypersensitivity

Immunoglobulin E

Mast Cells

VetBooks.ir

Response of Mast Cells to Antigen

Basophils

Eosinophils

30 Allergic Diseases

The Hygiene Hypothesis

Specific Allergic Diseases

Diagnosis of Type I Hypersensitivity

Treatment of Type I Hypersensitivity

31 Red Cell Antigens and Antibody-Mediated Hypersensitivity

Blood Groups

Blood Transfusion and Incompatible Transfusions

Hemolytic Disease of the Newborn

Blood Groups, Blood Transfusion, and Hemolytic Disease in Domestic Animals

Parentage Testing

Hemophagocytic Syndrome

Type II Hypersensitivity Reactions to Drugs

Type II Hypersensitivity in Infectious Diseases

32 Immune Complexes and Neutrophil-Mediated Hypersensitivity

Classification of Type III Hypersensitivity Reactions

Local Type III Hypersensitivity Reactions

Generalized Type III Hypersensitivity Reactions

Clinical Features of Glomerulonephritis

Other Immune Complex–Mediated Lesions

33 T Cell–Mediated Hypersensitivity

VetBooks.ir

The Tuberculin Reaction

Pathological Consequences of Type IV Hypersensitivity

Measurement of Cell-Mediated Immunity

34 Organ Graft Rejection and Pregnancy

Grafting of Organs

Allograft Rejection

Renal Allografts

Skin Allografts

Liver Allografts

Cardiac Allografts

Corneal Allografts

Bone Allografts

Bone Marrow Allografts

Xenografts

Allografts and the Reproductive System

35 Cancer Immunology and Immunotherapy

Tumors as Allografts?

Cellular Defenses

Failure of Immunity to Cancer Cells

Tumor Immunotherapy

Some Selected Tumors

Lymphoid Tumors

36 Autoimmunity

Induction of Autoimmunity

Normal Immune Responses

VetBooks.ir

Abnormal Immune Responses

Predisposing Factors

Mechanisms of Tissue Damage in Autoimmunity

37 Organ-Specific Autoimmune Diseases

Autoimmune Endocrine Disease

Autoimmune Neurological Disease

Autoimmune Eye Disease

Autoimmune Reproductive Diseases

Autoimmune Skin Diseases

Autoimmune Nephritis

Autoimmune Blood Diseases

Autoimmune Muscle Diseases

Dermatomyositis

Chronic Active Hepatitis

38 Immune-Mediated Inflammatory Diseases

Systemic Lupus Erythematosus

Discoid Lupus Erythematosus

Sjögren's Syndrome

Autoimmune Polyarthritis

Immune Vasculitis

39 Primary Immunodeficiencies

Inherited Defects in Innate Immunity

Inherited Defects in the Adaptive Immune System

Immunodeficiencies of Horses

Immunodeficiencies of Cattle

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Immunodeficiencies of Pigs

Immunodeficiencies of Dogs

Immunodeficiencies of Cats

Immunodeficiencies of Mice

Immunodeficiencies of Humans

40 Secondary Immunological Defects

Virus-Induced Immunosuppression

Retrovirus Infections in Cats

Other Causes of Secondary Immunodeficiency

Other Secondary Immunodeficiencies

41 Drugs and Other Agents That Affect the Immune System

Suppression of the Immune System

Nonspecific Immunosuppression

Selective Immunosuppression

Stimulation of the Immune System

42 Immunodiagnostic Techniques

Reagents Used in Serological Tests

Primary Binding Tests

Antibody Labels

The Flow Cytometer

Secondary Binding Tests

Assays in Living Systems

Molecular Methods

Diagnostic Applications of Immunological Tests

VetBooks.ir

43 Evolution of the Immune System

- Immunity in Invertebrates
- Immunity in Vertebrates
- Immunity in Cyclostomes
- Immunity in Jawed Fish
- Immunity in Amphibians
- Immunity in Reptiles
- Immunity in Birds
- Generation of Antibody Diversity
- Maternal Immunity
- Immunity in Monotremes and Marsupials
- Mammalian Phylogeny
- Fever

Appendix 1 Annotated List of Selected CD Molecules

Appendix 2 Some Selected Cytokines

Appendix 3 Some Important Abbreviations

Glossary

Index

Greek Letters

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Dedication

To Devon and Trevor

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Preface

This textbook was first published in 1977, at a time when veterinary immunology was relatively new and poorly understood. Nevertheless, it was recognized, even then, that immunology held the key to many of the significant issues in veterinary medicine. The importance of immunology has not declined since then, except among those tasked with establishing college curricula. The decline in basic science instruction at colleges of veterinary medicine in favor of more clinical training has resulted in graduating a generation of veterinarians whose knowledge of immunology is minimal. Yet immunology continues to be a key science. It is central to our understanding of major veterinary issues such as vaccination, cancer, infectious diseases, and allergies.

It has been tempting to dilute the contents of this text and its associated website to conform to current curricular trends. I have resisted this. Because the amount of knowledge of immunology required to obtain a veterinary degree has been progressively reduced, the need to document the science in depth has correspondingly increased. This book therefore continues to grow as the result of opening up new, exciting areas of the science. Veterinarians need to know much of this if they are to practice cutting-edge science in the 21st century. Read it, study it, and enjoy its wonderful complexity.

Over the many years that this textbook has been published we have seen many changes in the science of immunology. In most cases, these changes have been gradual in nature as investigators add detail to existing knowledge. Occasionally, however, major leaps are made that effectively revolutionize the discipline—so-called paradigm shifts. For example, in the late 1990s, the concept of innate immunity was accepted. Inflammation and other processes were finally recognized as being essential components of the

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immune system and the dual innate and adaptive immune systems were shown to complement each other. It is interesting to note that this was not a new discovery but a new way of looking at well recognized processes.

This new edition also reflects revolutionary changes in the way we think of immunology. Like the concept of innate immunity, these changes are not a result of a previously unknown process but a belated recognition of something known about since the dawn of microbiology, the body's normal microbiota. New methodologies and intensive studies have revealed that many body processes, especially immunity, are regulated by the diverse microbiota that colonizes all body surfaces. Much of immunology has had to be reassessed in the light of this new knowledge. Both innate and adaptive immunity are regulated by those organisms, especially bacteria, that live in the intestine and respiratory tract and on the skin. Many previously unexplained phenomena have now been shown to depend on the normal microbiota. As a result of this new information, the reader will encounter the microbiota at every turn in this book, plus a completely new chapter on the subject.

The second new chapter deals with allergic diseases. For many years, these were readily explained by the production of IgE against allergens. The most recent information, however, has demonstrated that allergies are much more complex than this. For example, atopic dermatitis, one of the most common conditions seen by small animal veterinarians, is likely a syndrome with multiple complex causative factors. As a result, allergic and inflammatory diseases deserve a new chapter.

These additions cannot, however, hide the fact that the rest of immunology also continues to move forward. Thus the mechanisms by which the body rejects gastrointestinal helminths has been clarified with the discovery of the importance of tuft cells and interleukin-33. Associated with this has been the recognition of the complex nature of innate lymphoid cells and their subpopulations.

Some of these advances can be considered routine, such as the identification of new cell surface molecules and many new cytokines. New disease syndromes such as swine SCID, bovine neonatal pancytopenia, and immune-mediated keratoconjunctivitis are now described, and the pathogenesis of others such as atopic

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dermatitis, type I diabetes mellitus, equine laminitis, and systemic lupus are shown to be more complex than previously believed.

New advances in therapy are covered, including the introduction of immune checkpoint therapy in cancer and the use of monoclonal antibody therapy and intravenous immunoglobulin therapy for immune-mediated diseases. The exciting new developments in the use of nanoparticles in vaccines and as adjuvants are now described. The significant roles of vitamins A and D as well as the aryl hydrocarbon receptor in immunity are also recognized.

Advances in basic science that are now discussed include new findings on the structure and genetics of bovine antibodies, epigenetics, RNA interference, microRNA, macrophage polarization, and type 1 and type 2 immune responses.

I make no apologies for the size and complexity of this text. Immunology is a complex subject that has a direct bearing on many of the most important areas of veterinary medicine. Students, as well as graduate veterinarians, ignore it at their peril.

Finally, readers are strongly encouraged to visit the Evolve website for this text. The site is also constantly evolving and improving. In it you will be able to find a collection of more than 450 multiple-choice questions (with the answers!) keyed to each chapter; a set of flashcards also linked to chapters; all the text figures available as PowerPoint slides; and a collection of animations kindly provided by Dr. Abul Abbas, as well as brand new animations created for this textbook, designed to clarify some complex processes and pathways where still figures are inadequate. All the chapter references are keyed to PubMed. There are also new hand-outs that students can print off and use as a reference, should you wish. It is my hope that these assets will help you remain current in this rapidly expanding and exciting field.

Ian Tizard

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I would also like to acknowledge the professionalism and assistance provided by the staff of Elsevier, especially my editor, Alexandra York, and my project manager, Tracey Schriefer.

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Ian Tizard

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Surviving in a Microbial World

CHAPTER OUTLINE

The Microbial World, 2
The Defenders, 2
Physical Barriers, 2
Innate Immunity, 3
Adaptive Immunity, 4
Antibody-Mediated Immunity, 5
Cell-Mediated Immunity, 6
Mechanisms of Adaptive Immunity, 7
Where to Go for Additional Information, 7

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Recognize that our environment is occupied by dense and complex populations of microbes, especially bacteria.
- Understand that these bacteria do not usually cause disease because the immune system excludes those that can cause damage.
- Understand the importance of physical barriers to exclude invaders, innate immunity to provide rapid initial protection, and adaptive immunity to provide prolonged effective resistance to infection and disease.
- Explain the differences between the innate and adaptive immune responses.

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- Explain why there are two major forms of adaptive immunity: humoral and cell mediated.
- Understand that adaptive immunity directed against bacterial invaders is mediated by antibodies produced by lymphocytes called B cells.
- Understand that adaptive immunity mediated by lymphocytes called T cells is called cell-mediated immunity.
- Explain how the adaptive immune system also generates memory cells and the importance of immunological memory.
- Define innate immunity, adaptive immunity, antibody-mediated immunity and cell-mediated immunity.

The animal body contains all the components necessary to sustain life. It is warm, moist, and rich in nutrients. As a result, animal tissues are extremely attractive to microorganisms that try to invade the body and exploit these resources for themselves. The magnitude of this microbial attack can be readily seen when an animal dies. Within a few hours, especially when warm, a body decomposes rapidly as bacteria invade its tissues. On the other hand, the tissues of living, healthy animals are highly resistant to invasion since their survival depends on preventing microbial invasion. The defense of the body is encompassed by the discipline of immunology and is the subject of this book.

Because effective resistance to infection is critical, the body dare not rely on a single defense mechanism alone. To ensure reliability, multiple defense systems must be available. Some may be effective against many different invaders. Others may destroy specific organisms. Some act at the body surface to exclude invaders. Others act deep within the body to destroy organisms that have breached the outer defenses. Some defend against bacterial invaders, some against viruses that live inside cells, and some against large invaders such as fungi or parasitic worms and insects. The protection of the body therefore depends upon a complex system of overlapping and interlinked defense networks using cells and molecules that collectively destroy or control almost all invaders. Any failure in these defenses, permitting invading organisms to overcome or evade them, will result in disease and possibly death.

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An effective immune system is therefore not simply a useful system to have around. It is essential to life itself.

The immune system can be thought of as a set of interactive cellular and molecular networks where the presence of foreign invaders triggers changes in cellular activities and generates an expanding set of cellular and molecular responses that eventually results in elimination of the invaders and increased resistance to infection. Most of the complexity of the immune system stems from the fact that none of its pathways are truly independent. Pathways interact and intersect. Cells talk to each other by means of hundreds of different signaling molecules. Microbial invasion results not in a single response but in multiple responses involving many different cell types producing many different molecules. Collectively, it is the responses of these cells and molecules that keep us alive in a microbial world.

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The Microbial World

Historically, our concerns regarding infectious diseases have caused us to regard all microbes as potential enemies. Dangerous microbial invaders include not just bacteria and viruses, but also fungi, protozoa, arthropods, and helminths (worms). Nevertheless, the real situation is much more complex. Bacteria find animal hosts to be a rich source of nutrients and a great place to shelter. As a result, enormous numbers colonize our body surfaces, especially within the intestine, in the airways, and on our skin. Most of these bacteria—our normal microbiota—do not even try to invade the body and do not normally cause damage. They share resources with us and so are regarded as commensal organisms.

The presence of this microbiota and the diversity of molecules it generates must either be tolerated or ignored if an animal is to remain healthy. An animal cannot afford to act aggressively toward its own microbiota. Any response must be carefully regulated and must not happen unless necessary for the defense of the body. The immune system is aware of the intestinal microbiota. Numerous bacterial molecules cross the intestinal epithelium and influence the immune responses. They do not, however, automatically trigger strong defensive responses unless tissue damage occurs. The response is measured, proportional, and carefully controlled. The immune system has to watch them warily, but they rarely cause trouble. In fact, they are needed for the proper digestion of food as well as a stimulus that keeps our defenses in peak operating condition.

A small number of other, more aggressive bacteria, try to invade animal tissues and do cause damage. This is normally prevented, or at least controlled, by our immune defenses. If these organisms succeed in invading the body and overcoming the immune defenses, they may cause sufficient damage that results in disease or death. On the other hand, organisms such as the viruses are intracellular parasites that can survive for only a limited time outside the animal body. These invaders will only survive if they can avoid the host's defenses for sufficient time to replicate and transmit their progeny to a new animal host. While it is essential for

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an animal to control invading organisms (or at least minimize damage), viruses are under even more potent selective pressure. They must find a host or die. Viruses that cannot evade or overcome the immune defenses will not survive and will be eliminated. Fungi, like bacteria, are opportunistic invaders that can take advantage of local circumstance to invade the host. They commonly exploit situations where the host's immune system is defective or suppressed in some way. Parasitic worms and protozoan parasites, like viruses, must be able to survive within a host or be eliminated. They have evolved numerous and complex strategies to evade immune destruction.

An organism that can cause sufficient damage to result in disease is said to be a pathogen. Remember, however, that only a small proportion of the world's microorganisms are associated with animals, and very few of these can overcome the body's defenses and become pathogens. Pathogenic microorganisms vary greatly in their ability to invade the body and cause damage. This ability is termed virulence. Thus a highly virulent organism has a greater ability to cause damage than an organism with low virulence. If a bacterium can cause significant damage almost every time it invades a healthy individual, even in low numbers, then it is considered a primary pathogen. Examples of primary pathogens include canine distemper virus; feline panleukopenia virus; and *Brucella abortus,* the cause of contagious abortion in cattle. Other pathogens may be of such low virulence that they will only cause disease if administered in very high doses or if the immune defenses of the body are impaired first. These are opportunistic pathogens. Examples of opportunistic pathogens include bacteria such as *Mannheimia hemolytica* and fungi such as *Pneumocystis jirovecii.* These organisms rarely cause disease in healthy animals.

For many years, it was believed that the role of the immune system was simply to ensure the complete exclusion of all invading microbes by distinguishing between self and not-self and eliminating foreign antigens. We now know, however, that this is insufficient to ensure health. The immune system must also determine the threat level posed by the microbes it encounters and adjust its response accordingly. It must maintain tolerance to the normal microbiota or food antigens while, at the same time, be

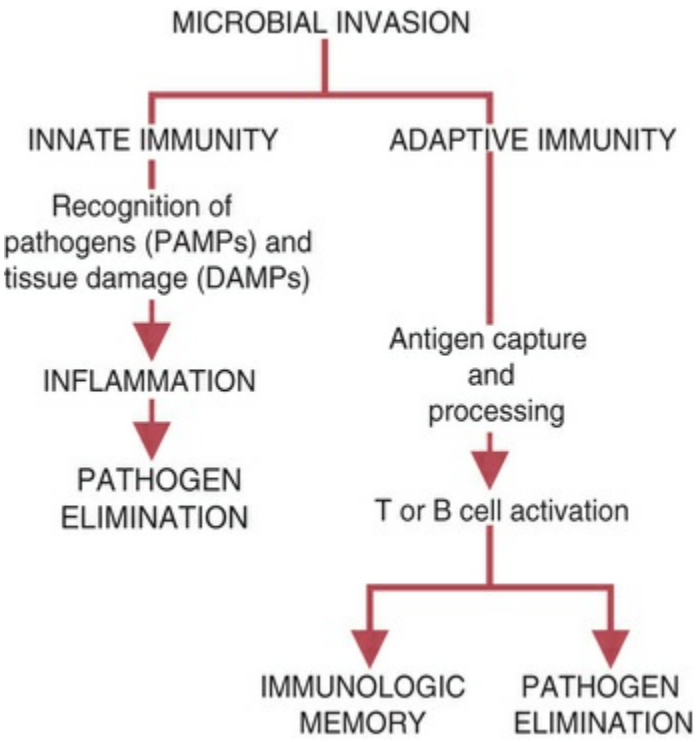
highly responsive to invading pathogens.

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The Defenders

The defenses of the body, collectively called the immune system, consist of interacting networks of cells and molecules. For descriptive purposes, it is convenient to divide these networks into discrete pathways (Fig. 1.1). Nevertheless, the reader should be aware that these biochemical and cellular pathways are extensively interlinked. No immune response is restricted to a single biochemical mechanism or pathway. The invasion of the animal body by microbes alters the behavior of many different cell types and the production of many different molecules. Understanding immunity requires an understanding of these dynamic immunological networks. These networks possess redundancies, regulatory mechanisms, and multiple simultaneous responses working together to ensure microbial destruction. In addition, the immune responses are adaptable and adjust their mechanisms depending upon the nature and severity of the threat. This of course maximizes their efficiency and minimizes the chances of any individual microbe successfully evading those defenses.



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FIG. 1.1 The basic arrangement of the innate and adaptive immune systems. Innate immunity is the first line of defense; adaptive immunity backs it up.

Physical Barriers

Because the successful exclusion of microbial invaders is essential for survival, it is not surprising that the animal body employs multiple, overlapping layers of defense (Fig. 1.2). For example, a microbe that has succeeded in breaking through the first layer of defenses is then confronted with the need to overcome a second, higher barrier, and so forth. The first and most obvious of these defenses are the physical barriers to invasion. Intact skin provides an effective barrier to microbial invasion. If skin is damaged, microbes may invade; but wound healing ensures that this is repaired promptly. On other body surfaces, such as in the respiratory and gastrointestinal tracts, simple physical defenses include the "self-cleaning" processes: coughing, sneezing, and mucus flow in the respiratory tract; vomiting and diarrhea in the gastrointestinal tract; and urine flow in the urinary system. The presence of huge populations of commensal bacteria on the skin, respiratory tract, and in the intestine also excludes many potential invaders. Well-adapted commensal organisms adapted to living on body surfaces can easily outcompete poorly adapted pathogenic organisms. The microbiota thus plays an essential role in resistance to invasion.

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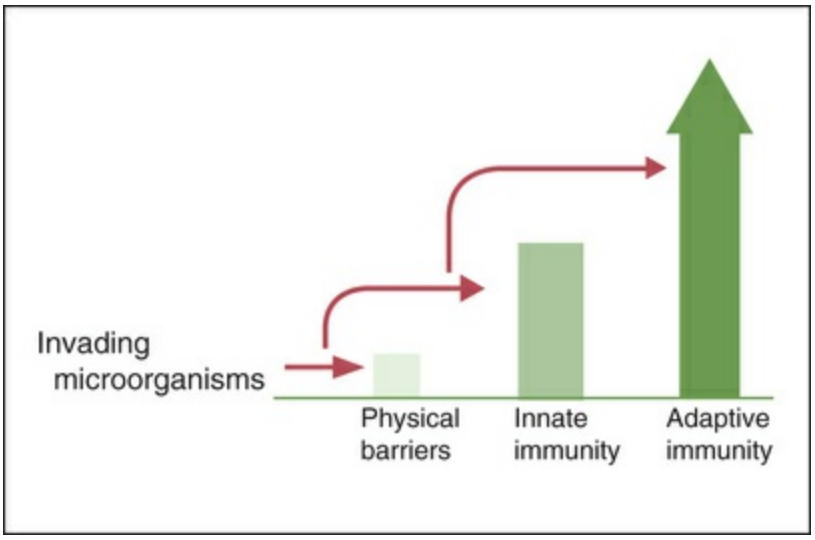


FIG. 1.2 The three major barriers that protect an animal's body against microbial invasion. Each barrier provides a more effective defense than the previous one. The adaptive immune system also improves with experience so that it presents a progressively greater barrier to invasion.

Innate Immunity

Physical barriers, though essential in excluding invaders, cannot be totally effective by themselves. Given time and persistence an invading microorganism will eventually overcome mere physical obstacles. Nevertheless, most microbial attempts at invasion are rapidly blocked before they can result in disease. All animals and plants, even the least evolved, need to detect and eliminate microbial invaders as fast and as effectively as possible. This immediate response is the task of the innate immune system. Many different innate defense mechanisms have evolved over time and the mammalian innate immune system is therefore a diverse collection of subsystems that work through many different mechanisms. Collectively, they all respond rapidly to block microbial invasion and minimize tissue damage (Fig. 1.3). Innate immune responses are activated immediately when a pathogen penetrates the epithelial barriers. These responses are generic; that is, they detect microbes such as bacteria and viruses because they differ structurally and chemically from normal animal tissues. Once the invaders are recognized, multiple innate responses are available to destroy them. For example, animals make many different antimicrobial proteins that either kill invaders directly or promote

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their destruction by defensive cells. Some of these molecules are present in normal tissues all the time, while others are produced in response to the presence of bacteria, viruses or cell and tissue damage.

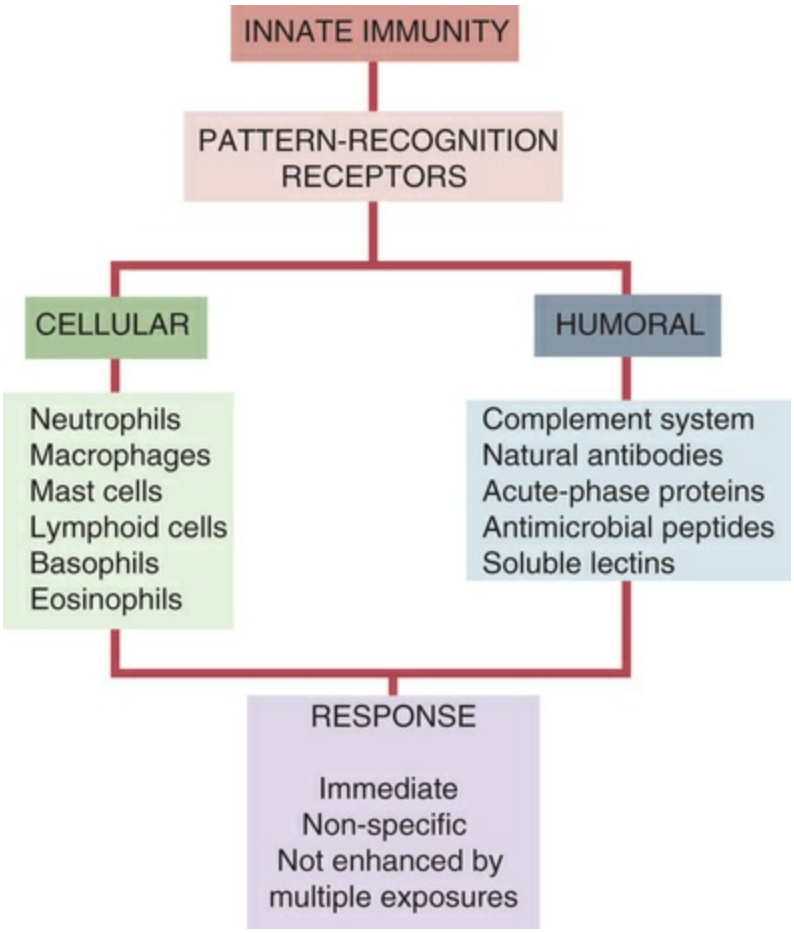


FIG. 1.3 The innate immune system consists of a collection of subsystems. They can be divided into the cellular mechanisms where populations of specialized cells detect, eat, and kill invaders; and the humoral mechanisms where diverse soluble molecules bind and kill the invaders.

Other innate subsystems rely on rapid cellular responses to invasion. Thus the body employs sentinel cells that can detect invading bacteria and viruses. Sentinel cells recruit other cells, called leukocytes, that converge on the invaders and destroy them in the process we call inflammation. Inflammation is central to the innate defenses of the animal body. Some of the cells involved in inflammation may also help repair damaged tissues once the invading microbes have been destroyed. It is the presence of a

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combination of microbial-induced tissue damage as well as inflammation that results in the set of animal behaviors that we call sickness.

The innate immune system is a mixture of "hard-wired" subsystems that lack any form of memory, and, as a result, each episode of infection is treated identically. The intensity and duration of innate responses such as inflammation therefore remain unchanged no matter how often a specific invader is encountered. These responses also come at a price: the pain of inflammation or the development of sickness largely result from the activation of innate immune pathways. On the other hand, the multiple subsystems of the innate immune system are "on call" and ready to respond immediately when invaders are detected (Fig. 1.4).

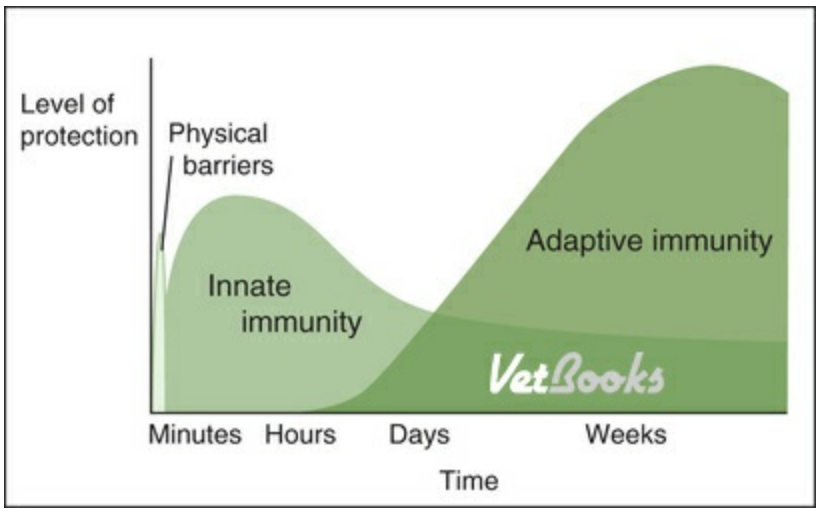


FIG. 1.4 The time course of innate and adaptive immunity. Surface barriers provide immediate protection. Innate mechanisms provide rapid protection that keeps microbial invaders at bay until adaptive immunity can develop. It may take several days or even weeks for adaptive immunity to become effective.

Adaptive Immunity

Inflammation and the other innate defenses are critical to the defense of the body. Animals that fail to mount innate responses will die from overwhelming infections. Nevertheless, these responses cannot offer the ultimate solution to the defense of the body. What is really needed is a defense system that can recognize

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and destroy specific invaders, and then learn from the process so that if they invade a second time, they will be destroyed even more effectively. In this system, the more often an individual encounters an invading bacterium or virus, the more effective will be its defenses against that organism. This type of "smart" response is the function of the adaptive immune system, so called since it adapts itself to ongoing threats to the animal. Although it develops slowly, when an animal eventually develops adaptive immunity, the chances of successful invasion by that organism decline precipitously, and the animal is said to be immune. The adaptive immune system provides the ultimate defense of the body. Its essential nature is readily seen when its loss leads inevitably to uncontrolled infections and death.

A key difference between the innate and adaptive immune systems lies in their use of cell surface receptors to recognize foreign invaders (Table 1.1). The cells of the innate system use a limited number of preformed receptors that bind to molecules expressed by many different microbes, and the response is therefore generic in nature. In contrast, the cells of the adaptive immune system generate enormous numbers of new, structurally unique receptors that bind specifically to those foreign molecules that induce them. Because the binding repertoire of these receptors is generated randomly, they are assured of recognizing at least some of the molecules found on almost any invading microorganism.

TABLE 1.1

A Comparison of Innate and Adaptive Immunity

	Innate Immunity **Always "on"**	**Adaptive Immunity** Turned on by Antigens
Cells engaged	Macrophages, dendritic cells Neutrophils, NK cells*	T and B cells
Cell receptors	Preformed and directed against common microbial molecules	Generated in response to foreign molecules
Evolutionary history	Ancient	Recent
Onset	Rapid (min-hr)	Slow (days-weeks)
Specificity	Common microbial structures	Unique antigens
Potency	May be overwhelmed	Rarely overwhelmed
Memory	None	Significant memory
Effectiveness	Does not improve	Improves with exposure

*Note: Natural killer (NK) cells are a family of innate cells that can kill tumors and virus-

infected cells. They are described in Chapter 19.

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The adaptive immune system not only recognizes invading microbes, but it also destroys them and retains the memory of the encounter. If the animal encounters the same organism a second time, the adaptive immune system responds more rapidly and much more effectively. Such a sophisticated system must, out of necessity, be complex.

Another reason for this complexity is the great diversity of potential invaders, including: bacteria, viruses, fungi, protozoa, and helminths (worms). These invaders may be classified into two broad categories. One category consists of the organisms that normally reside outside cells—extracellular invaders. This includes most bacteria and fungi, as well as many protozoa and invading helminths. The second category consists of organisms that originate or live within the body's own cells—the intracellular invaders. These include viruses and intracellular bacteria or protozoa. Each category requires a different defensive strategy.

The adaptive immune system thus consists of two major branches (Fig. 1.5). One branch is directed against the extracellular invaders. The other is directed against intracellular invaders. Both branches depend upon the use of specialized white blood cells called lymphocytes. There are two major lymphocyte populations, B cells and T cells. Immunity to extracellular invaders is mainly the function of B cells. They produce proteins called antibodies that promote the microbial destruction. This B-cell-mediated immune response is sometimes called the “humoral immune response” since antibodies are found in body fluids (or “humors”).

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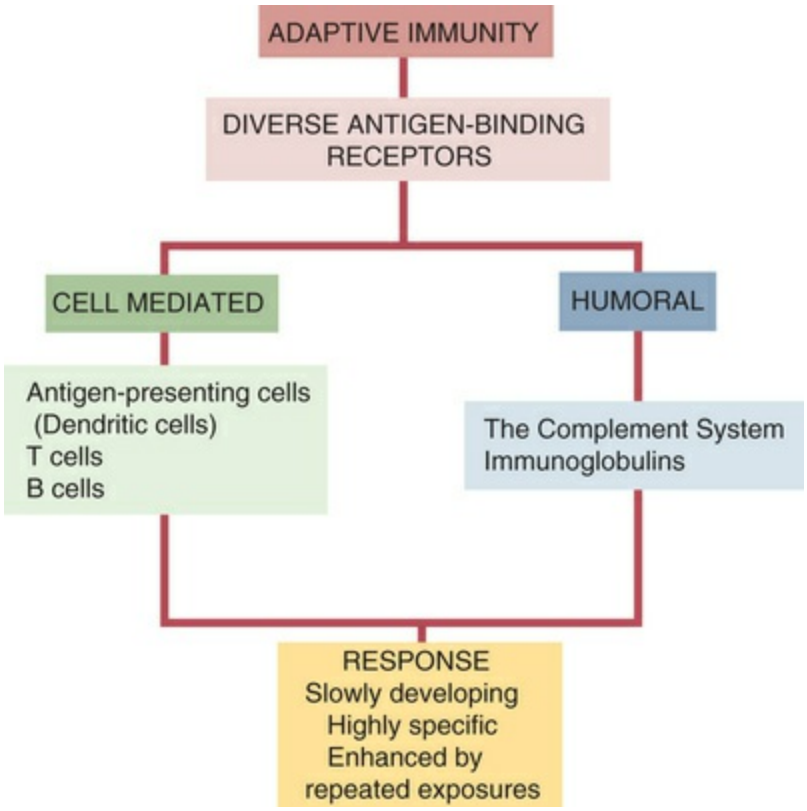


FIG. 1.5 A simple flow diagram showing the essential features of the humoral and cell-mediated adaptive immune responses.

Because antibodies do not work inside cells, immunity to intracellular invaders is the function of T cells. The T cells can destroy infected or abnormal cells. This type of response is therefore called the "cell-mediated immune response." There are three major types of T cell. One is responsible for killing abnormal cells and so consists of cytotoxic T cells. Another type is responsible for providing the signals that activate the adaptive immune responses and hence are called helper T cells. The third cell type regulates immune responses and so are called regulatory T cells. Most importantly, adaptive immune responses generate long-lived populations of memory T and B cells that ensure that this immunity persists for a long time, perhaps even as long as the animal's lifetime.

Antibody-Mediated Immunity

Soon after it was discovered that animals could be made immune to infectious agents by vaccination, (Chapter 24) it was recognized that the substances that provided this immunity could be found in blood serum. For example, if blood is taken from an immune horse

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that has been previously vaccinated against tetanus (or has recovered from tetanus), its serum separated and then injected into a normal horse, the recipient animal will become temporarily resistant to tetanus (Fig. 1.6).

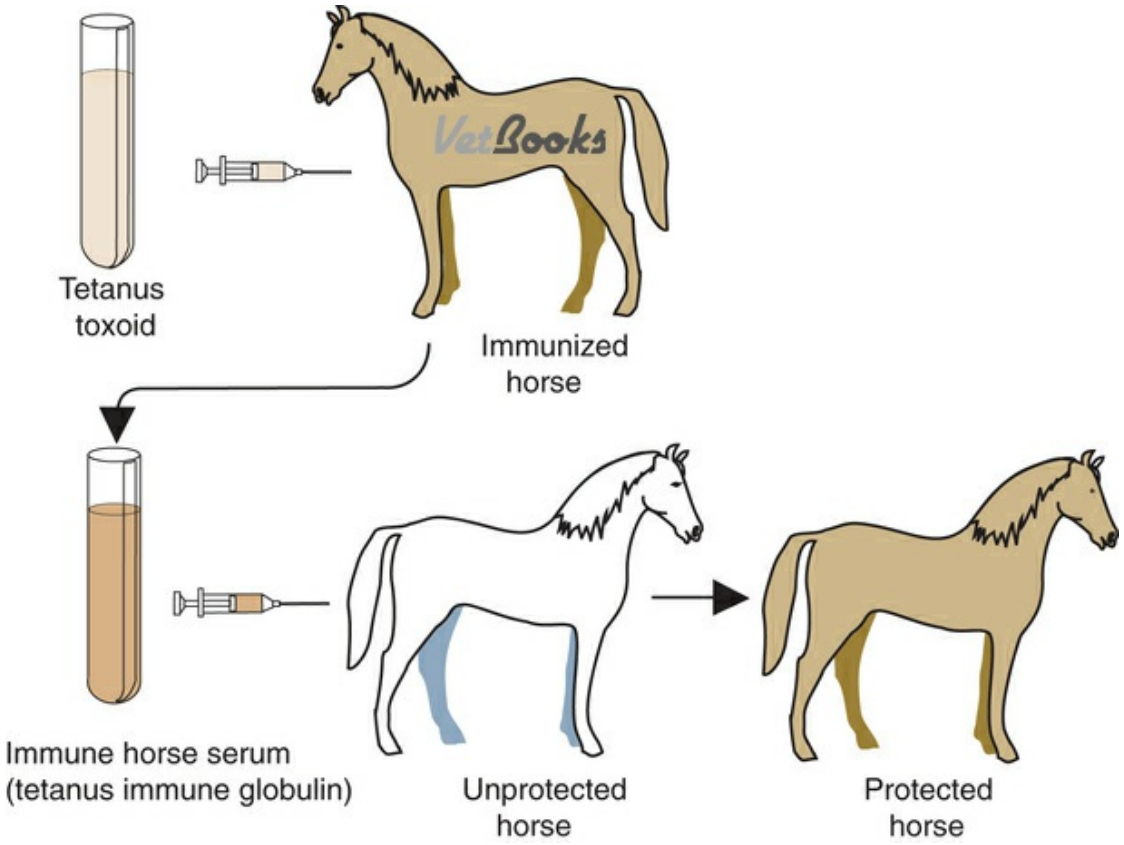


FIG. 1.6 Immunity to tetanus can be transferred to a normal horse by means of serum derived from an immunized horse. This clearly demonstrates that antibodies in serum are sufficient to confer immunity to tetanus in horses.

The protective molecules found in the serum of immune animals are proteins called antibodies. Antibodies against tetanus toxin are not found in normal horses but are produced following exposure to tetanus toxin as a result of infection or vaccination. Tetanus toxin is an example of a foreign substance that stimulates an adaptive immune response. The general term for such a substance is antigen. When an antigen enters an animal, the animal's B cells are stimulated to produce antibodies that bind to that antigen and ensure its destruction. Antibodies are highly specific and bind only to the antigen that stimulates their production. For example, the

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antibodies produced in response to tetanus toxin bind only to tetanus toxin. When the antibodies bind, they "neutralize" the toxin so that it is no longer toxic. In this way antibodies protect animals against lethal tetanus.

The time course of the antibody response to tetanus toxin can be examined by taking blood from a horse at intervals after injection of a low dose of the toxin. The blood is allowed to clot and the clear serum removed. The amount of antibody in the serum may be estimated by measuring its ability to neutralize a standard amount of toxin. Following a single injection of toxin into an unexposed horse, no antibody is detectable for several days (Fig. 1.7). This lag period lasts for about 1 week as responding B cell populations grow and begin to produce antibodies. When antibodies eventually appear their levels climb to reach a peak by 10 to 20 days before declining and disappearing within a few weeks. The amount of antibody formed, and therefore the amount of protection conferred, during this first or primary response is relatively small since there are few antibody-producing B cells. However, memory B cells are produced in large numbers.

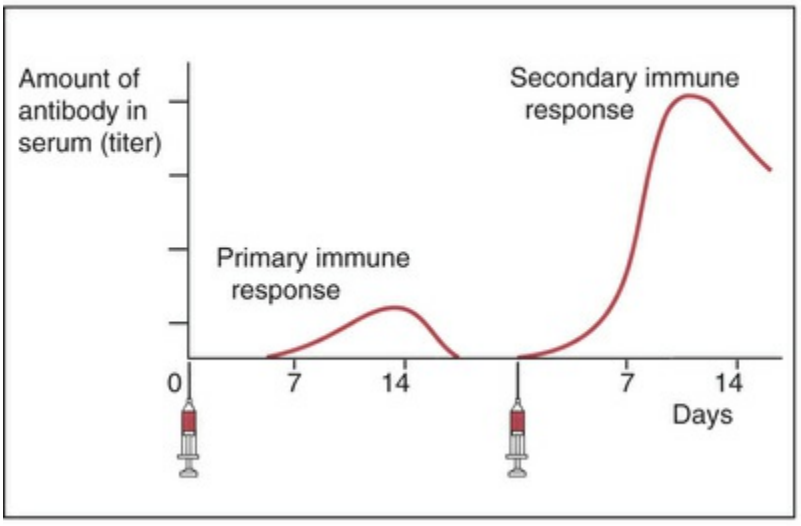


FIG. 1.7 The characteristic time course of the adaptive immune response to an antigen as measured by serum antibody levels. Note the differences between a primary and a secondary immune response. These are the reasons why the adaptive immune responses are so successful.

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If sometime later a second dose of toxin is injected into the same horse, it is recognized by this much larger population of memory B cells. As a result, the lag period lasts for no more than 2 or 3 days. The amount of antibody in serum then rises rapidly to a high level before declining slowly. Antibodies may be detected for many months or years after this injection. A third dose of the antigen given to the same animal results in an immune response characterized by an even shorter lag period and a still higher and more prolonged antibody response. As will be described later in this book, the antibodies produced after repeated injections are better able to bind and neutralize the toxin than those produced early in the immune response. This progressive improvement of adaptive immune responses to infectious agents by repeated injections of antigen effectively generates memory cells and forms the basis of vaccination.

The response of an animal to a second dose of antigen is very different from the first in that it occurs much more quickly, antibodies reach much higher levels, and it lasts for much longer. This secondary B cell response is specific in that it can be provoked only by a second dose of the same antigen. A secondary response may be provoked many months or years after the first injection of antigen, although its size tends to decline as time passes. A secondary response can also be induced even though the response of the animal to the first injection of antigen was so weak as to be undetectable. These features of the secondary response indicate that memory B cells possess the ability to "remember" previous exposure to an antigen. For this reason, the secondary immune response is sometimes called an anamnestic response (*anamnesko* is Greek for "remembering").

Cell-Mediated Immunity

If a piece of living tissue such as a kidney or a piece of skin is surgically removed from one animal and grafted onto another of the same species, it only survives for a few days before being rejected and destroyed by the recipient. This process of graft rejection is significant because it demonstrates a mechanism whereby foreign cells, differing only slightly from an animal's own normal cells, are rapidly recognized and destroyed. Even cells with

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minor structural abnormalities may be recognized as foreign by the immune system and destroyed, though they are otherwise apparently healthy. These abnormal cells include aged cells, virus-infected cells, and some cancer cells. The immune response to foreign cells as shown by graft rejection is mediated by cytotoxic T cells that identify and destroy the "abnormal" cells.

If a piece of skin is transplanted from one dog to a second, unrelated dog, it will survive for about 10 days. The grafted skin will initially appear to be healthy, and blood vessels will develop between the graft and its host. By 1 week, however, these new blood vessels will begin to degenerate, the blood supply to the graft will be cut off, and the graft will eventually die and be shed (Fig. 1.8). If the experiment is repeated and a second graft is taken from the original donor and placed on the same recipient, then the second graft will survive for no more than a day or two before being rejected. Thus the rejection of a first graft is relatively weak and slow and analogous to the primary antibody response, whereas a second graft stimulates very rapid and powerful rejection similar in many ways to the secondary antibody response. Graft rejection, like antibody formation, is a specific adaptive immune response in that a rapid secondary reaction occurs only if the second graft is from the same donor as the first. Like antibody formation, the graft rejection process also involves the generation of long-lived memory cells, since a second graft may be rapidly rejected many months or years after loss of the first.

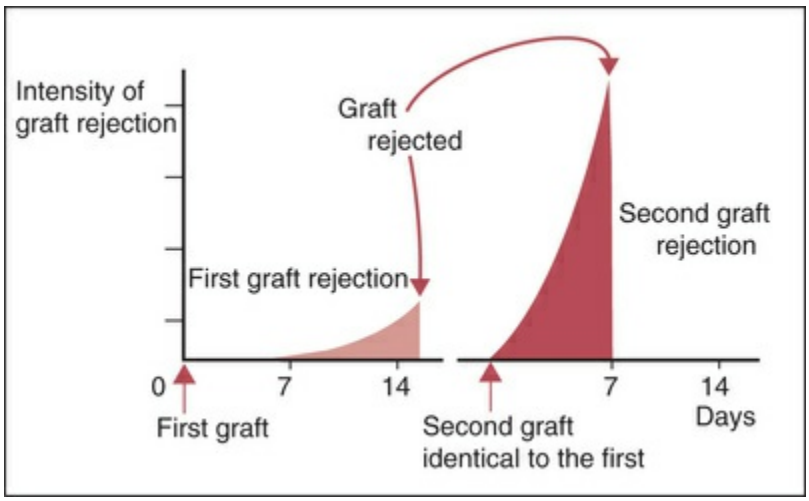


FIG. 1.8 The time course of the rejection of a foreign skin graft.

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The rejection of a first graft is slow and relatively mild. The rejection of a second graft is rapid and accompanied by severe inflammation. This accelerated rejection is due to the presence of memory cells. Notice the similarity of this response to that in Fig. 1.7.

However, graft rejection differs from antibody-mediated immunity in that it cannot be transferred from a sensitized to a normal animal by serum. The ability to mount a secondary reaction to a graft can only be transferred between animals by living T cells. These T cells are found in the spleen, lymph nodes, or blood, and they are responsible for organ graft rejection. It is a good example of a cell-mediated immune response.

Mechanisms of Adaptive Immunity

In some ways the adaptive immune system may be compared to systems in a totalitarian state in which foreigners are expelled, citizens who behave themselves are tolerated, but those who "deviate" are eliminated. While this analogy must not be carried too far, clearly such regimes possess a number of characteristic features. These include border defenses and a police force that keeps the population under surveillance and promptly eliminates dissidents. In the case of the adaptive immune system, the antibody-mediated responses would be responsible for keeping the foreigners out, whereas the cell-mediated responses would be responsible for stopping internal dissent. Organizations of this type also tend to develop a pass system, so that invading foreigners or dissidents not possessing certain identifying features are rapidly detected and dealt with.

Similarly, when foreign antigens enter the body, they first must be trapped and processed so that they can be recognized as being foreign. If so recognized, then this information must be conveyed either to the antibody-forming B cells or to the T cells of the cell-mediated immune system. These cells must then respond by producing specific antibodies and/or cytotoxic T cells that can eliminate the antigen. The adaptive immune system must also generate long-lived B or T memory cells that can remember this event so that the next time an animal is exposed to the same antigen, these cells will respond faster and with greater efficiency. In our totalitarian state analogy, the police force would be trained

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to recognize selected foreigners or dissidents, keep a file on them, and respond more promptly when they reappear.

It must be emphasized, however, that just as human societies and responses are very complex and involve the interactions of thousands of individuals, so too is the immune system. While, for reasons of simplicity and teaching, we discuss discrete cells, processes and pathways, the immune systems should be thought of as an interactive network. Thousands of different cells interact in many ways and are subject to multiple influences. The cells involved interact with each other, sometimes in a very complex manner. Likewise, the invading microbe, its virulence, its ability to evade defenses, and its interactions with other microbes, all lead to variations in a host's immune response.

For introductory purposes, we can consider the process of adaptive immunity to proceed by a series of steps (Fig. 1.9). Thus it is triggered by cells that can recognize, trap, and process antigen. The most important of these cells are dendritic cells and macrophages. These cells then present the antigen to the T and B cells of the immune system. The T and B cells can recognize and respond to this processed antigen since they possess specific antigen receptors on their surface. The B cells, once activated, will produce specific antibodies, while the T cells will participate in the cell-mediated immune responses. Long-lived B and T memory cells are generated at the same time. These cells retain the memory of these events and will react very rapidly to each specific antigen if it is encountered again. They are thus responsible for the enhanced immunity that develops in secondary immune responses. Finally, helper and regulatory T cells control these responses and ensure that they function at an appropriate level.

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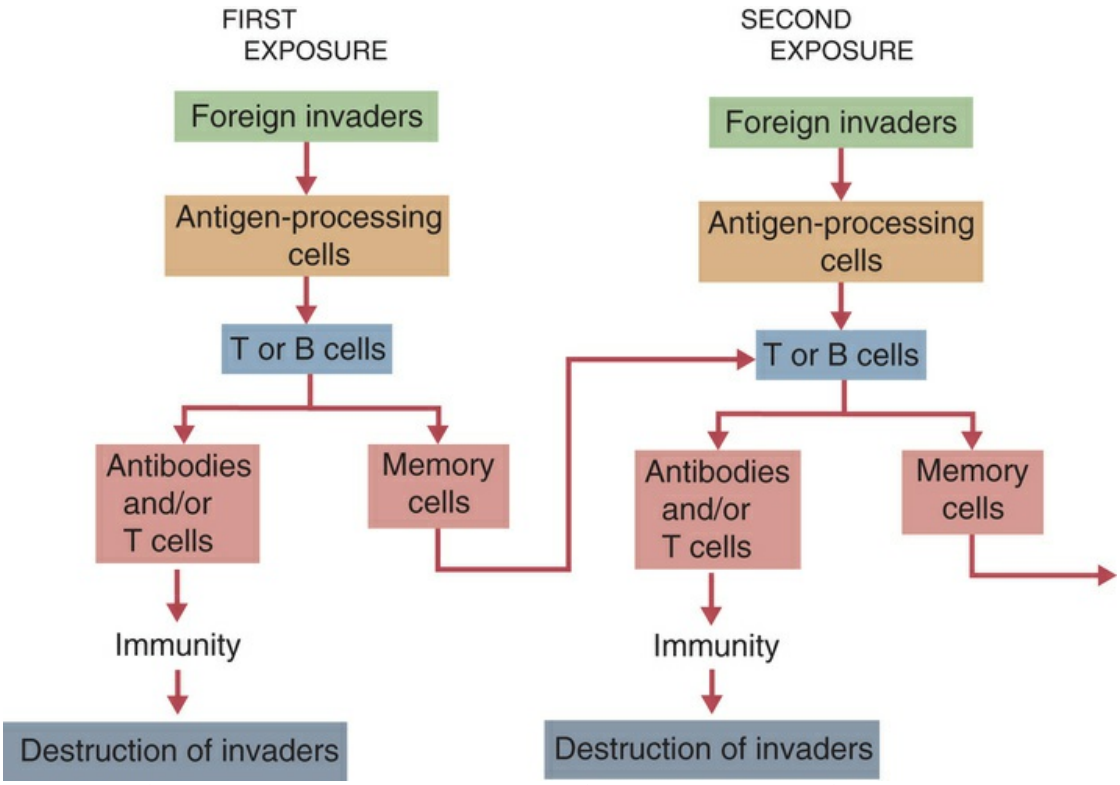


FIG. 1.9 In adaptive immunity, on first exposure to a foreign invader the body destroys the invaders but, in addition, generates memory cells. On second exposure to that invader, these memory cells act to mediate a much stronger immune response that results in much faster destruction of the invader.

In subsequent chapters we will first review the mechanisms involved in innate immunity. Following that, we will review adaptive immunity and examine each of its basic components in turn. We will then examine the role of the immune system in protecting animals against microbial invasion. Finally, we will look at the diseases that result when the immune system functions either excessively or inadequately.

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Where to Go for Additional Information

Many veterinary journals carry articles of interest to immunologists. Some of the most important ones used in this book include: *Animal Genetics, Australian Veterinary Journal, BMC Veterinary Research, Developmental and Comparative Immunology, Journal of Comparative Pathology, Journal of Veterinary Internal Medicine, Research in Veterinary Science, Trends in Parasitology, Vaccine, Veterinary Dermatology, Veterinary Immunology and Immunopathology, The Veterinary Journal,* and *Veterinary Pathology.*

For information on new developments on basic immunology (with occasional papers on subjects of veterinary interest), the reader should review journals such as *Cell, Cell Host and Microbe, Clinical and Vaccine Immunology, Infection and Immunity, Immunity, Immunogenetics, Immunology, Journal of Immunology, Journal of Leukocyte Biology, Molecular Immunology, Nature, Nature Immunology, Nature Reviews Immunology, Proceedings of the National Academy of Sciences, Science, Trends in Immunology, Trends in Microbiology, Trends in Molecular Medicine,* and *Vaccine.*

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Innate Immunity

How to Detect Invaders

CHAPTER OUTLINE

How Invaders Are Recognized, 9
- Pattern-Recognition Receptors, 10
- Toll-Like Receptors, 10
- RIG-1-Like Receptors, 13
- NOD-Like Receptors, 13

Pathogen-Associated Molecular Patterns, 14
- Bacterial Lipopolysaccharides, 14
- Bacterial Peptidoglycans, 14
- Bacterial DNA, 14
- Viral Nucleic Acids, 15

Damage-Associated Molecular Patterns, 15

Soluble Pattern-Recognition Receptors, 16

Sentinel Cells, 17
- Macrophages, 17
- Dendritic Cells, 17
- Mast Cells, 17

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LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Understand how the body detects both invading microbes and tissue damage by using specific receptors that bind conserved microbial molecules (PAMPs) as well as molecules released by broken cells (DAMPs).
- Understand how PAMPs and DAMPs bind to pattern-recognition receptors (PRRs) found on cell surfaces or inside cells.
- Understand how microbial nucleic acids can be detected by innate receptors.
- Describe how other triggering signals come from damaged tissues or broken cells.
- Understand that the most important family of PRRs consists of the toll-like receptors (TLRs).
- Recognize the significance of lipopolysaccharides and other conserved microbial molecules in triggering innate immune responses.
- List four major populations of sentinel cells.
- List some microbial molecules that trigger responses by toll-like receptors.
- Define pattern recognition receptors; damage-associated molecular patterns; pathogen-associated molecular patterns; caspases; lectins; selectins.

Bacteria and viruses multiply very rapidly. A single bacterium with a doubling time of 50 minutes can produce about 500 million offspring within 24 hours. When these microbes invade the body they must be destroyed before they can overwhelm its defenses. Time is of the essence, and delay can be fatal. The body must therefore employ fast-acting response mechanisms as its first line of defense against invaders. These mechanisms need to be on constant standby and respond to the first signs of microbial invasion. They constitute the innate immune system.

Because all multicellular organisms are subject to microbial attack, innate immunity has evolved in animals and plants, in vertebrates, and in invertebrates. Innate immune mechanisms have evolved in different ways and at different times in response to different threats. As a result, the innate immune system consists of many diverse subsystems or modules. The most important of these

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subsystems is the process we call inflammation.

Inflammation is a process whereby defensive cells and antimicrobial molecules converge on sites of microbial invasion and tissue damage. These defensive cells are the white blood cells (leukocytes) that circulate constantly in the bloodstream. Inflammation triggers their emigration from the bloodstream into tissues where they attack and destroy invaders. Likewise, many protective proteins, such as antibodies and complement components, are normally found only in blood and only enter tissues during inflammation. These defensive cells and antimicrobial proteins together destroy the invaders and repair any subsequent tissue damage (Fig. 2.1).

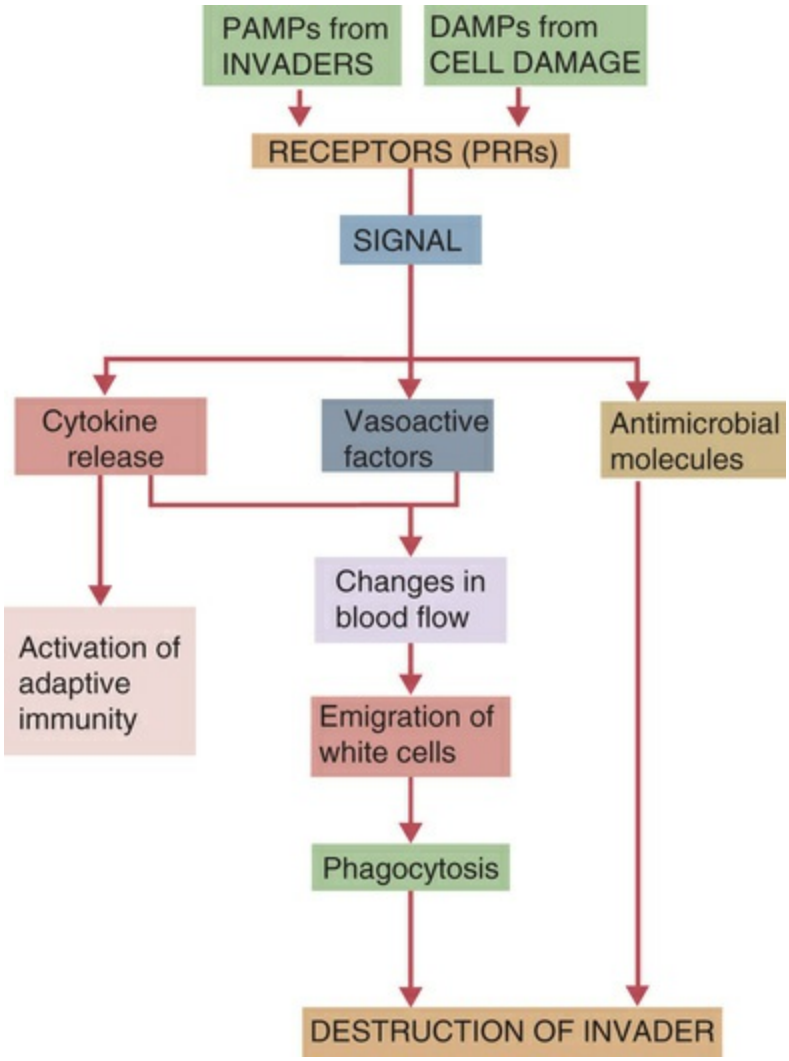


FIG. 2.1 An overview of the essential features of acute inflammation, an innate mechanism for focusing cells and other defensive molecules at sites of microbial invasion. Inflammation is

triggered by microbial invasion and tissue damage.

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How Invaders Are Recognized

The innate immune system is activated when the body senses that it is under attack. It uses alarm signals generated either by the presence of invading microorganisms or by dead, damaged, and dying cells. Microbial invaders express a diverse mixture of molecules that can be recognized by the body as foreign. Collectively, these are called pathogen-associated molecular patterns (PAMPs). Likewise, molecules released from damaged cells, collectively called damage-associated molecular patterns (DAMPs) also generate alarm signals. Together, the PAMPs and DAMPs bind to pattern-recognition receptors (PRRs) found on sentinel cells located throughout the body. Triggering of inflammation requires the binding of either PAMPs or DAMPs to PRRs.

Pattern-Recognition Receptors

Microbes not only grow very fast but also are highly diverse and can alter their surface molecules very rapidly. For this reason, the PRRs of the innate immune system cannot recognize all possible microbial molecules. Rather, the PRRs recognize abundant, essential molecules. Because they are essential, these molecules tend be structurally conserved and may be shared by entire classes of pathogens. They form, in effect, conserved molecular patterns. For example, the walls of Gram-positive bacteria are largely composed of peptidoglycans (chains of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid cross-linked by short peptide side chains) and lipoteichoic acids (Fig. 2.2). Likewise, the walls of Gram-negative bacteria consist of peptidoglycans covered by a layer of lipopolysaccharide (LPS). Acid-fast bacteria are covered in glycolipids. Yeasts have a mannan- or β-glucan-rich cell wall. Viruses have unique nucleic acids. PRRs can recognize all of these molecules.

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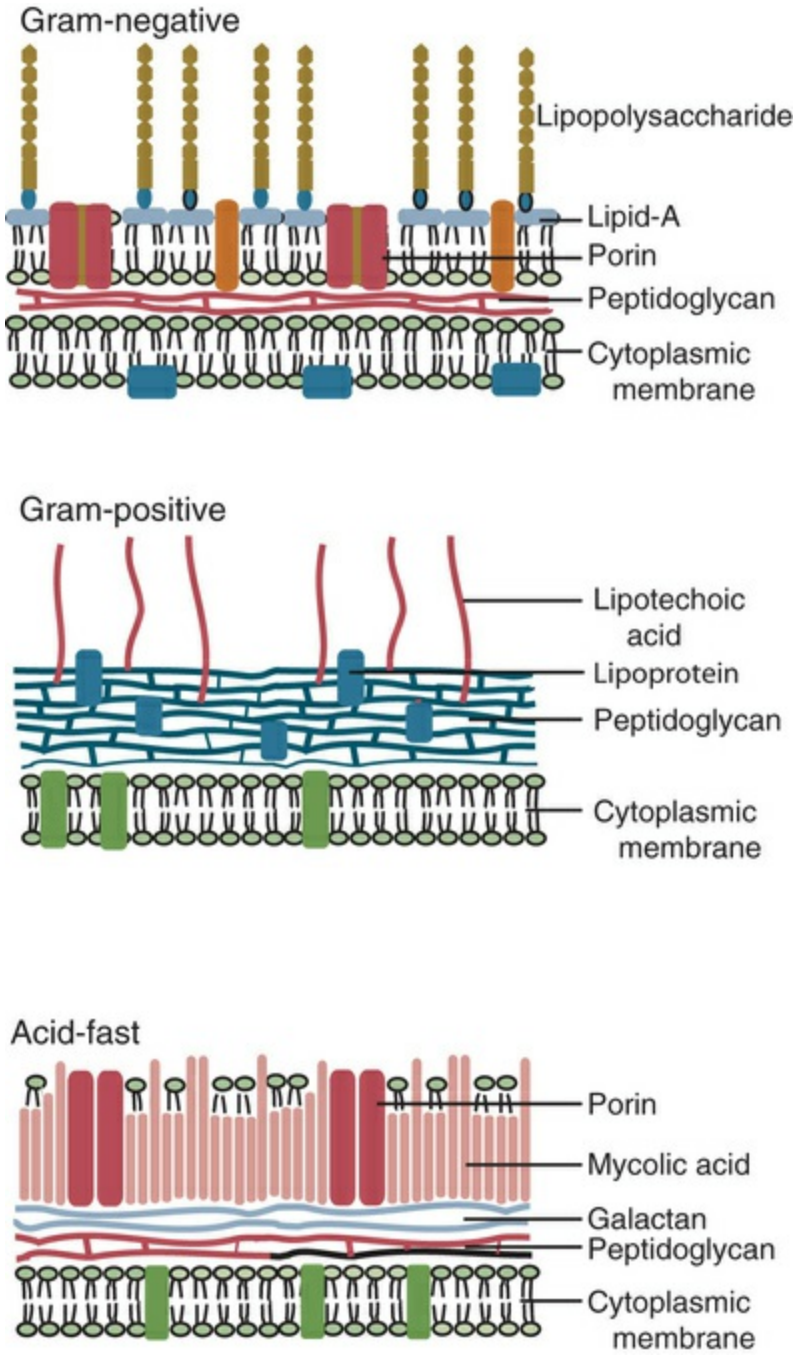


FIG. 2.2 The major structural features of the cell walls of Gram-negative, Gram-positive, and acid-fast bacteria. It is these conserved structural molecules that serve as pathogen-associated molecular patterns and are recognized by pattern-recognition receptors such as the toll-like receptors.

Animals use many different PRRs to ensure detection of as many PAMPs as possible. Most PRRs are cell associated. They are found on cell membranes, within the cytosol, and within cytoplasmic vesicles, while other soluble PRRs circulate in the bloodstream (Fig. 2.3).

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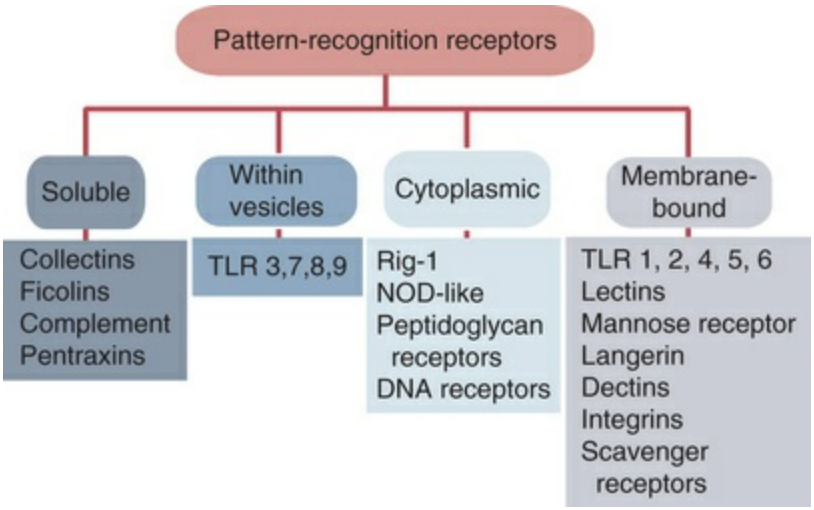


FIG. 2.3 There are many different pattern-recognition receptors used by the animal body. Most are found on or within cells. Others are soluble molecules that circulate in the bloodstream.

Toll-Like Receptors

The most significant family of PRRs consists of the toll-like receptors (TLRs) (Box 2.1). Some TLRs are located on cell surfaces, where they can bind PAMPs from extracellular invaders such as bacteria and fungi. Other TLRs are found inside cells, where they bind PAMPs from intracellular invaders such as viruses.

Box 2.1

Toll-like Receptors

Their name alludes to the original discovery of a protein called "toll" in fruit flies *(Drosophila).* This protein was necessary for proper embryological development. In its absence, the flies developed abnormally, and the German researchers who first saw these abnormal flies exclaimed "Toll!" (amazing, or weird!). Toll protein was subsequently found to be necessary for antifungal immunity in *Drosophila* species. When the first pattern-recognition receptor was identified in mammals, it was found to be similar in sequence and structure to the *Drosophila* toll protein, hence "toll-like receptors."

TLRs are found on sentinel cells of the innate immune system

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such as macrophages, neutrophils, mast cells, and dendritic cells. They are also found on T and B cells of the adaptive immune system, as well as on nonimmune cells such as the epithelial cells that line the respiratory and intestinal tracts. When activated they turn on genes involved in producing proinflammatory factors. Thus they trigger inflammation when they sense the presence of invaders or tissue damage.

Mammals possess 10 or 12 different functional TLRs (TLRs 1 to 10 in humans, sheep and cattle and TLRs 1 to 9 and 11 to 13 in mice) (Table 2.1). The cell surface TLRs (TLRs 1, 2, 4, 5, 6, and 11) mainly bind bacterial and fungal proteins, lipoproteins, and lipopolysaccharides. The intracellular TLRs (TLRs 3, 7, 8, 9, and 10), bind viral and bacterial nucleic acids. For example, TLR4 on the cell surface binds lipopolysaccharides from Gram-negative bacteria. TLR2 binds peptidoglycans and lipoproteins from Gram-positive bacteria and a glycolipid called lipoarabinomannan from *Mycobacterium tuberculosis.* TLR5 binds flagellin, the major protein of bacterial flagella. TLR9, in contrast, is an intracellular sensor of bacterial DNA and is therefore triggered by intracellular bacteria. Other intracellular receptors, such as TLR3, bind viral double-stranded (ds) ribonucleic acid (RNA), whereas TLR7 and TLR8 bind viral single-stranded (ss) RNA (Fig. 2.4).

TABLE 2.1

Pathogen-Associated Molecular Patterns and the Functions of the Mammalian Toll-like Receptors

TLR	Cell Location	Ligand	Pathogens Recognized
TLR1	Cell surface	Triacylated lipoprotein	Bacteria
TLR2	Cell surface	Lipoproteins	Bacteria, viruses, parasites
TLR3	Intracellular	dsRNA	Viruses
TLR4	Cell surface	LPS	Bacteria, viruses
TLR5	Cell surface	Flagellin	Bacteria
TLR6	Cell surface	Diacylated lipoprotein	Bacteria, viruses
TLR7	Intracellular	ssRNA, guanosine	Viruses, bacteria
TLR8	Intracellular	ssRNA	Viruses, bacteria
TLR9	Intracellular	CpG DNA, dsDNA	Viruses, bacteria, protozoa
TLR10	Intracellular	Regulates TLR2 responses	Suppresses inflammation
TLR11	Cell surface	Profilin and flagellin	Protozoa, bacteria
TLR12	Cell surface	Profilin	Protozoa
TLR13	Intracellular	Unmethylated RNA	Bacteria

CpG, cytosine-guanosine; *LPS*, lipopolysaccharide; *TLR*, toll-like receptor.

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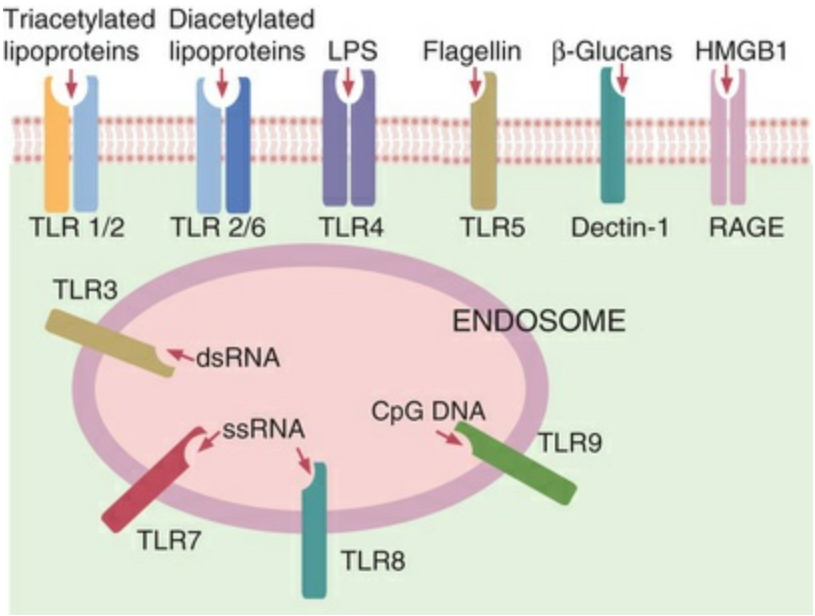


FIG. 2.4 The toll-like receptors (TLRs). These receptors, either alone or in pairs, can bind a diverse array of microbial molecules (PAMPs). The TLRs expressed on the outer cell membrane are generally optimized to bind bacterial molecules. The TLRs expressed inside the cell in endosomes are optimized to bind viral and bacterial nucleic acids.

All TLRs are transmembrane glycoproteins. Most are homodimers formed by two identical paired peptide chains. They may also form heterodimers by using two different chains. For example, TLR2 can associate with TLR6, and this dimer can then bind bacterial diacylated lipopeptides. TLR2 can also associate with TLR1 to recognize mycobacterial triacylated lipopeptides. Given the number of possible TLR chain pairs, it is believed that the presently known TLRs can collectively bind almost all PAMPs. TLR11 differs from the others in that it is found only on dendritic cells, macrophages, and epithelial cells in the mouse urinary tract, where it binds PAMPs from bacteria and protozoan parasites.

When a PAMP binds to its corresponding TLR, signals are passed to the cell. As a result, multiprotein signaling complexes form, signal transduction cascades are initiated, and proinflammatory molecules are produced by the cell. Each step in the process involves multiple biochemical reactions involving many different proteins. Additionally, the cell surface TLRs use different signaling pathways than do the intracellular TLRs. All extracellular TLRs (except TLR3) use an adaptor protein called MyD88 to activate the transcription factors, nuclear factor kappa-B (NF-κB), and IRF3 (Fig.

2.5). NF-κB activates the genes for three proteins, interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α). IRF3 activates the gene for interferon-β(IFN-β). (For additional details on these signal transduction pathways, see Chapter 8.)

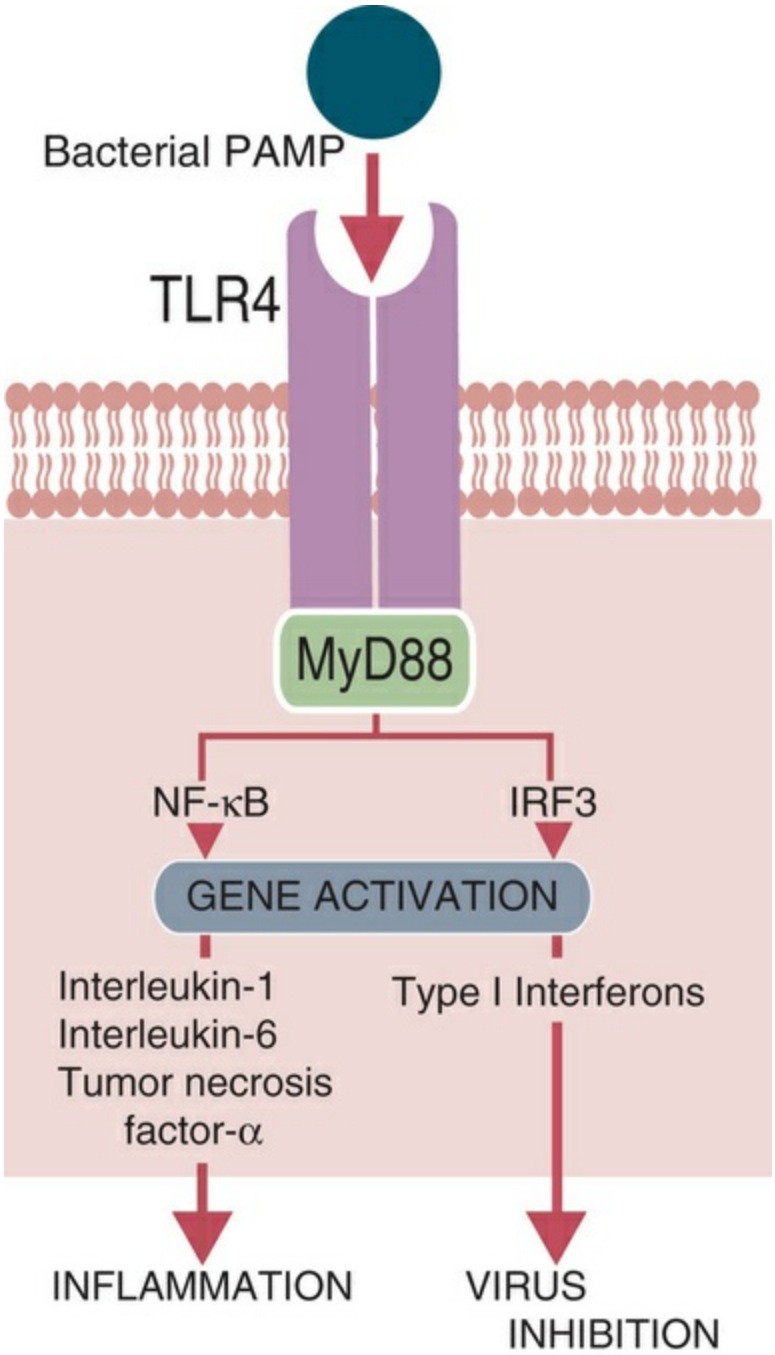


FIG. 2.5 Binding of a pathogen-associated molecular pattern such as lipopolysaccharide to a toll-like receptor generates a signaling cascade that eventually activates two transcription factors, NF-κB and IRF3. These transcription factors activate the genes for the three major cytokines, IL-1, IL-6 and TNF-α. The TRIF complex

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turns on another transcription factor, IRF3, that activates the gene for IFN-β.

The proteins produced in response to TLR-ligation on sentinel cells are known as cytokines, proteins that regulate the cells involved in the defense of the body. These cytokines are produced as inactive precursors and then activated by an enzyme called caspase-1. The production of caspase-1 is triggered by a protein complex called an inflammasome (Box 2.2). Caspases are proteases (**c**ysteinyl **aspa**rtate-**s**pecific proteinas**es**). Many, such as caspase-1, -4, -5, and -12, are activated by signals generated through TLRs. Caspase-1 is most important because it acts on inactive precursors to generate the active cytokines.

Box 2.2

Inflammasomes

When PAMPs and DAMPs bind to NOD-like receptors, they initiate the assembly of large intracellular multiprotein complexes called inflammasomes. These inflammasomes then activate two proteolytic enzymes, caspase-1 and caspase-11. The caspase-11 triggers cell death by a process called pyroptosis. The caspase-1 acts on pro-IL-1 and pro-IL-18 to generate the active forms of these two cytokines that are then released as cells die (see Fig. 2.9). Four different types of inflammasome have been characterized, each generated by different PAMPs and DAMPs, and containing slightly different components, and thus presumably generating different cytokines and proinflammatory molecules. In humans, inherited defects in some inflammasome components are linked to uncontrolled inflammation. Inflammasome-mediated responses are important in controlling microbial infections as well as in regulating some metabolic processes and immune responses, especially in the intestinal mucosa. Caspase-1 deficient mice are more susceptible to bee and snake venoms, suggesting that inflammasomes also play a role in defense against envenomation.

Different TLRs trigger the production of different cytokine mixtures, and different PAMPs trigger distinctly different responses

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even within a single cell type. For example, TLRs that bind bacterial PAMPs tend to trigger the production of cytokines optimized to combat bacteria, while those that bind viral PAMPs produce antiviral cytokines, and so forth. TLRs not only trigger innate responses such as inflammation but also begin the process of "turning on" the adaptive immune system. For example, DAMPs binding to TLR4 trigger macrophages and their close relatives, the dendritic cells, to produce cytokines that are potent stimulators of lymphocytes (Chapter 8). The intracellular TLRs detect the presence of viral nucleic acids. When triggered, they synthesize antiviral cytokines, collectively called type I interferons (IFNs). These interferons turn on antiviral pathways and "interfere" with viral growth.

TLRs are mainly expressed on the cells most likely to encounter invaders. These include neutrophils, macrophages, dendritic cells, and epithelial cells, such as the enterocytes that line the intestine. However, dendritic cells and macrophages include many different cell types. Their TLR expression may differ between subpopulations and depends on the degree of cellular activation.

TLRs are also expressed on bone marrow stem cells, the source of blood leukocytes. Bacterial lipopolysaccharides binding to TLR4 on these stem cells stimulates increased leukocyte production. An increase in leukocyte numbers in the blood (the white cell count) is therefore a consistent feature of bacterial diseases.

The TLRs of the major domestic species have been examined in detail. They appear to be similar to the TLRs of humans and rodents. They also vary in structure (they are "polymorphic"), and these variations influence an animal's resistance to infections. Thus some bovine TLR polymorphisms are associated with resistance to mastitis and Johne's disease (Box 2.3).

Box 2.3

TLRs and Diarrhea in German Shepherd Dogs

TLRs trigger the initial steps in resistance to microbial invaders. If they are ineffective, an animal may show increased susceptibility to infections. For example, chronic enteric disease is especially

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common in German Shepherd dogs. Genetic analysis of a large number of affected dogs showed that several single-nucleotide polymorphisms (SNPs) in the TLR4 and TLR5 genes were associated with the occurrence of this disease. It is likely that in German Shepherd dogs, mutations in their TLR4 and TLR5 genes reduced their ability to defend against intestinal bacterial invasion. This predisposes to enteric infections, as shown by diarrhea and vomiting.

Kathrani A, House A, Catchpole B, et al: Polymorphisms in the TLR4 and TLR5 gene are significantly associated with inflammatory bowel disease in German Shepherd dogs, *PloS One* 5:e15740, 2010.

RIG-1-Like Receptors

Retinoic acid inducible gene (RIG)-like receptors (RLRs) are another family of PRRs expressed within cells. They detect viral double-stranded (ds) RNA molecules. Because dsRNA molecules do not occur in uninfected cells, their detection by RLRs activates caspases and triggers the production of type I interferons.

NOD-Like Receptors

Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are a family of PRRs that detect intracellular PAMPs (Table 2.2). Although TLRs and NLRs differ in their location and function, they both react to microbial PAMPs and trigger innate responses to invaders. NOD1 binds bacterial peptidoglycans, whereas NOD2 binds muramyl dipeptide and serves as a general sensor of intracellular bacteria. Binding to either NLR activates the NF-κB pathway and triggers the production of proinflammatory cytokines (Fig. 2.6). NOD2 binding also triggers the production of the antimicrobial proteins called defensins (Chapter 3). NOD3 binds diverse ligands including many viral nucleic acids and inorganic matter such as silica, asbestos, and alum.

TABLE 2.2

Other Mammalian Pattern-Recognition Receptors

Receptor	Location	Ligand	Source of Ligand

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RLRs			
RIG-1	Intracellular	Short dsRNA	RNA viruses
NLRs			
NOD1	Cytoplasm	Peptidoglycans	Bacteria
NOD2	Cytoplasm	Muramyl dipeptide	Bacteria
CLRs			
Dectins	Cell surface	Glucans	Fungi
Mannose receptor (CD206)	Cell surface	Glycoproteins	Bacteria
Others			
CD14	Cell surface	LPS	Bacteria
Peptidoglycan recognition proteins	Cell surface	Peptidoglycans	Bacteria
CD1	Cell surface	Glycolipids	Bacteria
CD36	Cell surface	Lipoproteins	Bacteria
CD48	Cell surface	Fimbria	Bacteria

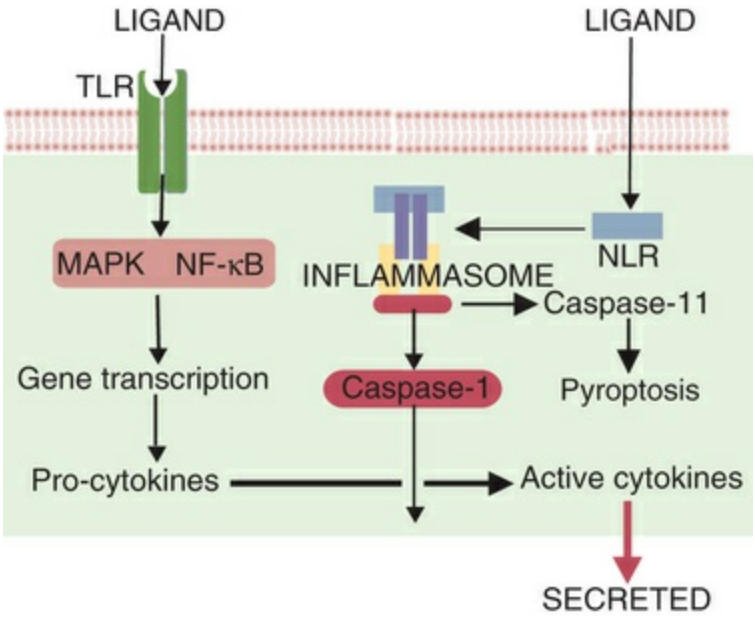


FIG. 2.6 PAMPs signaling through NOD-like receptors (NLR) generate large multiprotein complexes called inflammasomes. Inflammasomes generate caspase-1 that activates cytokines such as IL-1 and IL-18. They also generate caspase-11, an enzyme that causes cell death by pyroptosis.

Macrophages, mast cells, and dendritic cells express many other receptors that can recognize microbial molecules and trigger innate responses. These include C-type lectin receptors (CLRs) that bind carbohydrates, a molecule called CD36 that binds lipoproteins, and one called CD1 that binds glycolipids (Box 2.4).

Box 2.4

The CD Nomenclature System

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When advances in immunology made it possible to produce highly specific antibodies against individual cell surface proteins (Chapter 9), it was shown that mammalian cells expressed hundreds of different proteins on their surface. Initially, each protein was given a specific name and often an acronym as well. It was soon apparent, however, that such a system was unworkable. In an attempt to classify these proteins, a system has been established that assigns each protein to a numbered cluster of differentiation (CD). In many cases, a defined CD denotes a protein (or related proteins) of specific function. For example, the protein CD14 binds bacterial lipopolysaccharides and controls TLR4 responses. As of March 2016, numbers up to CD371 have been assigned. Unfortunately, CD numbers provide no clue to a molecule's function. In practice, therefore, immunologists tend to use a mixed system employing both a CD number and an abbreviation that denotes the function of a molecule. For example, CD32 is also called FcγR1. A list of selected CD molecules can be found in Appendix 1.

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Pathogen-Associated Molecular Patterns

As described earlier, PAMPs are common, essential, conserved molecular structures (or patterns) that are produced by a diverse range of potential microbial invaders. Thus, they include lipopolysaccharides, peptidoglycans, and nucleic acids.

Bacterial Lipopolysaccharides

Lipopolysaccharides are structural components of the cell walls of many bacteria, especially Gram-negative ones. They are recognized by TLR4. TLR4 does not bind LPS directly but only when linked to three other proteins. These proteins are MD-2 (myeloid differentiation factor-2), LPS-binding protein (LBP), and CD14. The CD14 interacts with TLR4 in such a way that it decreases the specificity of these reactions and enables both rough and smooth strains of bacteria to be recognized (Fig. 2.7). Binding of LPS to the CD14/TLR4/MD-2 complex activates macrophages and triggers cytokine production. The LPS subsequently dissociates from CD14 and binds to lipoproteins, where its toxic activities are lost. CD14 also binds many other bacterial molecules, including lipoarabinomannans from mycobacteria, mannuronic acid polymers from *Pseudomonas,* and peptidoglycans from *Staphylococcus aureus.*

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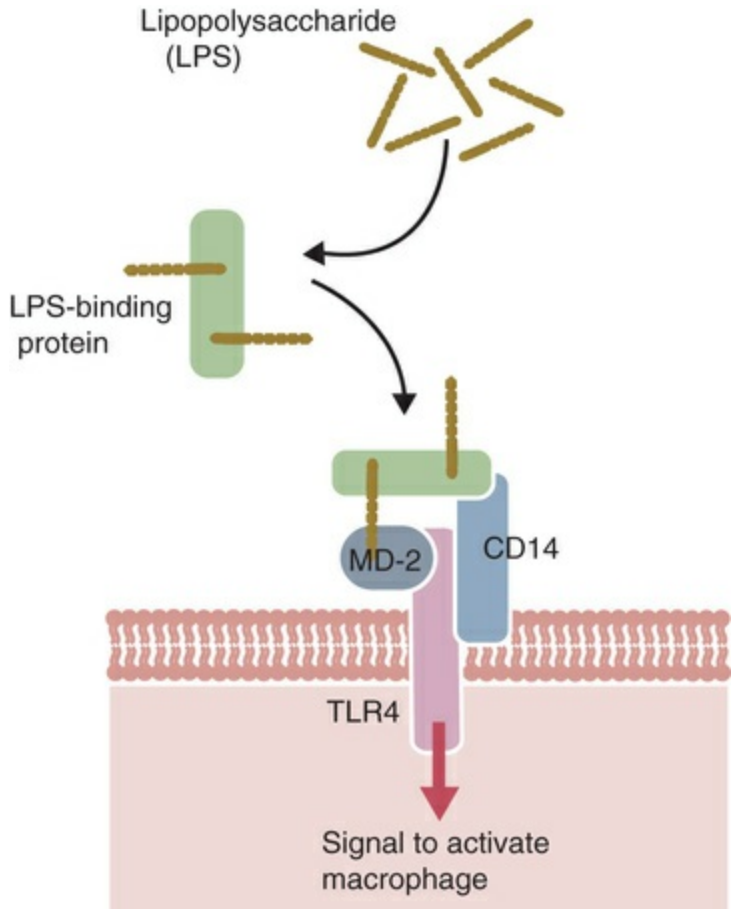


FIG. 2.7 Bacterial lipopolysaccharide cannot bind TLR4 directly. It must first bind to lipopolysaccharide-binding protein and then to two other proteins, MD-2 and CD14, before it can bind and activate cells such as macrophages.

Bacterial Peptidoglycans

Peptidoglycans are polymers of alternating *N*-acetylglucosamine and *N*-acetylmuraminic acid that are major constituents of the cell walls of both Gram-positive and -negative bacteria. PRRs that can bind these peptidoglycans include some TLRs, NODs, and CD14. Peptidoglycan recognition proteins (PGRPs) are PRRs that induce the production of proinflammatory and antimicrobial peptides. They are found in humans, mice, cattle, and pigs. In pigs, PGRPs are expressed constitutively in the skin, bone marrow, intestine, liver, kidney, and spleen. Bovine PGRP-S can kill microorganisms in which the peptidoglycan is either buried (Gram-negative bacteria) or absent *(Cryptococcus),* raising questions about its precise ligand. PGRP-S also binds bacterial lipopolysaccharides and lipoteichoic acids. It is found in neutrophil granules, and these

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neutrophils release PGRP-S when exposed to bacteria. Thus PGRP-S probably plays a significant role in the resistance of cattle to bacterial infections.

Bacterial DNA

Bacterial deoxyribonucleic acid (DNA) can stimulate innate immunity because it is structurally different from eukaryotic DNA. Much of it consists of the dinucleotide, unmethylated cytosine-guanosine (CpG). (The cytosine in eukaryotic DNA is normally methylated, but not in prokaryotes such as bacteria.) Unmethylated CpG dinucleotides bind and activate TLR9. Bacterial DNA also contains deoxyguanosine (dG) nucleotides. These dG nucleotides form molecular structures that differ from the usual DNA double helix. They also bind to TLR9 and trigger production of cytokines such as TNF-α, IL-6, and IL-12.

Viral Nucleic Acids

Viruses are simple structures, usually consisting of a nucleic acid core surrounded by a layer of proteins, the capsid, and possibly a lipid envelope (see Fig. 9.2). Viruses have few characteristic molecular signatures. However, their nucleic acids are structurally different from those in animals so they also bind to intracellular PRRs. TLR9 binds DNA from viruses and intracellular bacteria, whereas TLR7 and TLR8 bind ssRNA from viruses. TLR3, in contrast, mainly binds viral dsRNA, but it can also recognize some ssRNA and some dsDNA viruses. Intracellular RLRs also bind and respond to viral dsRNA. TLR7 and TLR9 activate the MyD88-mediated signaling pathways and trigger production of inflammatory cytokines and type I IFNs. TLR3 uses another signaling molecule, the TIR-domain-containing adaptor protein inducing IFN-β (TRIF). The TRIF pathway activates the transcription factor IRF3 that then activates the genes for inflammatory cytokines and IFN-β.

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Damage-Associated Molecular Patterns

Inflammation can be triggered not only by microbial infection but also by physical trauma and tissue damage. Thus the TLRs recognize not only PAMPs from invading microorganisms but also molecules escaping from dead, dying, and damaged tissues. These molecules, collectively called DAMPs or "alarmins," may be released when cells die (intracellular) or generated when connective tissue is damaged (extracellular) (see Fig. 2.7). Other DAMPs may be produced by stimulated sentinel cells. Some of these DAMPs have potent antimicrobial properties. Others recruit and activate cells of the innate immune system and promote adaptive immune responses (Box 2.5).

Box 2.5

How Cells Die

Cellular death is a feature of many innate and adaptive immune responses. The body has to get rid of 200 billion cells daily. Cells can die in several different ways. Irreparable damage to an essential pathway will kill a cell in an uncontrolled manner. However, there are ways in which a cell can participate in its own death. These forms of programmed cell death can benefit the body by stopping microbial infections, sparing uninfected nearby cells, and generating alarmins and inflammatory mediators, and are essentially "suicide."

Apoptosis

This is the "normal" way unwanted healthy cells are eliminated. It occurs through two pathways. An extrinsic pathway where the cell receives signals from extracellular molecules that bind to cell surface receptors. This generates a cascade of activated caspases that cause mitochondrial permeability. The intrinsic pathway is activated by internal cellular events such as DNA damage or microbial infection and also leads to mitochondrial

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permeabilization, the release of cytochrome *c* into the cytoplasm, the formation of an apoptosome and activation of lethal caspase-9. Apoptotic cells are characterized by distinctive morphological changes. They form apoptotic bodies. These cell fragments are destroyed by other phagocytes before their plasma membranes rupture and, as a result, apoptosis does not trigger inflammation.

Pyroptosis

Pyroptosis is initiated in response to infections and other irritants that generate inflammasomes. These inflammasomes activate the inflammatory caspases, caspase-1 and caspase-11. (These are distinct from the caspases that mediate apoptosis) (see Fig. 2.9). There is no loss of mitochondrial integrity, no DNA cleavage, and apoptotic bodies are not formed. Instead, the plasma membrane ruptures and releases cellular contents including IL-1 and IL-18 into the tissues and thus causes inflammation.

Necroptosis

Necroptosis is triggered by death receptors such as TNFR, nucleic acid sensors, or by TLR-signaling. They activate receptor activating kinases and form molecular complexes called necrosomes. Together with proteases and kinases, they generate a pore-forming protein that inserts itself into the cell wall and allows the escape of cellular contents. Mitochondria are not involved in necroptosis. Like pyroptosis, necroptosis causes inflammation because of the escape of intracellular DAMPs such as HMGB1 and interleukin-33 (IL-33).

Mitochondria provide a link between PAMPs and DAMPs. Mitochondria are cytoplasmic organelles that generate energy for cells. They evolved from intracellular bacteria and retain many of their original bacterial features. Indeed, in many respects they act like intracellular bacteria. For example, they have their own DNA that is rich in unmethylated CpG. When cells die, broken mitochondria may release large amounts of this DNA that can bind TLR9 and trigger inflammation. Mitochondria, like bacteria, also contain proteins with a formyl group at their amino termini. When these formylated proteins escape, they bind and activate neutrophil receptors. These neutrophils leave blood vessels and move into

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tissues such as the lung, where they release their proteases and cause damage. Thus in animals with severe trauma, mitochondrial DNA and formyl peptides are released from damaged tissues and flood into the bloodstream. The resulting inflammatory cascade is an initiating factor for the systemic inflammatory response syndrome (Chapter 7).

One of the most important intracellular DAMPs is high mobility group box protein-1 (HMGB1) (Fig. 2.8). HMGB1 normally binds DNA molecules and ensures that they fold correctly. However, HMGB1 is also a potent trigger of inflammation. It is secreted by macrophages that have been activated by lipopolysaccharides or by cytokines such as IFN-γ. HMGB1 also escapes from broken cells. HMGB1 binds both TLR2 and TLR4 and sustains and prolongs inflammation. It stimulates the secretion of inflammatory cytokines from macrophages, monocytes, neutrophils, and endothelial cells. Administration of HMGB1 to animals causes fever, weight loss, anorexia, acute lung injury, arthritis, and even death. HMGB1 stimulates the growth of new blood vessels and tissue repair. It also has potent antimicrobial activity. The cytokine IL-33 is also stored within the nucleus and released when cells die (Chapter 29). It too is a potent DAMP.

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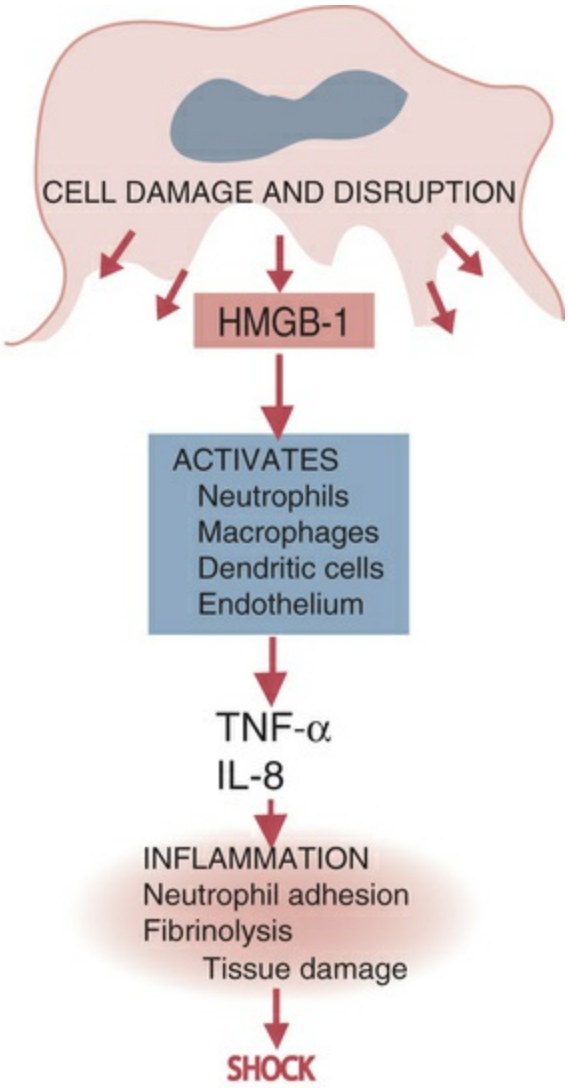


FIG. 2.8 The properties of HMGB1. Released from "broken" cells, HMGB1 activates many of the cells associated with inflammation and triggers systemic responses causing septic shock.

Many molecules released from broken cells act as intracellular DAMPs. These include adenosine and adenosine triphosphate, uric acid, S100 proteins (a family of calcium-binding proteins involved in cell growth and tissue injury), and heat-shock proteins (Chapter 26). An important extracellular DAMP is heparan sulfate. This molecule is normally found in cell membranes and the extracellular matrix but is shed into tissue fluids following injury. Heparan sulfate binds and triggers TLR4. Other extracellular DAMPs include hyaluronic acid, fibronectin, and peptides from collagen and elastin (Fig. 2.9).

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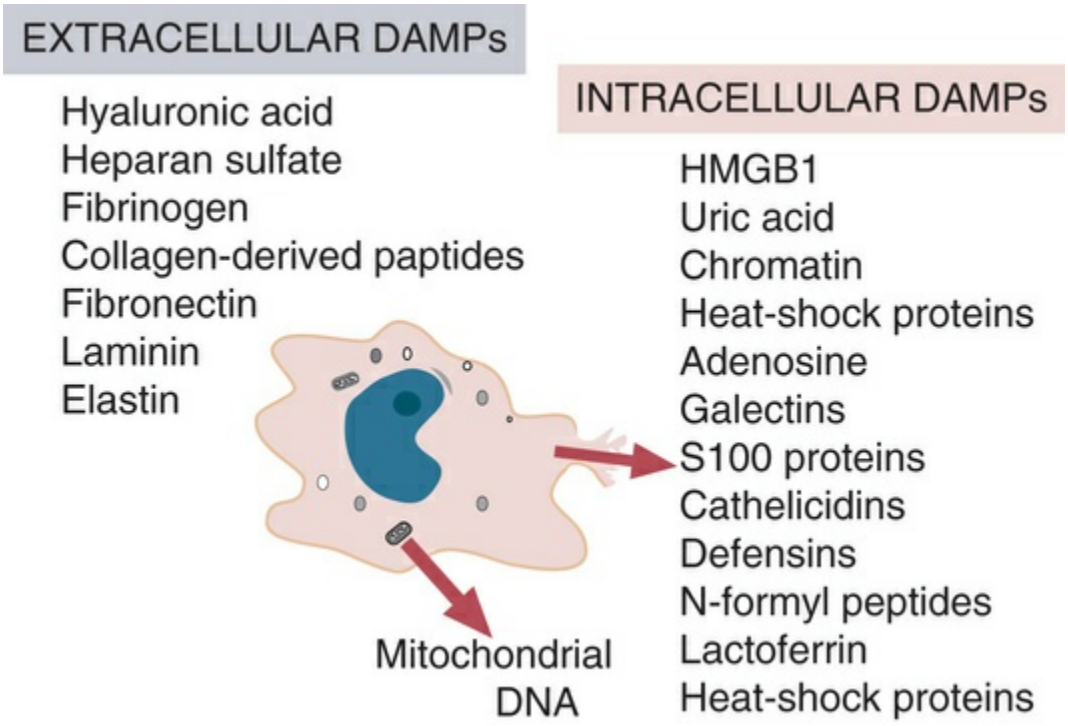


FIG. 2.9 Damage-associated molecular patterns that trigger innate immune responses. They are derived from both intracellular and extracellular sources.

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Soluble Pattern-Recognition Receptors

Although the TLRs, NLRs, and RLRs are expressed on cell surfaces, soluble PRRs also bind PAMPs. Because these molecules function in the extracellular fluid, they generally serve to promote destruction (phagocytosis) of any organisms they encounter. In general they do not induce the expression of inflammatory cytokines. That task is accomplished by the cell-surface PRRs.

Since many bacterial PAMPs are glycoproteins and polysaccharides, circulating carbohydrate-binding proteins called lectins play important roles in innate immunity. Three extracellular lectin families, the P-, S-, and C-type lectins, are involved in innate immunity.

P-type lectins are also called pentraxins. Pentraxins are formed by five protein subunits arranged in a ring. Two pentraxins, C-reactive protein (CRP) and serum amyloid P (SAP), are important acute-phase proteins (Chapter 7). (They are called acute-phase proteins because their blood levels climb greatly during acute infections or trauma.) Pentraxins have multiple biological functions, including activation of complement and stimulation of leukocytes. They bind to bacterial LPS in a calcium-dependent manner and activate the classical complement pathway by interacting with C1q (Chapter 4). They also interact with neutrophils, monocyte-macrophages, and NK cells and augment their activities.

Galectins are extracellular S-type lectins. Their name derives from their specificity for galactosides. They play a role in inflammation by binding leukocytes to the extracellular matrix.

C-type lectins (CLRs) are a large family of carbohydrate-binding proteins with many different roles. (At least 1000 have been identified.) All require calcium to bind to carbohydrates. Each end of a CLR has a distinct function; the C-terminal domain binds to carbohydrates, whereas the N-terminal domain interacts with cells or complement components, thereby exerting their biological effect. There are both soluble and membrane-bound CLRs. The most important of the soluble CLRs is mannose-binding lectin (MBL).

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MBL is found in high levels in blood. It has multiple sites that bind oligosaccharides, such as *N*-acetylglucosamine, mannose, glucose, galactose, and *N*-acetylgalactosamine. MBL thus binds strongly to bacteria such as *Salmonella enterica* and *Listeria monocytogenes.* It binds to *Escherichia coli* with moderate affinity. It binds strongly to yeasts such as *Candida albicans* and *Cryptococcus neoformans.* It can also bind viruses such as influenza A as well as parasites such as *Leishmania.* Bacteria coated by MBL are readily ingested by phagocytic cells. MBL plays an important role in activating the complement system (Chapter 4). There are two forms of MBL in the pig: MBL-A and MBL-C. These can bind to *Actinobacillus suis* and *Haemophilus parasuis.* Some European pig breeds may express very low levels of MBL-C and hence, suffer from increased disease susceptibility.

Multiple CLRs, such as the surfactant proteins SP-A and SP-D, are produced in the lungs. Six different soluble CLRs (conglutinin, MBL, pulmonary surfactant proteins [SP-A, SP-D], and collectins-46 [CL-46 and CL-43]) have been identified in mammals. However, conglutinin, CL-46, and CL-43 have been identified only in Bovidae.

Some CLRs are cell surface PRRs that can bind bacteria, fungi, and some viruses. The major ones are the dectins and DEC-205. The dectins bind β-glucans on fungal cell walls and play an important role in antifungal defense by promoting their intracellular destruction. Dectin-1 (also called CD369) is expressed by bovine macrophages, monocytes, and dendritic cells. Bovine dectin-2 is expressed on Langerhans cells in the skin (Chapter 10). DEC-205 is expressed on bovine dendritic cells. Another cell-associated lectin is the macrophage mannose receptor CD206. CD206 recognizes carbohydrates on diverse pathogens, such as the fungi *Candida albicans* and *Pneumocystis,* the protozoa *Leishmania,* and viruses such as bovine viral diarrhea virus.

Selectins are CLRs expressed on vascular endothelial cells that play a key role in the emigration of leukocytes from the bloodstream into the tissues in inflammation (Chapter 5).

Other soluble collectins include the ficolins (H-, L-, and M-ficolins), a family of lectins produced by the liver and some lung cells. These too can bind bacterial carbohydrates and activate the complement system (Chapter 4). There are also many cell-

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associated lectins. DC-SIGN, for example, is a lectin expressed on macrophages and dendritic cells (Chapter 10). Not only does DC-SIGN recognize bacterial carbohydrates but it also recognizes carbohydrates expressed on T cells. It is used by dendritic cells to interact with T cells. The collectins are especially important in the defense of young animals whose immature adaptive immune system is not capable of mounting an efficient response.

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Sentinel Cells

The cells whose primary function it is to recognize and respond to invading microbes are called sentinel cells. The major sentinel cell types, namely macrophages, dendritic cells, and mast cells, are scattered throughout the body but are found in highest numbers just below body surfaces, where invading microorganisms are most likely to be encountered. All these cells are equipped with multiple, diverse PRRs, so they can detect and then respond rapidly to both PAMPs and DAMPs. Other cells scattered throughout the body, such as epithelial cells, endothelial cells, neutrophils, and fibroblasts, may serve as sentinel cells when opportunity arises.

Macrophages

The most important sentinel cells are macrophages. Macrophages scattered throughout the body can capture, kill, and destroy microbial invaders. Macrophages are described in detail in Chapter 6.

Dendritic Cells

The second major population of sentinel cells consists of dendritic cells, so called because many possess long, thin cytoplasmic processes called dendrites that can trap invaders. Dendritic cells encompass several distinct cell types, many of which are closely related to, or derived from, macrophages. They are discussed in detail in Chapter 10.

Mast Cells

A third population of professional sentinel cells are the mast cells. These cells, strategically located close to epithelial and endothelial surfaces, are among the first to detect pathogens and danger signals. They express multiple PRRs and are packed with granules that store a complex mixture of inflammatory mediators. When released in response to appropriate stimuli, these mediators

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promote the clearance of pathogens. Long known to play a key role in allergies, it has now been recognized that they also trigger inflammation in conventional situations. Mast cells are described in detail in Chapter 29.

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Humoral Innate Immunity

Inflammatory Mediators

CHAPTER OUTLINE

Products of Sentinel Cells, 18
Cytokines, 19
Tumor Necrosis Factor-α, 19
Interleukin-1, 19
Interleukin-6, 20
Chemokines, 20
Inflammatory Mediators, 22
Vasoactive Amines, 23
Vasoactive Peptides, 23
Vasoactive Lipids, 23
The Coagulation System, 24
Antimicrobial Molecules, 24
Peptides, 24
Lysozyme, 25
Complement, 25

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LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Understand how stimulation of toll-like receptors (TLRs) and other pattern-recognition receptors (PRRs) activates sentinel cells and triggers secretion of cytokines.
- Recognize that sentinel cells and damaged cells produce many other molecules that trigger and maintain inflammation.
- Be aware that some inflammatory mediators are produced by nerves.
- Explain how these molecules trigger local increases in blood flow to cause the redness and swelling associated with inflammation.
- Discuss how these molecules also attract defensive cells such as neutrophils that can kill invading microorganisms.
- Identify the differences between acute and chronic inflammation.
- List the basic steps of the process of acute inflammation.
- Describe the basic properties and sources of interleukin-1, tumor necrosis factor α, and type I interferons.
- Explain the antimicrobial properties of defensins.
- Define cytokine, chemokine, defensin, prostanoids, prostaglandin, and kinins.
- List two important vasoactive amines.
- List the major antimicrobial peptides.

Acute inflammation develops within minutes after tissues are damaged. The damaged tissue generates three types of signal. First, broken cells release molecules (or damage-associated molecular patterns [DAMPs]) that trigger the release of cytokines, chemokines, and enzymes from sentinel cells. Second, invading microbes provide molecules (pathogen-associated molecular patterns [PAMPs]) that trigger additional sentinel cell responses. Third, pain due to tissue damage causes sensory nerves to release bioactive peptides. This complex mixture of molecules collectively attracts defensive white blood cells (leukocytes) and at the same time acts on blood vessels, resulting in increased local blood flow and leading to redness and swelling.

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Products of Sentinel Cells

Macrophages, dendritic cells, and mast cells are activated when PAMPs or DAMPs bind to their pattern-recognition receptors (PRRs). As a result, they synthesize and secrete molecules that trigger inflammation, inhibit microbial growth, and initiate the first steps in adaptive immunity. Mediators released by the sentinel cells diffuse to others nearby, where they bind to specific receptors and trigger their responses. The cells of the immune system can synthesize and secrete hundreds of different proteins that control the immune responses in this way. These proteins are called cytokines. Cytokines affect many different cell types, and cells rarely secrete a single cytokine at a time. This generates a cytokine network; a web of different signals transmitted among the cells of the immune system mediated by complex mixtures of cytokines.

Cytokines

When exposed to infectious agents or PAMPs through PRRs, sentinel cell signaling pathways activate the genes that result in the synthesis and secretion of three major cytokines. These are called tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and IL-6. TNF-α is produced very early in inflammation and is followed by waves of IL-1 and then by IL-6. Activated sentinel cells also secrete a mixture of small chemotactic proteins called chemokines. These chemokines attract defensive cells to sites of microbial invasion. At the same time, stimulated sentinel cells synthesize enzymes such as nitric oxide synthase 2 (NOS2) that in turn generates nitric oxide (NO), a powerful and lethal oxidant. They also make the enzyme cyclooxygenase-2 (COX-2) that generates inflammatory lipids such as the prostaglandins and leukotrienes. When these molecules reach the brain and liver, they cause a fever and sickness behavior and promote an acute-phase response (Chapter 7). If the sentinel cells detect the presence of damaged or foreign DNA or RNA, such as that from viruses, they will also secrete the antiviral type I interferons, IFN-α and IFN-β (Chapter 27).

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Tumor Necrosis Factor-α

TNF-α is a protein of 17 Da produced by sentinel cells in response to TLR stimulation. TNF-α can also be produced by endothelial cells, T cells, B cells, and fibroblasts. It is initially membrane-bound but then cleaved from the cell surface by a protease called TNF-α convertase. Soluble TNF-α triggers the release of chemokines and cytokines from nearby cells and promotes the adherence, migration, attraction, and activation of leukocytes (Fig. 3.1). Later, TNF-α facilitates the transition from innate to adaptive immunity. TNF-α production is stimulated not only through the TLRs but also by molecules secreted by nerves such as the neurotransmitter neurokinin-1.

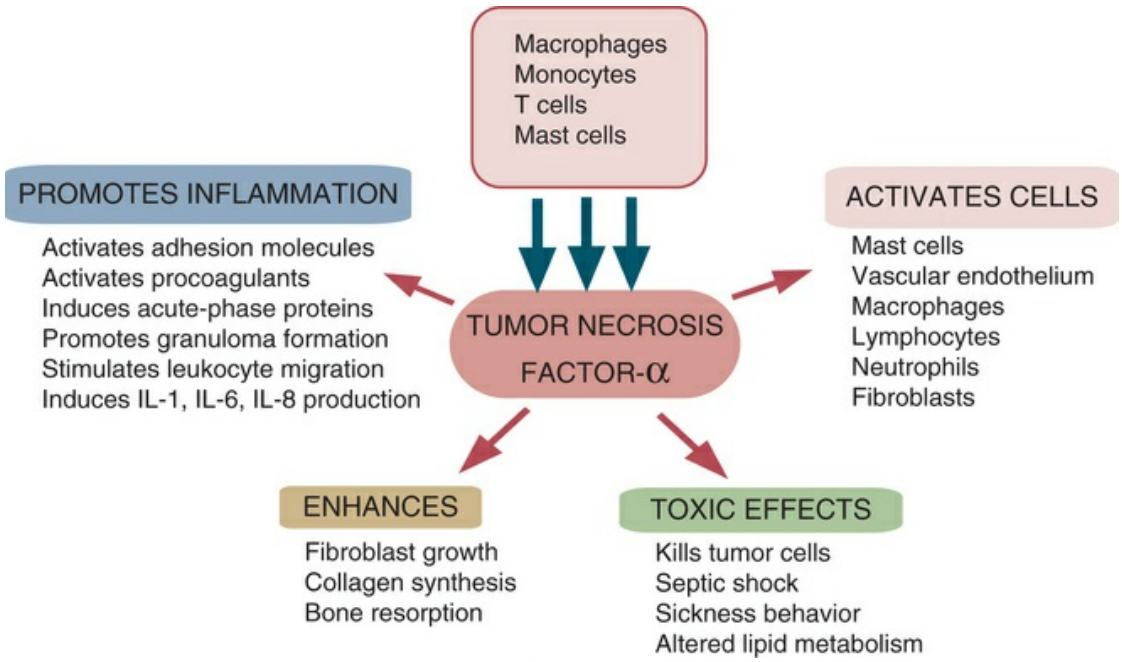


FIG. 3.1 The origins and some of the biological activities of tumor necrosis factor-α.

TNF-α is an essential mediator of inflammation because, in combination with IL-1, it triggers changes in small blood vessels. A local increase in TNF-α causes the classic signs of inflammation, including heat, swelling, pain, and redness. Circulating TNF-α can depress cardiac output, induce microvascular thrombosis, and cause capillary leakage. TNF-α acts on neutrophils (key defensive cells in inflammation; see Chapter 5) to enhance their ability to kill

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microbes. It attracts neutrophils to sites of tissue damage and increases their adherence to vascular endothelium. It stimulates macrophage phagocytosis and oxidant production. It amplifies and prolongs inflammation by promoting macrophage synthesis of important enzymes such as nitric oxide synthase and cyclooxygenase, and it also activates mast cells. TNF-α induces macrophages to increase its own synthesis together with that of IL-1. As its name implies, TNF-α can kill some tumor cells as well as virus-infected cells. In high doses, TNF-α may cause septic shock (Chapter 7). It mediates all these actions through two receptors: TNFR1 is found on many different cells, where it can bind both soluble and membrane-bound TNF-α; and TNFR2 is restricted to the cells of the immune system and responds only to membrane-bound TNF-α.

TNF-α is a member of a family of related molecules. Important members of this family include TNF-β (or lymphotoxin) (Chapter 19); CD40L (Chapter 20); and FasL (CD95L) (Chapter 18).

Interleukin-1

When stimulated through CD14 and TLR4, sentinel cells such as macrophages also synthesize cytokines belonging to the interleukin-1 family. The most important of these are IL-1α and IL-1β. IL-1β is produced as a large precursor protein that is cleaved by caspase-1 to form the active 17.5-kDa molecule. Ten- to 50-fold more IL-1β is produced than IL-1α, and whereas IL-1β is secreted, IL-1α remains attached to the cell surface. As a result, IL-1α only acts on cells that directly contact macrophages (Fig. 3.2). Transcription of IL-1β messenger RNA (mRNA) occurs within 15 minutes of ligand binding. It reaches a peak 3 to 4 hours later and levels off for several hours before declining. Like TNF-α, IL-1β acts on nearby cells to initiate and amplify inflammation. For example, it acts on vascular endothelial cells to make them adhesive for neutrophils. IL-1 also acts on macrophages to stimulate their synthesis of NOS2 and COX-2.

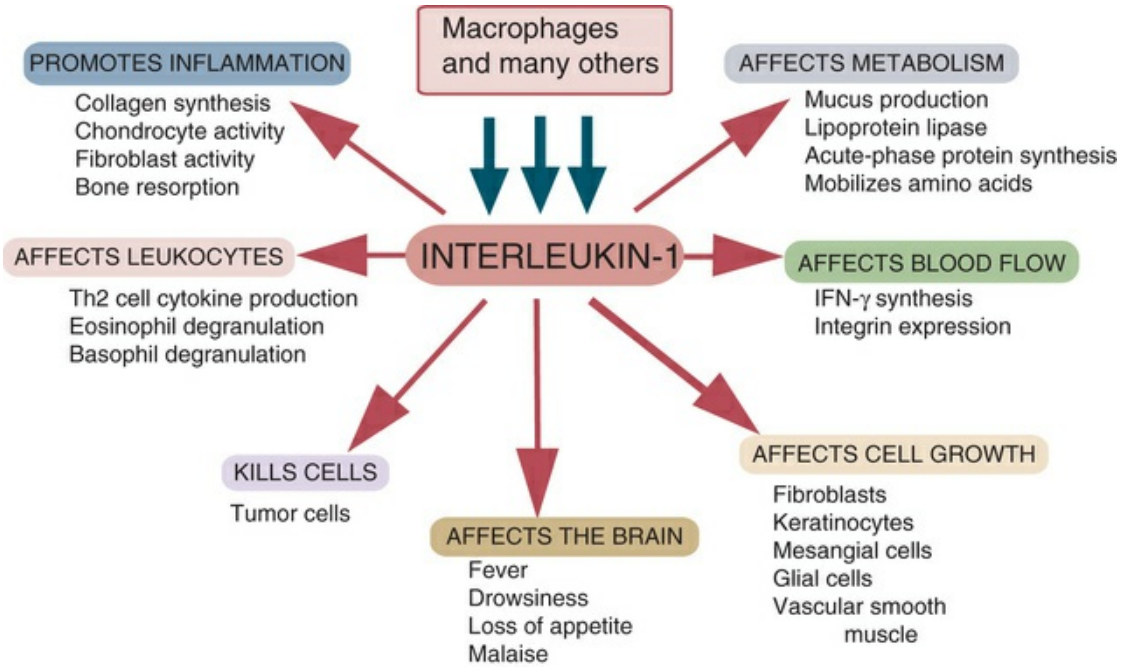


FIG. 3.2 The origins and some of the biological activities of interleukin-1.

During severe infections, IL-1β circulates in the bloodstream where, in association with TNF-α, it is responsible for sickness behavior. Thus it acts on the brain to cause fever, lethargy, and malaise. It acts on muscle cells to mobilize amino acids, causing pain and fatigue. It acts on liver cells to induce the production of new proteins, called acute-phase proteins, that also assist in the defense of the body (Chapter 7).

The most important IL-1 receptors are CD121a and CD121b. CD121a is a signaling receptor, whereas CD121b is not. CD121b thus binds IL-1, but nothing more happens. If soluble CD121b binds IL-1, it acts as an antagonist. IL-1 activity is also regulated by IL-1 receptor antagonist (IL-1RA), a protein that binds and blocks CD121a. IL-1RA is therefore an important regulator of IL-1 activity and inflammation. It reduces mortality in septic shock and graft-versus-host disease and has antiinflammatory effects (Chapter 8).

IL-1 is a member of a large family of cytokines that regulate innate immune responses. Other important family members include IL-1RA, IL-18, IL-33, IL-36, IL-37, and IL-38 (Chapter 8 and Appendix 3). All these cytokines signal through closely related receptors. Some, like IL-36, have a proinflammatory effect, while others such as IL-37 have antiinflammatory effects.

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Interleukin-6

Interleukin-6 is a glycoprotein produced by macrophages, T cells, and mast cells. Its production is triggered by bacterial endotoxins, as well as by IL-1, and TNF-α. IL-6 affects both inflammation and adaptive immunity (Fig. 3.3). It promotes some aspects of inflammation, especially in response to tissue damage and severe infections, and it is a major mediator of the acute-phase reaction and septic shock (Chapter 7). It has been suggested that IL-6 regulates the transition from a neutrophil-dominated process early in inflammation to a macrophage-dominated process later. IL-6 is also produced by muscles during exercise. IL-6 has an antiinflammatory role in that it inhibits some activities of TNF-α and IL-1 and promotes the production of IL-1RA as well as a suppressive cytokine called IL-10 (Chapter 8). The IL-6 receptor is a heterodimer consisting of two proteins, gp130 and IL-6R, found on T cells, neutrophils, macrophages, hepatocytes, and neurons.

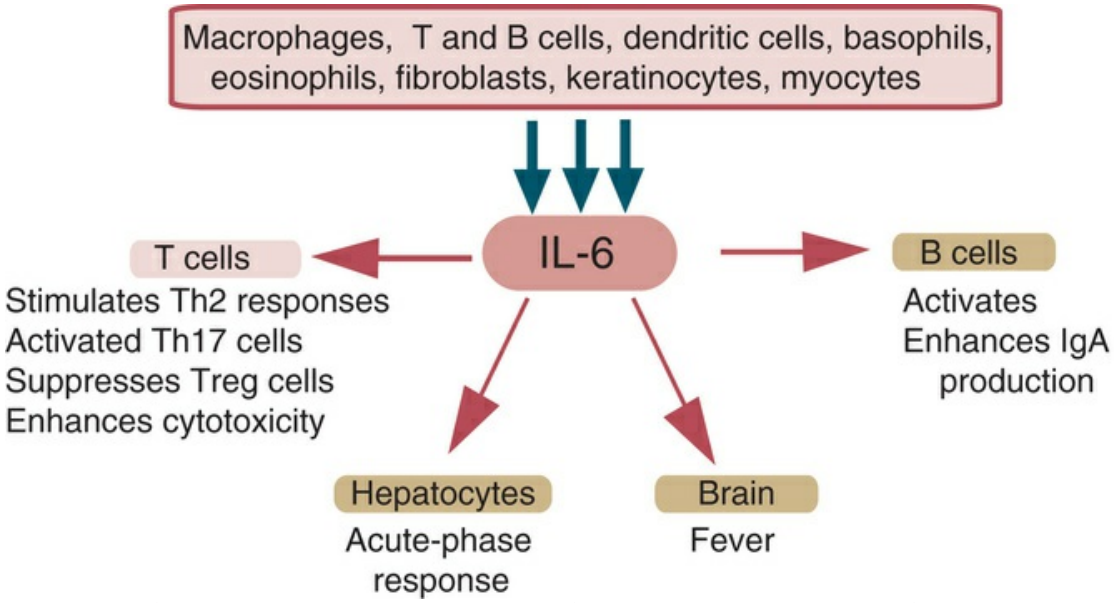


FIG. 3.3 The origins and some of the biological activities of interleukin-6.

Chemokines

Chemokines are a family of at least 50 small (8 to 10 kDa) proteins. They coordinate the migration of leukocytes and hence dictate the

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course of many inflammatory and immune responses (Table 3.1). Chemokines are produced by sentinel cells, including macrophages and mast cells. They are classified into four subfamilies on the basis of their amino acid sequences (Fig. 3.4). For example, the alpha (or CXC) chemokines have two cysteine (C) residues separated by another amino acid (X), whereas the beta (or CC) chemokines have two contiguous cysteine residues. (Chemokine nomenclature is based on this classification, with each molecule or receptor receiving a numerical designation. In addition, ligands have the suffix "L" [e.g., CXCL8], whereas receptors have the suffix "R" [e.g., CXCR1].)

TABLE 3.1

Nomenclature of Some Selected Chemokines and Their Receptors

Current Name	Alternative Name	Receptor
α Family		
CCL2	MCP-1	CCR2
CCL3	MIP-1α	CCR1, CCR5
CCL4	MIP-1β	CCR5
CCL5	RANTES	CCR1, CCR3, CCR5
CCL7	MCP-3	CCR3
CCL8	MCP-2	CCR3
CCL11	Eotaxin	CCR3
CCL13	MCP-4	CCR3
CCL20	MIP-3α	CCR6
CCL22	MDC	CCR4
CCL26	Eotaxin 3	CCR3
CCL28	MEC	CCR3
β Family		
CXCL1	GRO1	CXCR2
CXCL7	MDGF	CXCR2
CXCL8	IL-8	CXCR1, CXCR2
CXCL12	SDF	CXCR4
CXCL13	BCA-1	CXCR5
γ Family		
XCL1	Lymphotactin	XCR1
δ Family		
CX3CL1	Fractalkine	CX3CR1

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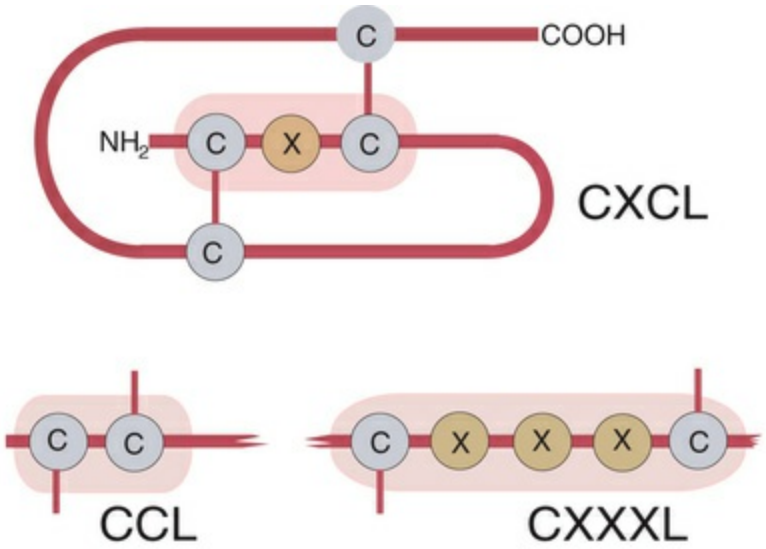


FIG. 3.4 The classification of chemokines is based on the location and spacing of their cysteine (C) residues and their separation by other (X) amino acids.

In addition to structural criteria, chemokines may be classified functionally. Thus inflammatory chemokines are upregulated in inflammation and are mainly involved in leukocyte recruitment. Others promote new blood vessel growth. Some are homeostatic and are found in normal tissues where they regulate cellular migration and homing, and many have overlapping functions.

One of the most important chemokines is CXCL8 (also called interleukin-8). CXCL8 attracts and activates neutrophils, releasing their granule contents and stimulating their respiratory burst (Chapter 5). Another important CXC chemokine is CXCL2 (macrophage inflammatory protein-2, MIP-2), which is secreted by macrophages and also attracts neutrophils.

CC chemokines act predominantly on macrophages and dendritic cells. Thus CCL3 and CCL4 (MIP-1α and -1β) are produced by macrophages and mast cells. CCL4 attracts CD4+ T cells, whereas CCL3 attracts B cells, eosinophils, and cytotoxic T cells. CCL2 (monocyte chemotactic protein-1, MCP-1) is produced by macrophages, T cells, fibroblasts, keratinocytes, and endothelial cells. It attracts and activates monocytes, stimulating their respiratory burst and lysosomal enzyme release. CCL5 (also called RANTES) is produced by T cells and macrophages. It attracts monocytes, eosinophils, and some T cells. It activates eosinophils and stimulates histamine release from basophils.

Two chemokines fall outside the CC and CXC families.

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Lymphotactin (XCL1) is a C (only one cysteine residue) or γ chemokine, that is chemotactic for lymphocytes. Its receptor is XCR1. Fractalkine (CX3CL1) is a CXXXC (two cysteines separated by three amino acids) or δ chemokine, that triggers adhesion by T cells and monocytes. Its receptor is CX3CR1.

Most chemokines are produced in infected or damaged tissues and attract other cells to sites of inflammation or microbial invasion. It is likely that the chemokine mixture produced by damaged or infected tissues regulates the precise composition of incoming inflammatory cell populations. In this way, the body can adjust the inflammatory response to optimize the destruction of different microbial invaders. Many chemokines, such as CXCL4, CCL20, and CCL5, are structurally similar to the antimicrobial proteins called defensins and, like them, have antibacterial activity.

Chemokines play a major role in infections and inflammation in domestic animals. Cattle possess fewer cytokines than humans but also have others not found in humans. For example, regakine-1 is a CC chemokine found in bovine serum that acts with CXCL8 and C5a to attract neutrophils and enhance inflammation. Chemokines can be detected in many inflammatory diseases, including bacterial pneumonia, mastitis, arthritis, and endotoxemia. Impaired neutrophil migration is associated with certain specific CXCR2 genotypes and this may result in increased susceptibility to mastitis in some cattle.

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Inflammatory Mediators

In its classic form, acute inflammation is said to have five major symptoms (or cardinal signs): heat, redness, swelling, pain, and loss of function. These symptoms result from changes in small blood vessels brought about by "vasoactive" molecules (Fig. 3.5). Immediately after injury, the blood flow through small capillaries at the injection site decreases. This gives leukocytes an opportunity to bind to the blood vessel walls. Shortly thereafter, the small blood vessels in the damaged area dilate, and blood flow to the injured tissue increases greatly.

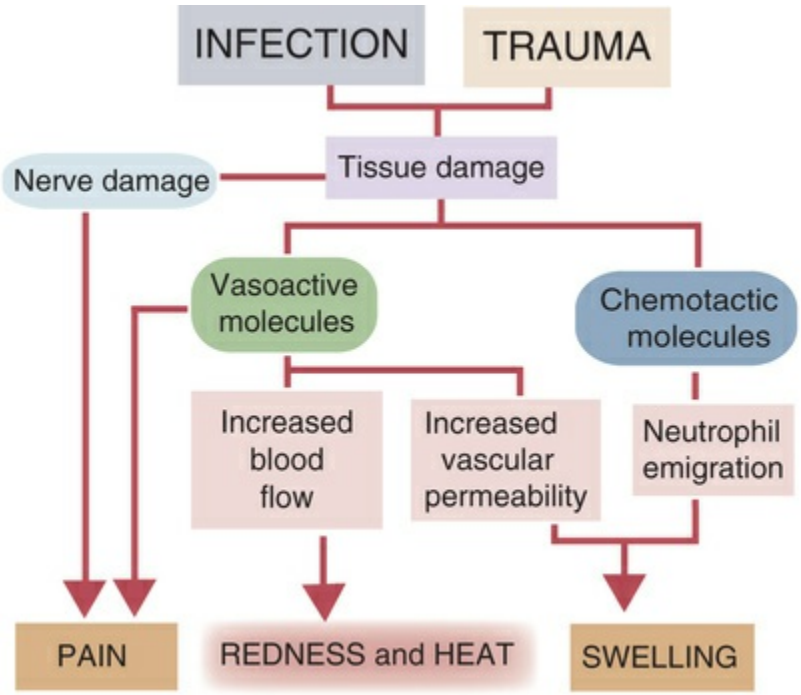


FIG. 3.5 The major signs of acute inflammation and how they are generated.

At the same time as these changes in blood flow occur, cellular responses are also taking place. Changes in nearby vascular endothelial cells permit neutrophils and monocytes to adhere. If the blood vessels are damaged, platelets may bind to injured endothelium and release vasoactive and clotting molecules. Inflamed tissues swell as a result of leakage of fluid from blood

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vessels. This leakage occurs in two stages. First, there is an immediate increase caused by vasoactive molecules produced by sentinel cells, damaged tissues, and nerves (Table 3.2). The second phase of leakage occurs several hours after the onset of inflammation, at a time when the leukocytes are beginning to emigrate. Endothelial and perivascular cells contract so that they pull apart and allow fluid and cells to escape through the intercellular spaces. After the invaders are eliminated, the inflammation is terminated, and blood flow returns to normal.

TABLE 3.2

Some Vasoactive Molecules Produced During Acute Inflammation

Mediator	Major Source	Function
Histamine	Mast cells and basophils, platelets	Increased vascular permeability, pain
Serotonin	Platelets, mast cells, basophils	Increased vascular permeability
Kinins	Plasma kininogens and tissues	Vasodilation Increased vascular permeability, pain
Prostaglandins	Arachidonic acid	Vasodilation Increased vascular permeability
Thromboxanes	Arachidonic acid	Increased platelet aggregation
Leukotriene B_4	Arachidonic acid	Neutrophil chemotaxis Increased vascular permeability
Leukotrienes C, D, E	Arachidonic acid	Smooth muscle contraction Increased vascular permeability
Platelet-activating factor	Phagocytic cells	Platelet secretion Neutrophil secretion Increased vascular permeability
Fibrinogen breakdown products	Clotted blood	Smooth muscle contraction Neutrophil chemotaxis Increased vascular permeability
C3a and C5a	Serum complement	Mast cell degranulation Smooth muscle contraction Neutrophil chemotaxis (C5a)

Vasoactive molecules come from multiple sources. Some are derived from inactive precursors in plasma. Others are derived from sentinel cells such as macrophages and mast cells; from leukocytes such as neutrophils, basophils, and platelets; or from damaged tissue cells. Stimulated sensory nerves may also produce neurotransmitters that cause vasodilation and increased permeability.

Vasoactive Amines

One of the most important of the vasoactive molecules released by mast cells is histamine (Fig. 3.6). Histamine receptors are expressed

on nerve cells, smooth muscle cells, endothelial cells, neutrophils, eosinophils, monocytes, dendritic cells, and T and B cells. When histamine binds to these receptors, it stimulates endothelial cells to produce nitric oxide, a potent vasodilator. At the same time, the histamine causes blood vessel leakage, leading to fluid escape and tissue edema. Histamine also upregulates TLR expression on sentinel cells.

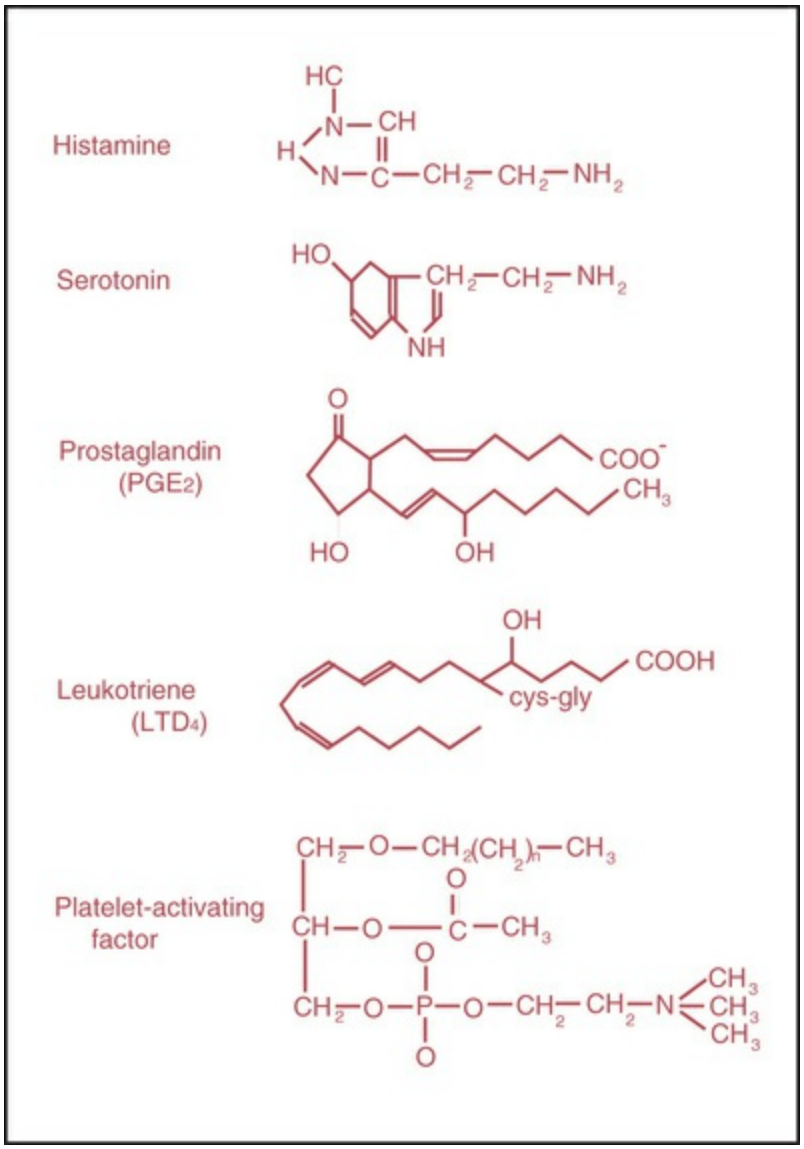


FIG. 3.6 Structure of some of the major vasoactive molecules generated during acute inflammation.

Serotonin (5-hydroxytryptamine, 5-HT), a derivative of the amino acid tryptophan, is another amine found in the mast cells of rodents

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and large domestic herbivores. Serotonin normally causes a vasoconstriction that results in a rise in blood pressure (except in cattle, in which it is a vasodilator). It has little effect on vascular permeability, except in rodents, in which it induces acute inflammation.

Vasoactive Peptides

Vasoactive peptides are generated by proteolysis of inactive precursors. For example, mast cell proteases act on the complement component C5 to generate a small (15-kDa) peptide called C5a (Chapter 4). This molecule promotes histamine release from mast cells. C5a is also a potent attractant for neutrophils and monocytes. Mast cell granules contain proteases called kallikreins. These act on proteins called kininogens to generate small vasoactive peptides called kinins. The most important of the kinins is bradykinin. Kinins not only increase vascular permeability, they also stimulate neutrophils and trigger pain receptors, and they may have defensin-like antimicrobial activity. Neuropeptides such as substance P and neurokinin produced by sensory nerves also cause pain and trigger vasodilation and increased permeability. A peptide called calcitonin gene-related peptide is the most abundant of these neurotransmitters. It is a potent, painful vasodilator.

Vasoactive Lipids

When tissues are damaged or sentinel cells stimulated, inflammasomes activate phospholipases that act on cell wall phospholipids to produce arachidonic acid. The enzyme 5-lipoxygenase then converts this arachidonic acid to biologically active lipids called leukotrienes (Fig. 3.7). Another enzyme, called cyclooxygenase, converts the arachidonic acid to a second family of vasoactive lipids called prostaglandins. The collective term for all these complex lipids is eicosanoids. They act as local inflammatory hormones.

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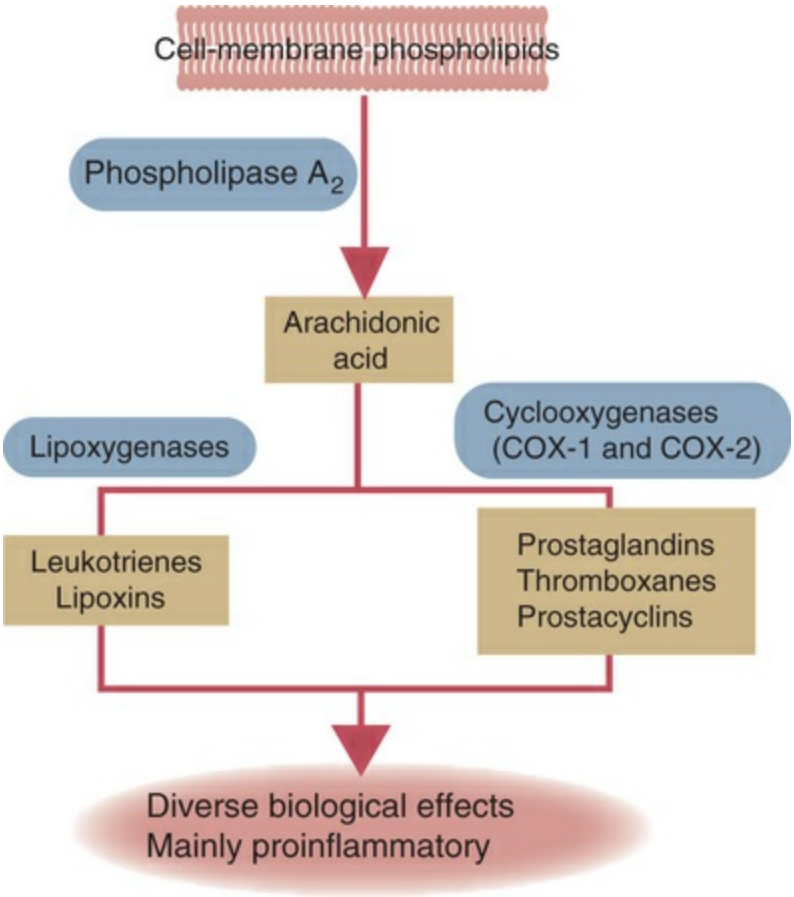


FIG. 3.7 Production of leukotrienes and prostaglandins by the actions of lipoxygenase and cyclooxygenase on arachidonic acid. Both prostaglandins and leukotrienes may have proinflammatory or antiinflammatory activity depending on their chemical structure.

Four leukotrienes promote leukocyte recruitment, survival, and activation. The most important of these, leukotriene B_4 (LTB_4) is a neutrophil attractant and activator produced by neutrophils, macrophages, and mast cells. LTB_4 also stimulates eosinophil chemotaxis and random motility. Leukotrienes C_4, D_4, and E_4, in contrast, increase vascular permeability and cause slow smooth muscle contraction. All three contain the amino acid cysteine conjugated to the lipid backbone, so they are called cysteinyl leukotrienes. They are produced by mast cells, eosinophils, and basophils. The cytokine interleukin-13 upregulates production of LTD_4 and its receptor, whereas LTD_4 upregulates IL-13 production. This feedback loop is a major contributor to severe inflammation.

There are four groups of proinflammatory prostaglandins: PGE_2, PGF_2, the thromboxanes (TxA_2, PGA_2), and the prostacyclins (PGI_2). Although prostaglandins can be generated by most nucleated cells,

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the prostacyclins are produced by vascular endothelial cells and the thromboxanes come from platelets. The biological activities of the prostaglandins vary widely, and since many different prostaglandins are released in inflamed tissues, their net effect on inflammation may be complex.

As neutrophils enter inflamed tissues, their 15-lipoxygenase generates lipoxins from arachidonic acid. These oxidized eicosanoids inhibit neutrophil migration. Thus, as inflammation proceeds, there is a gradual switch in production from proinflammatory leukotrienes to antiinflammatory lipoxins. The rise in PGE_2 in tissues also inhibits 5-lipoxygenase activity and eventually suppresses inflammation.

Activated neutrophils, mast cells, platelets, and eosinophils also produce a phospholipid called platelet-activating factor (PAF). PAF makes endothelial cells even stickier and thus enhances neutrophil adhesion and emigration. PAF aggregates platelets and makes them release their vasoactive molecules and synthesize thromboxanes. It acts on neutrophils in a similar fashion. Thus it promotes neutrophil aggregation, degranulation, chemotaxis, and release of oxidants.

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The Coagulation System

When blood vessels dilate and fluid leaks from the bloodstream into the tissues, the blood coagulation system is activated. Platelet aggregation accelerates this process. Activation of the coagulation system generates large quantities of thrombin, the main clotting enzyme. Thrombin acts on fibrinogen in tissue fluid and plasma to produce insoluble fibrin. Fibrin is therefore deposited in inflamed tissues, where it forms a barrier to the spread of infection. Activation of the coagulation cascade also initiates the fibrinolytic system. This leads to activation of plasminogen activator, which in turn generates plasmin, a potent fibrinolytic enzyme. In destroying fibrin, plasmin releases peptide fragments that attract neutrophils.

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Antimicrobial Molecules

The products of sentinel cells do two things: they increase vascular permeability and blood flow and at the same time attract white blood cells (leukocytes) to sites of microbial invasion and/or tissue damage. These leukocytes initially consist primarily of neutrophils, but these are followed by a wave of macrophages (Chapter 6). Their function is to kill microbial invaders as fast and as completely as possible. To this end, these cells produce a multitude of antimicrobial molecules.

Peptides

Antimicrobial peptides are widely distributed throughout the plant and animal kingdoms, and more than 800 have been identified to date. They include defensins, cathelicidins, C-type lectins, and S100 proteins. Different animal species employ their own specific set of peptides that have evolved in response to microbial invasion (Box 3.1). Although structurally diverse, these peptides usually contain multiple arginine and lysine residues, making them cationic, and they can form amphipathic structures; that is, they have both hydrophobic and hydrophilic regions. The hydrophobic regions can bind and insert themselves into the lipid-rich membranes of bacteria, whereas the other regions form channel-like pores or simply cover the membrane. This results in bacterial disruption and death. These cationic antimicrobial peptides kill both Gram-positive and -negative bacteria as well as some fungi, protozoa, enveloped viruses, and tumor cells. However, specific defensins may have different antimicrobial specificities.

Box 3.1

The Big Picture

The complete bovine genome has been sequenced, and unexpectedly, it was found to contain unusually large numbers of genes associated with innate immunity. For example, cattle have 10 cathelicidin genes, compared with only 1 in humans and mice.

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They have about 106 defensin genes, compared with 30 to 50 in humans and mice. They have many more interferon genes than other species, including a hitherto undescribed family, IFN-X (Chapter 27). It has been suggested that this duplication and divergence of genes involved in innate immunity may be a consequence of the huge load of microorganisms in the rumen and the resulting increased need to prevent microbial invasion. Alternatively, these new genes may be necessary since living within dense herds may promote infectious disease transmission between individuals and thus requires a more effective immune system. Additionally, cattle show substantial differences from other mammals in the genes related to lactation. Many of these lactation-associated genes, such as those for serum amyloid A, β_2-microglobulin, and the cathelicidins, are also related to innate immunity. Finally, the cattle genome contains 10 lysozyme genes, largely expressed in the abomasum and gastrointestinal tract. It is speculated that they may play a role in killing bacteria entering the intestine from the rumen.

Data from Elsik CG, Tellam RL, Worley KC, et al: The genome sequence of taurine cattle: a window to ruminant biology and evolution, *Science* 324:522-527, 2009.

Antimicrobial peptide production is concentrated in sites where microbes are most likely to be encountered. These include organelles within neutrophils and macrophages (Chapter 5) and sites within secondary lymphoid organs (Chapter 12). Epithelial cells of the skin (keratinocytes) and respiratory, alimentary, and genitourinary tracts also synthesize many antimicrobial peptides.

The defensins are typical antimicrobial peptides containing 28 to 42 amino acids arranged in a β-sheet that contains three or four disulfide bonds. More than 50 different mammalian defensins have been identified. The vertebrate defensins are classified as α-, β-, or θ- defensins based on their origin and on the number and position of these disulfide bonds. The α-defensins account for about 15% of the total protein in neutrophil granules. In cattle, at least 13 different α-defensins are produced by neutrophils alone. They are also found in the granules of Paneth cells in the small intestine (see Fig. 22.7). The β-defensins are expressed in the epithelial cells that line the airways, skin, salivary gland, and urinary system. Theta

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defensin is a circular peptide found only in primate neutrophils. Defensins may be produced at a constant rate (constitutively) or in response to microbial infection. Some defensins attract monocytes, immature dendritic cells, and T cells. All defensins identified so far can kill or inactivate some bacteria, fungi, or enveloped viruses. They act by disrupting the microbial cell membrane or envelope. Some defensins can neutralize microbial toxins such as those from *Bacillus anthracis, Corynebacterium diphtheriae,* and staphylokinase from *Staphylococcus aureus.* Although present in normal tissues, defensin concentrations increase in response to infections. For example, calves infected with *Cryptosporidium parvum* or *Mycobacterium paratuberculosis* show a significant increase in cryptdin production. *Mannheimia hemolytica* infection in bovine lungs induces increased defensin expression in airway epithelium. The equine defensin DEFA1 is an enteric defensin exclusively produced in Paneth cells. It has potent activity against the major horse pathogens, especially *Rhodococcus equi* and *Streptococcus equi.*

The second major group of antibacterial peptides found in neutrophil granules are the cathelicidins. These are 12 to 80 amino acids in size. They are stored in an inactive form attached to a precursor and released from cells following cleavage of the precursor. They are named using acronyms or amino acid symbols followed by the number of amino acids they contain. Humans and mice have only one cathelicidin gene, whereas the pig, cow, and horse have multiple cathelicidin genes. Porcine cathelicidin PR-39 has been shown to promote wound repair, angiogenesis, and neutrophil chemotaxis. (Because of their high cationic charge, porcine cathelicidins efficiently bind bacterial nucleic acids. They can then deliver these nucleic acids to dendritic cells [Chapter 10] and as a result, trigger a very strong IFN-α response.) The bovine cathelicidin BMAP-28 induces apoptosis in some cells and may serve to get rid of unwanted cells. Canine cathelicidin K9CATH has broad-spectrum activity against both Gram-positive and Gram-negative bacteria. Many cathelicidins have been given specific names such as protegrins, novispirin, and ovispirin.

Serprocidins are antimicrobial serine proteases found in the granules of neutrophils. Granulysins are peptides produced by cytotoxic T cells and natural killer (NK) cells (Chapters 18 and 19).

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In addition to their antibacterial functions, granulysins attract and activate macrophages. Two other important antibacterial proteins are bactericidal permeability-increasing protein (BPI) and calprotectin. BPI is a major constituent of the primary granules of human and rabbit neutrophils. It kills Gram-negative bacteria by binding to lipopolysaccharides and damaging their inner membrane. Calprotectin is found in neutrophils, monocytes, macrophages, and epidermal cells. It forms about 60% of neutrophil cytosolic protein and is released in large amounts into blood and tissue fluid in inflammation. It belongs to the S100 family of antimicrobial proteins. Calprotectin sequesters zinc and manganese during bacterial infections and thus makes them unavailable for bacterial growth.

The production of some antimicrobial proteins by epithelial cells is regulated by cytokines. In particular, the two cytokines produced by Th17 cells, IL-17 and IL-22, are crucial regulators of antimicrobial peptide production in the intestine and lungs (Chapter 22). Likewise, IL-1 stimulates epithelial cells to produce antimicrobial proteins. Antimicrobial peptides regulate cytokine production and can serve as immunomodulators. For example, lactoferrin stimulates macrophage production of IL-18, whereas some cathelicidins stimulate production of IL-6, IL-8, and IL-10.

Lysozyme

The enzyme lysozyme cleaves the bond between *N*-acetyl-muraminic acid and *N*-acetylglucosamine and destroys cell wall peptidoglycans in Gram-positive bacteria. Lysozyme is found in all body fluids except cerebrospinal fluid and urine. It is present in large amounts in inflammatory tissue fluid. While absent from bovine neutrophils and tears, it is present in high concentrations in the tears of other mammals. Although many of the bacteria killed by lysozyme are nonpathogenic, it might reasonably be pointed out that this susceptibility could account for their lack of pathogenicity. Lysozyme is found in high concentrations in neutrophil granules and accumulates in areas of acute inflammation, including sites of bacterial invasion. Lysozyme is also a potent opsonin, binding to bacterial surfaces and facilitating phagocytosis in the absence of

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specific antibodies and under conditions in which its enzyme activity is ineffective (Chapter 5).

Complement

The complement system is an essential innate defense subsystem that consists of a complex mixture of enzymes, regulatory proteins, and receptors that plays a major role in innate antimicrobial immunity. This system, described in detail in the next chapter, can be activated simply by exposing microbial cell walls to serum proteins since the complement components can also recognize PAMPs. It can also be activated when antibodies bind to microbial cell walls. Once activated, complement components, especially C3, the third component, bind irreversibly to bacteria and initiate bacterial killing or phagocytosis.

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Humoral Innate Immunity

The Complement System

CHAPTER OUTLINE

Complement Proteins, 27
Activation Pathways, 27
The Alternative Pathway, 27
The Lectin Pathway, 29
The Classical Pathway, 30
The Amplification Pathway, 31
Regulation of Complement Activation, 32
Complement Receptors (CR), 32
Other Consequences of Complement Activation, 33
Opsonization, 33
Removal of Apoptotic Cells, 33
Inflammation, 33
Blood Coagulation, 33
Chemotaxis, 34
Immune Regulation, 34
Complement Genes, 35
Complement Deficiencies, 35

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Canine C3 Deficiency, 35
Porcine Factor H Deficiency, 36
Other Complement Deficiencies, 37

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Explain why the complement system is one of the most important innate defense systems.
- Describe the three different pathways by which the complement system is activated: two innate pathways, the alternative pathway and the lectin pathway; and one adaptive pathway, the classical pathway.
- Explain how the innate complement pathways are activated by PAMPs, while the classical pathway is activated when antibodies bind foreign antigens.
- Explain the significance of complement components, especially C3b, binding covalently (and hence irreversibly) to invading microbes and opsonizing them.
- Explain how terminal complement complexes are constructed and their biological significance.
- Understand how the complement system triggers inflammation by generating a potent chemoattractant called C5a.
- Explain why deficiencies of some complement components result in increased susceptibility to infections.
- Define complement, properdin, and anaphylatoxins.

The complement system is an essential innate defense system. Although its main role is to kill pathogens immediately when they enter the body, the complement system also alerts the immune systems to the presence of invaders, regulates inflammation, removes damaged or altered cells, and regulates adaptive immune responses. It is involved in the clearance of antigen-antibody complexes, blood vessel formation, mobilization of stem cells, tissue

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regeneration, and lipid metabolism.

Protection from infection requires that the body respond to invaders as rapidly as possible. A critical component of this early response is the complement system. It can detect and kill invaders before other defenses have had a chance to respond. The complement system is a network of interacting pattern-recognition proteins, proteases, serum proteins, receptors, and regulators that kills invaders fast (Fig. 4.1). The major complement proteins bind covalently (and hence irreversibly) to the surface of invading microbes and then destroy them. The complement system is activated by the presence of either pathogen-associated molecular patterns (PAMPs) or by antigen-bound antibodies. Because the complement system is so potent, it must be tightly regulated and controlled. Disturbances in this system may result in inflammation or autoimmunity.

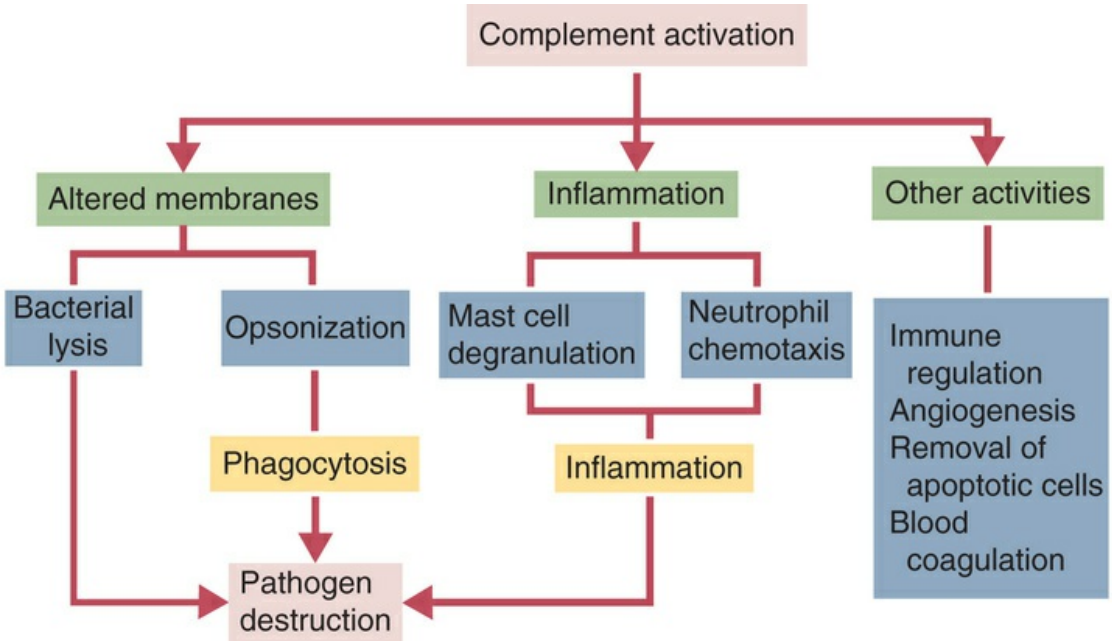


FIG. 4.1 The functions of the complement system. Complement may either alter microbial membranes or trigger inflammation. Either way, it hastens the elimination of microbial invaders and is thus a key component of the innate immune system. It has multiple other functions as well as those noted here.

The complement system consists of multiple proteins that are activated in sequence. Once activated, the system generates multiple effector molecules. The first step, complement activation,

occurs by three different pathways, referred to as the alternative, the lectin, and the classical pathways (Fig. 4.2). The alternative and lectin pathways are activated by microbial carbohydrates and are thus typical pattern-recognition pathways that trigger innate responses. The classical pathway, in contrast, is activated by antibodies and thus works in association with adaptive immune responses.

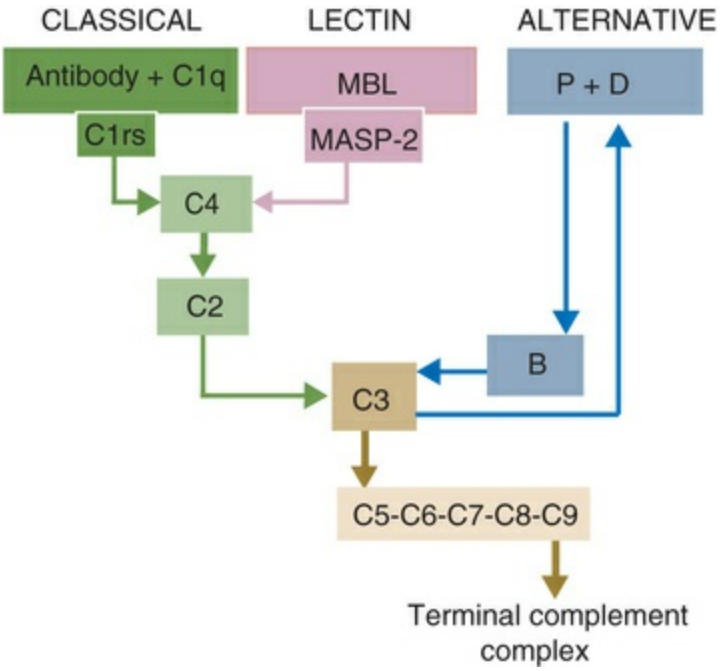


FIG. 4.2 The three pathways by which the complement system can be activated. The alternate and lectin pathways are innate, the classical pathway is adaptive since it is triggered by antibodies. All three activate C3, which in turn initiates the amplification pathway and generates the terminal complement complex.

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Complement Proteins

Complement proteins are either labeled numerically with the prefix C (e.g., C1, C2, C3) or designated as "factors" by letters of the alphabet (FB, FD, FP, and so forth). Some are found free in serum, whereas others are cell-surface receptors. Collectively, complement components account for 5% to 10% of the proteins in blood serum—a reflection of the critical importance of this system. Their size varies from 24 kDa for factor D (FD) to 460 kDa for C1q. Their serum concentrations in humans vary between 20 μg/mL of C2 and 1300 μg/mL of C3 (Table 4.1). Complement proteins are synthesized at multiple sites. C3, C6, C8, and FB are made in the liver, whereas C2, C3, C4, C5, FB, FD, FP, and FI are made by macrophages. C1q is produced by mast cells. Neutrophil granules may store large quantities of C6 and C7. As a result, these proteins are readily available for defense at sites where neutrophils accumulate.

TABLE 4.1

Complement Components

Name	MW (kDa)	Serum Concentration (mg/mL)
Classical Pathway		
C1q	460	80
C1r	83	50
C1s	83	50
C4	200	600
C2	102	20
C3	185	1300
Alternate Pathway		
FD	24	1
FB	90	210
Terminal Components		
C5	195	70
C6	120	65
C7	120	55
C8	160	55
C9	70	60
Control Proteins		
C1-INH	105	200
C4BP	550	250
FH	150	480
FI	88	35
Ana INH	310	35
FP	4 × 56	20
S	83	500

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Activation Pathways

The Alternative Pathway

The alternative pathway is an evolutionary ancient innate pathway. It is triggered when microbial cell walls meet complement proteins in the blood.

The most important complement protein is called C3. C3 is a disulfide-linked heterodimer with α and β chains. It is synthesized by liver cells and macrophages and is the most abundant complement component in serum. C3 has a reactive thioester side-chain, that, when activated, binds to microbes and marks them for destruction by immune cells. Activation of this thioester side-chain must be carefully regulated to ensure that C3 does not bind to normal cells. To prevent such accidents, the thioester group in inactive C3 is hidden within the folded molecule like a pocket knife.

In healthy normal animals, C3 spontaneously breaks down into two fragments called C3a and C3b (Fig. 4.3). This breakdown exposes the reactive thioester group in C3b. The thioester then generates a carbonyl group that covalently binds the C3b to carbohydrates and proteins on nearby cell surfaces (Fig. 4.4). The breakdown of C3 also exposes binding sites for a protein called factor H (FH). When FH binds to these sites, a protease called factor I (FI) can then cleave the C3b, preventing further activation and generating two fragments, iC3b and C3c. iC3b binds receptors on circulating leukocytes (Fig. 4.5). It stimulates these cells to engulf pathogens and it activates inflammatory cells. The final breakdown product of C3, C3dg, targets pathogens to surface receptors on B cells and so promotes antibody production (Chapter 15).

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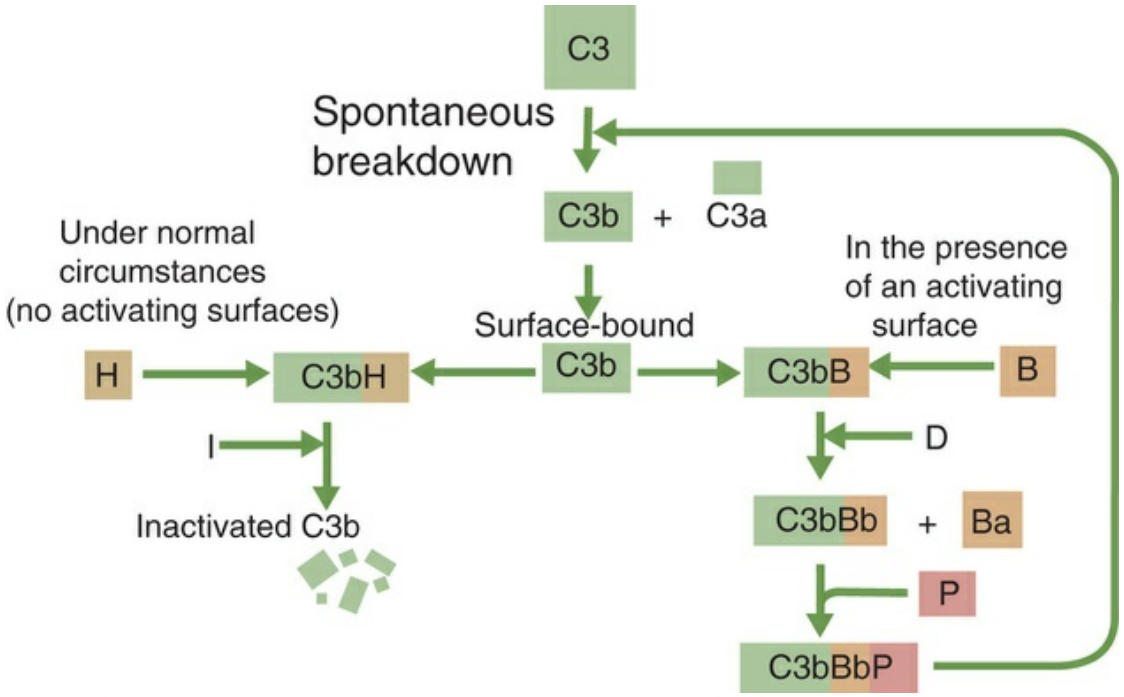


FIG. 4.3 The alternative complement pathway. Surface-bound C3b may either be destroyed, as normally happens, or activated by the presence of an activating surface.

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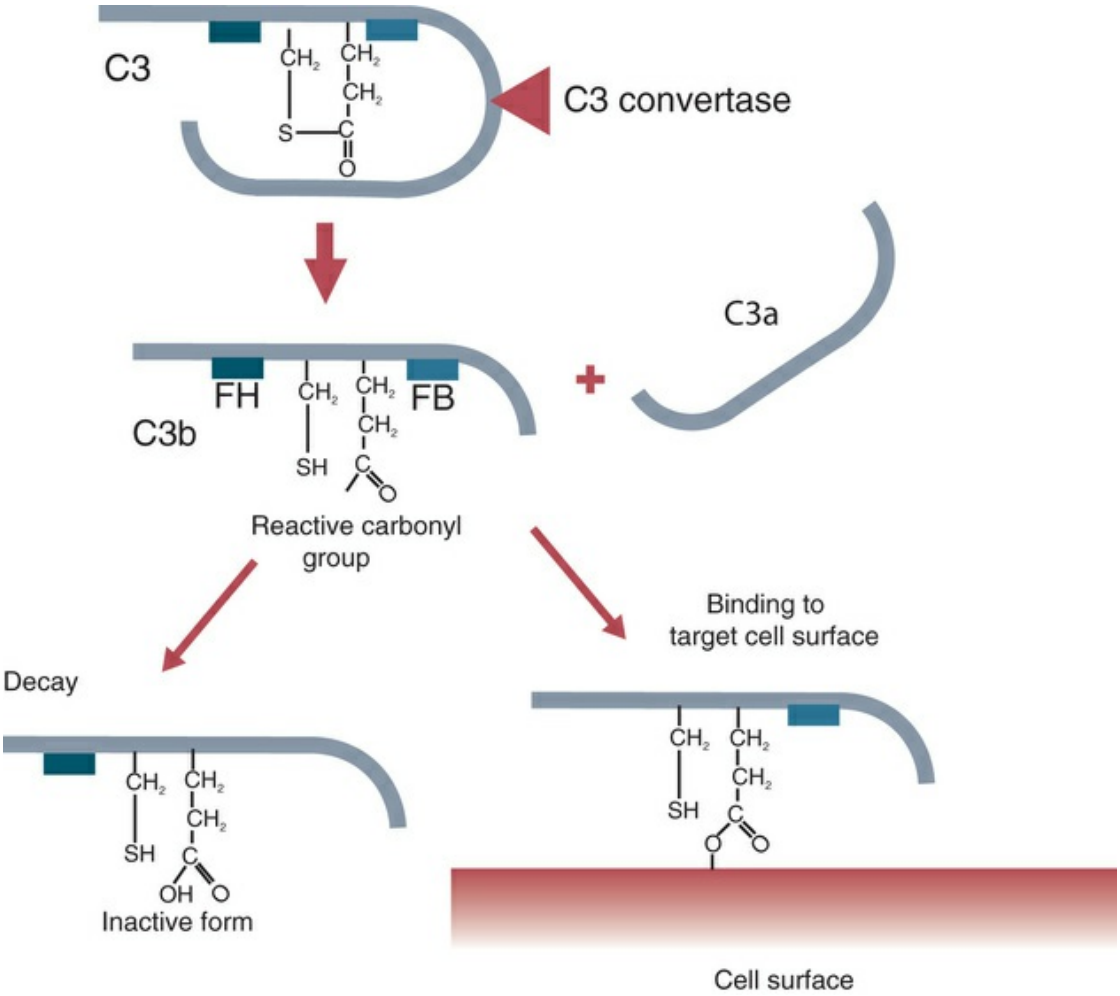


FIG. 4.4 Activation of C3 involves its cleavage by C3 convertase. This exposes a thioester bond between a cysteine and a glutamine. This bond breaks to form a reactive carbonyl group that enables the molecule to bind covalently (and hence irreversibly) to target cell surfaces. Removal of C3a also reveals the binding sites for FH and FB.

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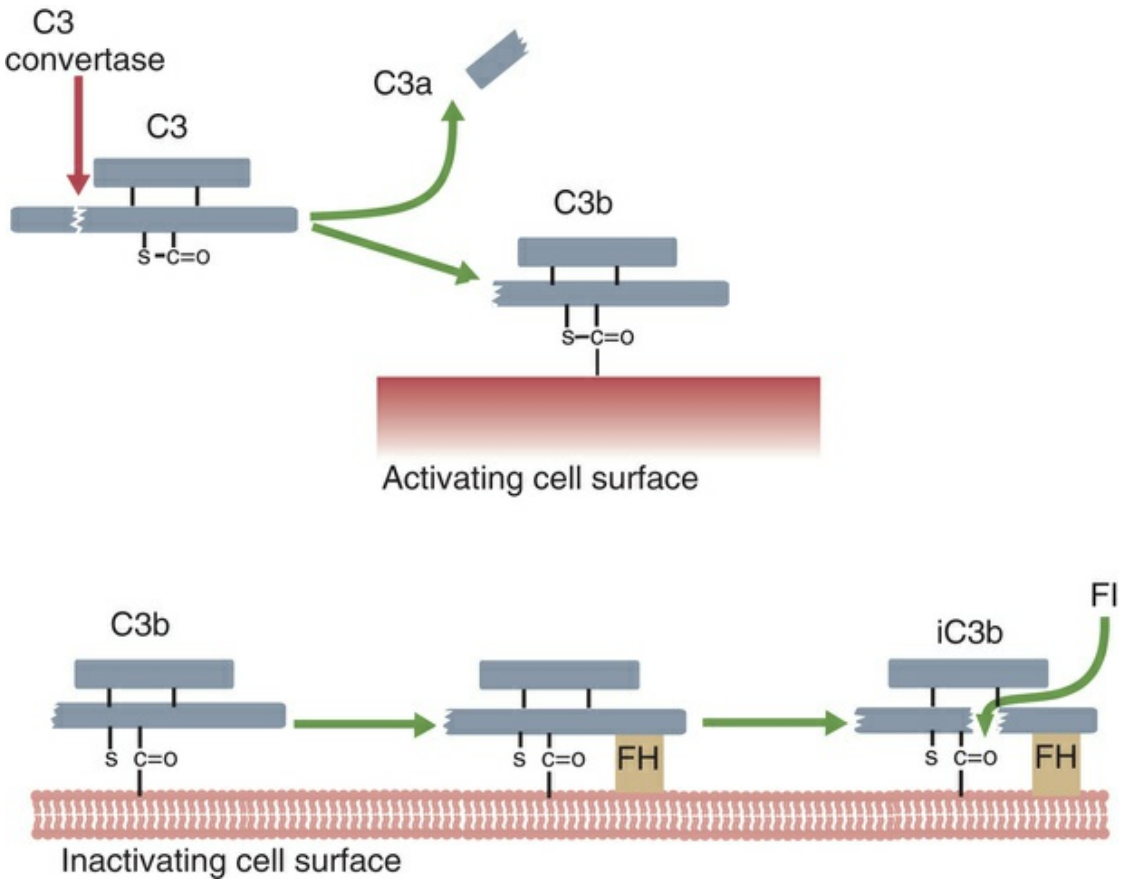


FIG. 4.5 Activated C3 binds to a cell surfaces. This C3b is normally inactivated by the actions of FH and FI. However, FH must first be activated by binding to the surface. In the absence of FH, FI will not work. In this case, C3b persists and activates the terminal complement pathway.

The consequences of the breakdown of cell-bound C3b depend on FH binding. This binding depends in turn on the nature of the target surface. When FH interacts with normal cells, glycoproteins rich in sialic acid (*N*-acetylneuraminic acid) and other neutral or anionic polysaccharides enhance FH binding to C3b. As a result, FI is activated, and the C3b is destroyed. In a healthy animal, therefore, there is a continuous low-level activation of C3, but FH and FI destroy C3b as fast as it is generated.

In contrast, bacterial cell walls lack sialic acid. When C3b binds to bacteria, FH cannot bind, FI is inactivated, and the C3b remains attached to the microbial surface. This bound C3b exposes a binding site for another complement protein called factor B (FB) and as a result, a complex called C3bB forms. The bound FB is then cleaved by a protease called factor D (FD), releasing a soluble fragment called Ba and leaving C3bBb attached to the bacteria. This

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C3bBb complex is a protease whose preferred substrate is C3. (It is therefore called the alternative C3 convertase.) FD can act only on FB after it has bound to C3b but not before. This constraint is called substrate modulation and regulates several reactions in the complement pathways. It ensures that the activities of enzymes such as FD are confined to the correct molecules.

The alternative C3 convertase, C3bBb, cleaves bound C3 to generate more C3b. C3bBb is, however, very unstable, with a half-life of only 5 minutes. If a protein called factor P (FP or properdin) binds to the complex, it forms a stable C3bBbP complex with a half-life of 30 minutes. Since C3b generates more C3bBbP, the net effect of all this is that a positive feedback loop is established where increasing amounts of C3b are produced and irreversibly bound to the surface of the invading organism. Despite its name, the alternative complement pathway accounts for 80% to 90% of all complement activation.

The Lectin Pathway

A second method of activating complement is triggered by the binding of soluble pattern-recognition molecules (lectins) to microbial carbohydrates. When these lectins bind to microbes, they activate proteases that activate complement. Like the alternative pathway, this is an innate pathway triggered simply by the presence of bacterial PAMPs (Fig. 4.6).

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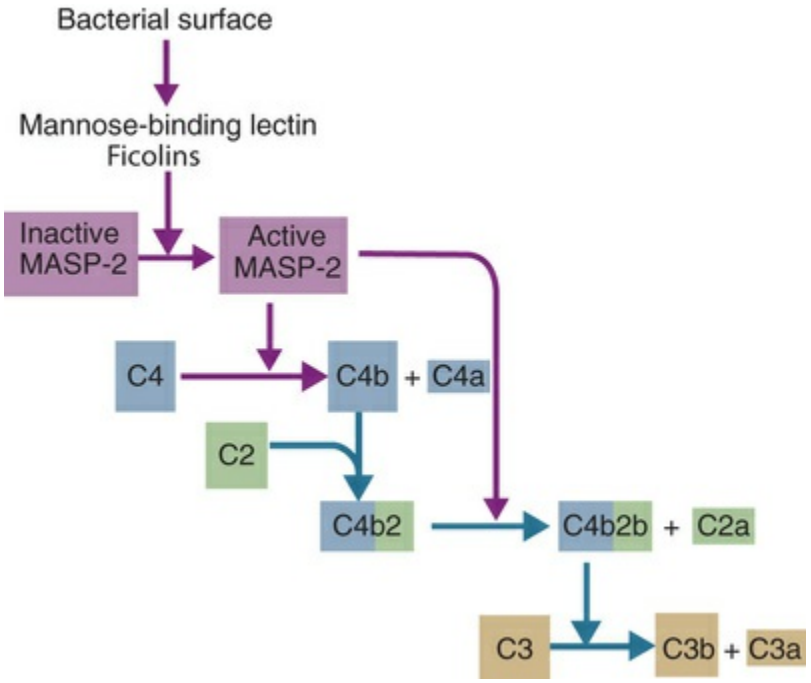


FIG. 4.6 Complement activation by the lectin pathway.

Complement-activating lectins include mannose-binding lectin (MBL) and a family of proteins called ficolins. MBL attaches to mannose or *N*-acetylglucosamine on the walls of bacteria, fungi, and protozoa. It does not bind to mammalian glycoproteins. Once bound, MBL activates a serum protease called MASP-2. (MASP stands for MBL-associated serine protease.) Activated MASP-2, in turn, acts on the complement component C4, splitting it into C4a and C4b. This exposes a thioester group on the C4b that generates a reactive carbonyl group that binds the C4b to the microbial surface (Fig. 4.7). Another complement component, C2, then binds to the C4b to form a complex, C4b2. The bound C2 is then cleaved by MASP-2 to generate C4b2b (Box 4.1).

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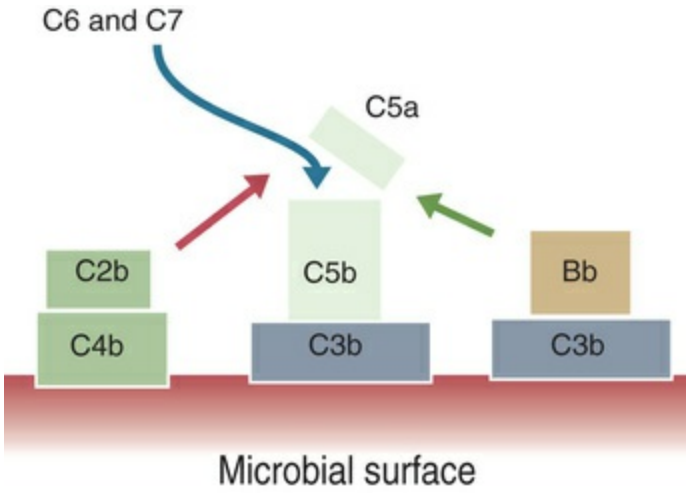


FIG. 4.7 The two C3 convertases, C4b2b and C3bBb, act on C5 when it is linked to C3b and cleave off a small peptide called C5a. In so doing they reveal a site that binds C6 and C7.

Box 4.1

The Defense Collagens

C1q and mannose-binding lectin (MBL) are members of a unique protein family called the defense collagens. Other members of this family include surfactant protein A, adiponectin, conglutinin, and ficolin. These are soluble lectins characterized as containing a conserved collagen-like region as well as a carbohydrate recognition domain. Like C1q, they commonly polymerize. These proteins serve as soluble pattern-recognition receptors. They can bind to foreign pathogens and subsequently interact with phagocytic cells or complement. Thus MBL recognizes mannose-containing carbohydrates. On binding to their ligands, they trigger an immediate protective response such as activation of complement or promotion of phagocytosis.

Cell-bound C4b2b is a protease that acts on C3 to generate C3a and C3b and exposes the thioester group on the C3b. The activation of C3b by C4b2b is a major step because each C4b2b complex can generate as many as 200 C3b molecules. Since these reactions are confined to the microenvironment close to microbial surfaces, newly formed C3b will bind to nearby microbes. The bound C3b can also bind C5 and cleave it to generate C5a and C5b. The

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complement pathway then can proceed to completion and the killing of the organism by terminal complement complexes.

The lectin pathway is ancient, having existed for at least 300 million years (It is present in many invertebrates (see Chapter 43). Although in many ways it duplicates the alternative pathway, it is an example of the way the body uses redundant mechanisms to ensure protection.

The Classical Pathway

The classical complement pathway (Fig. 4.8) is triggered when the complement component C1q encounters antibody molecules bound to an invading microorganism.

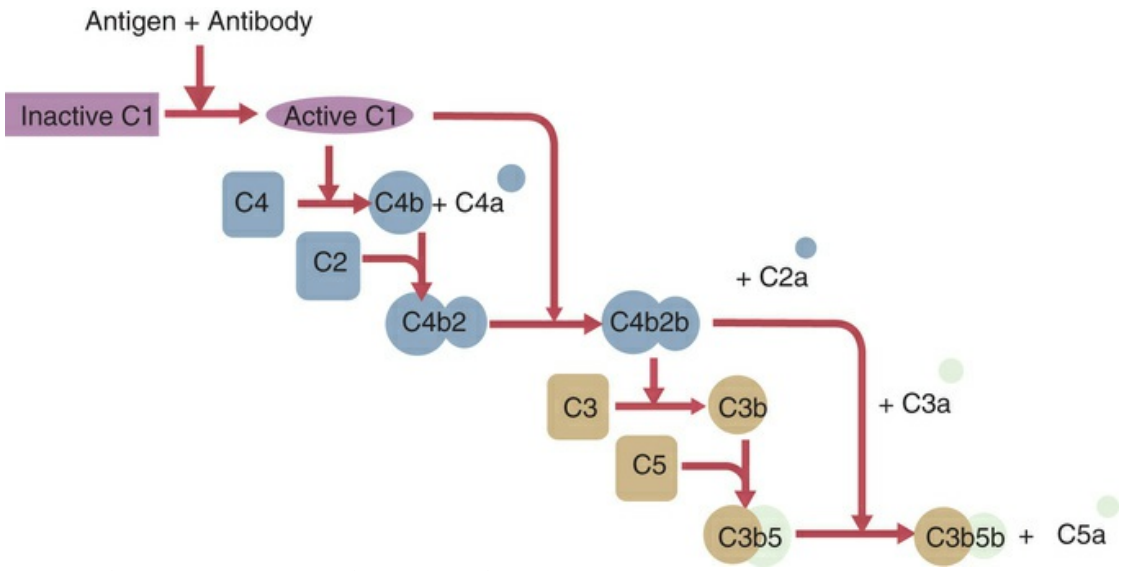


FIG. 4.8 The basic features of the classical complement pathway.

Unlike the alternate and lectin pathways, the classical pathway cannot be activated until antibodies are made and immune complexes form, which may take 7 to 10 days after onset of infection. When antibody molecules bind to an invader, active sites on their Fc regions are exposed. When multiple antibody molecules bind to an organism, these active sites collectively trigger the classical complement pathway.

The first component of the classical pathway is a protein complex called C1. C1 consists of three subunits (C1q, C1r, C1s) bound

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together by calcium. The completely assembled C1q looks like a six-stranded whip when viewed by electron microscopy (Fig. 4.9). Two molecules each of C1r and C1s form a complex located between the C1q strands. C1q is activated when its strands bind to activating sites on clustered antibody molecules. This binding triggers a conformational change in C1q that permits C1r to interact with C1s, and C1s is converted into an active protease.

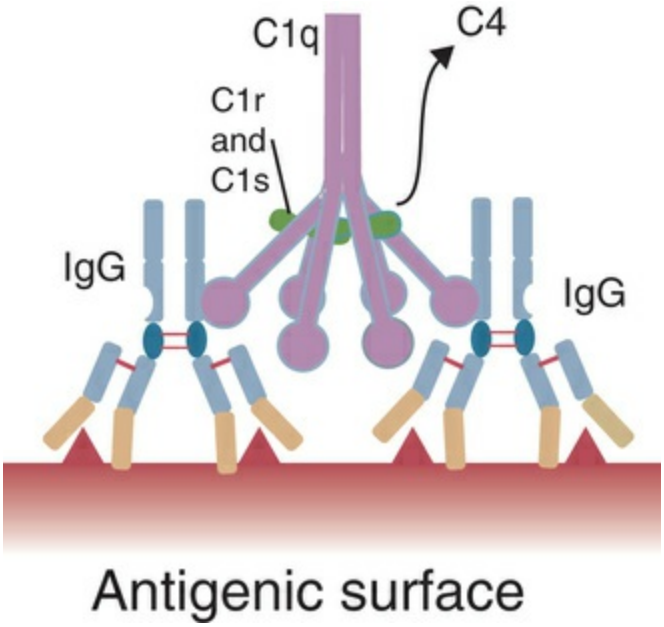


FIG. 4.9 The structure of C1 and its role in interacting with antibodies to initiate the classical complement pathway.

Single, antigen-bound molecules of immunoglobulin (Ig) M or paired, antigen-bound molecules of IgG are required to activate C1 (Chapter 16). The polymeric IgM structure readily provides several closely spaced complement-activating sites. In contrast, two IgG molecules must be located close to each other to have the same effect. Thus IgG is much less efficient than IgM in activating the classical pathway.

Active C1s cleaves C4 into C4a and C4b. C2 then binds to C4b to form the complex C4b2. Activated C1s then splits the bound C2, generating a small peptide fragment C2a and the C4b2b complex. C1s cannot act on soluble C2; the C2 must first be bound to C4b before it can be split (another example of substrate modulation). The C4b2b complex is a potent protease whose target is C3, and it is

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therefore called classical C3 convertase. The newly generated C3b binds and activates C5. Subsequent reactions lead to formation of the terminal complement complex and microbial killing.

In addition to binding immune complexes, C1 can also be activated directly by some viruses, or by bacteria such as *Escherichia coli* and *Klebsiella pneumoniae*. C1q can also bind to apoptotic and necrotic cells, extracellular matrix proteins, pentraxins such as C-reactive protein, amyloid and prion proteins, and DNA. However, all these substances (with the exception of immune complexes) can also bind the complement inhibitors C1-BP and FH so that full complement activation does not occur. If these inhibitory processes are blocked, uncontrolled complement activation may lead to unwanted inflammation.

The Amplification Pathway

All surface-bound C3 convertases, regardless of their origin, can induce the next steps in complement activation, the amplification pathway (Fig. 4.10). Once C5 binds to C3b, substrate modulation occurs, and the C5 is then cleaved by C3bBb (Fig. 4.11). The convertases break C5 (195 kDa) into a small fragment called C5a, leaving a large fragment C5b attached to the C3b. This cleavage also exposes a site on C5b that can bind two new proteins, C6 and C7, to form a multimolecular complex called C5b67 (Fig. 4.12). The C5b67 complex can then insert itself into the microbial cell wall. Once inserted in the surface of an organism, the complex first binds a molecule of C8 to form C5b678. Twelve to 18 C9 molecules then polymerize with the C5b678 complex to form a tubular structure called the terminal complement complex (TCC), (also called the membrane attack complex [MAC] or C5b6789). The TCC inserts into microbial cell membranes and punches a hole in the invader. If sufficient TCCs are formed on an organism, it will be killed by osmotic lysis. These TCCs can be seen by electron microscopy as ring-shaped structures on the microbial surface with a central electron-dense area surrounded by a lighter ring of poly C9 (see Fig. 4.12).

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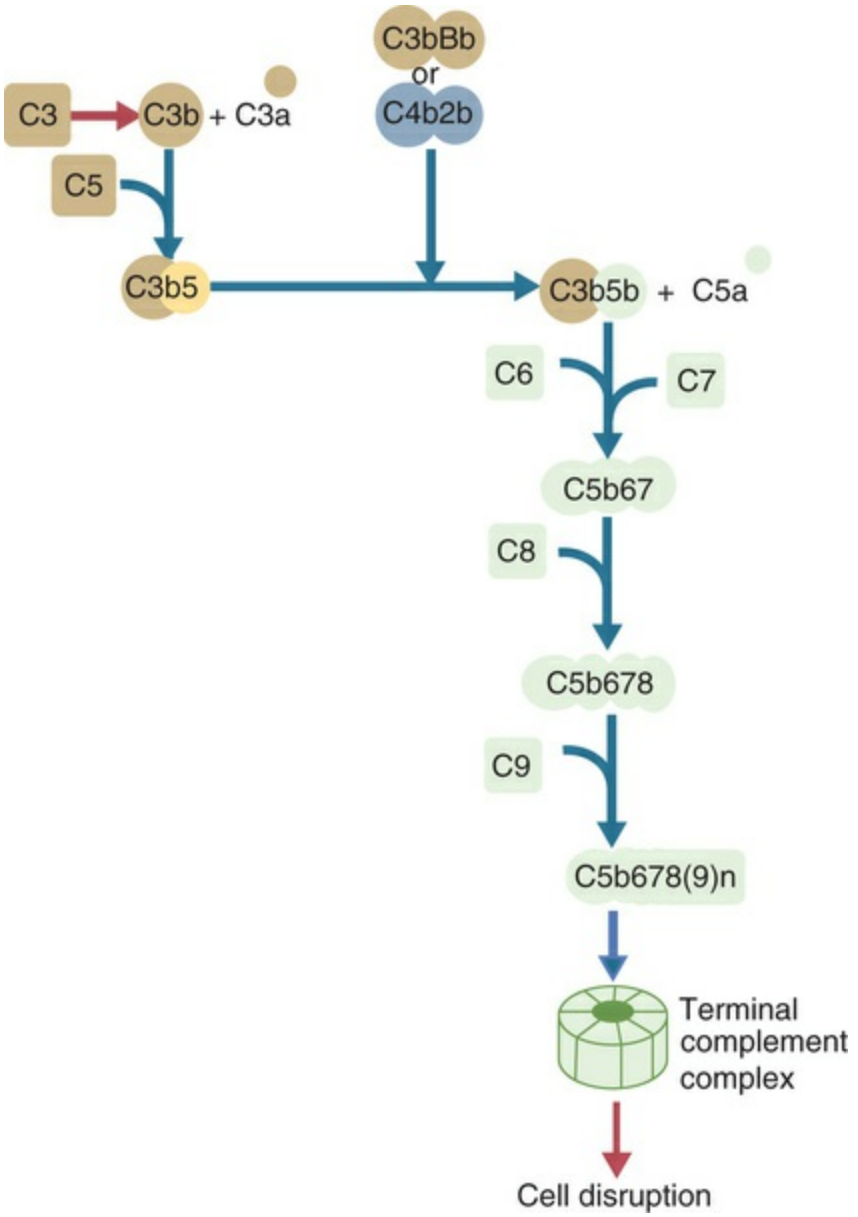


FIG. 4.10 The amplification pathway. The progressive aggregation of the terminal complement components eventually leads to the polymerization of C9 and the assembly of a membrane attack complex.

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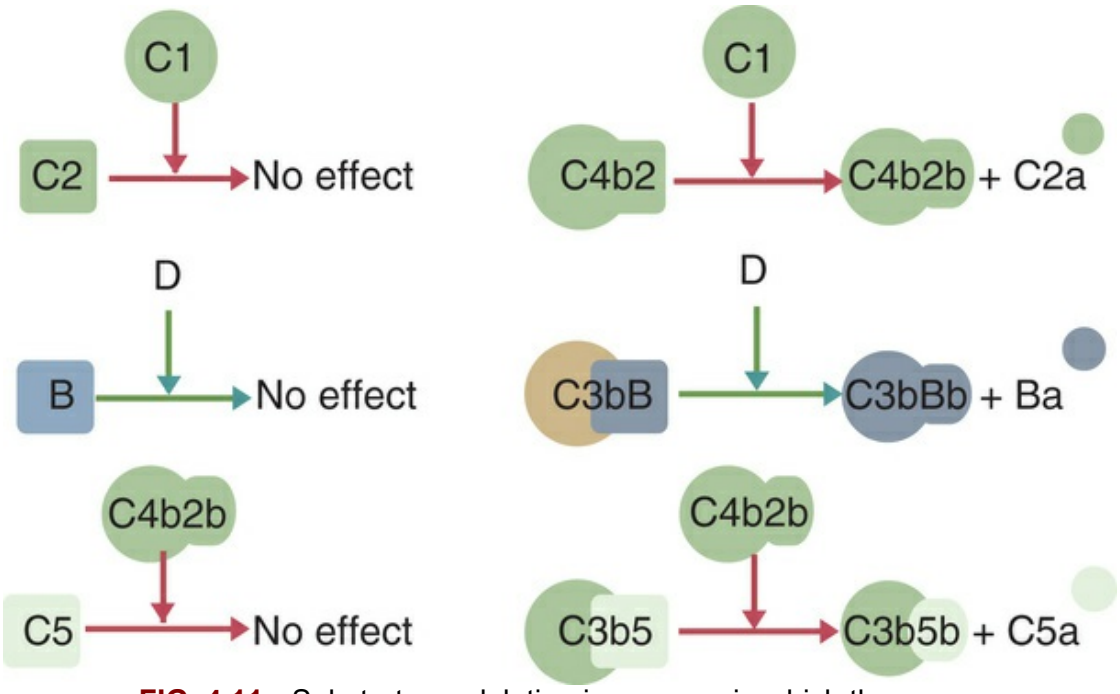


FIG. 4.11 Substrate modulation is one way in which the complement system is regulated. The target for a protease cannot be cleaved unless it is first bound to another protein. Examples of substrate modulation include the cleavage of factors C2, B, and C5 only after they have bound to C4, C3, and C3, respectively.

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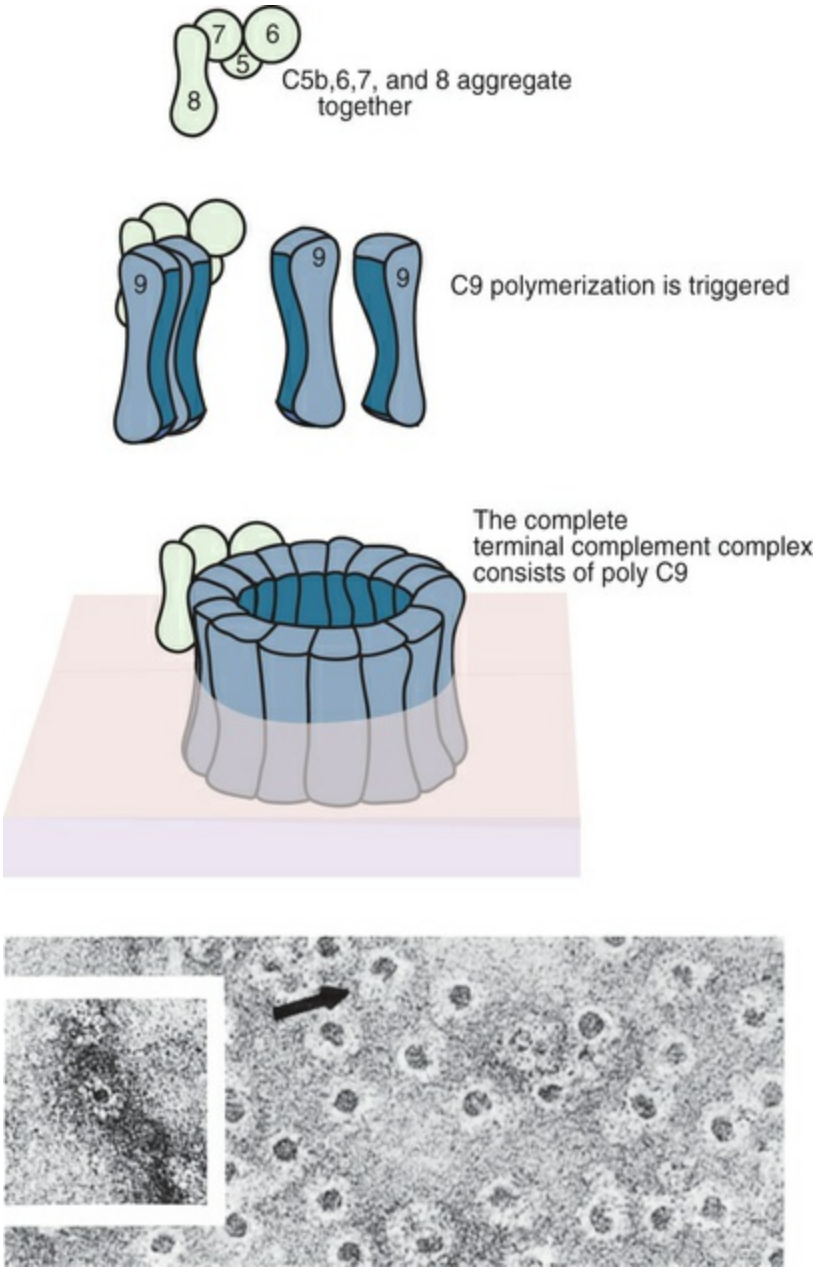


FIG. 4.12 Formation of poly C9 by the amplification pathway and an electron micrograph of poly C9-complement lesions on an erythrocyte membrane. The *insert* shows a mouse complement lesion. The *arrow* points to a possible C5b678 complex. Compare these lesions to the T cell polyperforins in Fig. 18.9. (From Podack ER, Dennert G: Assembly of two types of tubules with putative cytolytic function by cloned natural killer cells, *Nature* 307:442, 1983.)

More important than direct TCC-mediated lysis are the potent inflammatory effects of the small released peptide C5a. C5a can degranulate mast cells and stimulate platelets to release histamine and serotonin. It also triggers inflammation through its cell surface receptor. C5a is a powerful attractant for neutrophils and macrophages. It increases vascular permeability, causes lysosomal

enzyme release from neutrophils and thromboxane release from macrophages, and regulates some T cell responses (Fig. 4.13). The other small complement peptide, C3a, can kill bacteria such as *E. coli, Pseudomonas aeruginosa, Enterococcus faecalis,* and *Streptococcus pyogenes.* C3a acts like other antimicrobial peptides by disrupting bacterial membranes. (C3a and C5a are also called anaphylatoxins since, when injected in sufficient amounts, they can kill an animal in a manner similar to anaphylaxis [Chapter 30].)

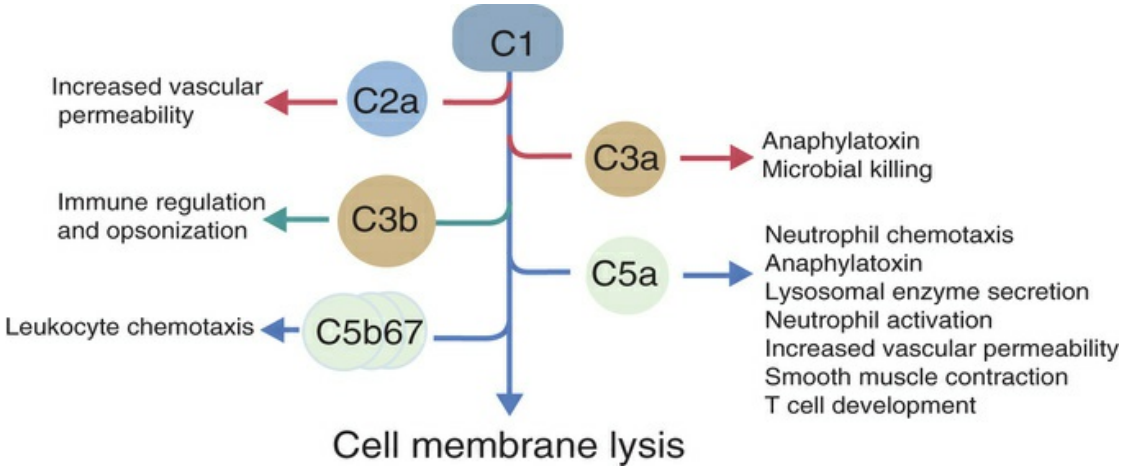


FIG. 4.13 Some of the biological consequences of complement activation.

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Regulation of Complement Activation

The consequences of complement activation are so significant and potentially dangerous that each of the activation pathways must be carefully regulated by soluble and cell-bound regulatory proteins (Fig. 4.14).

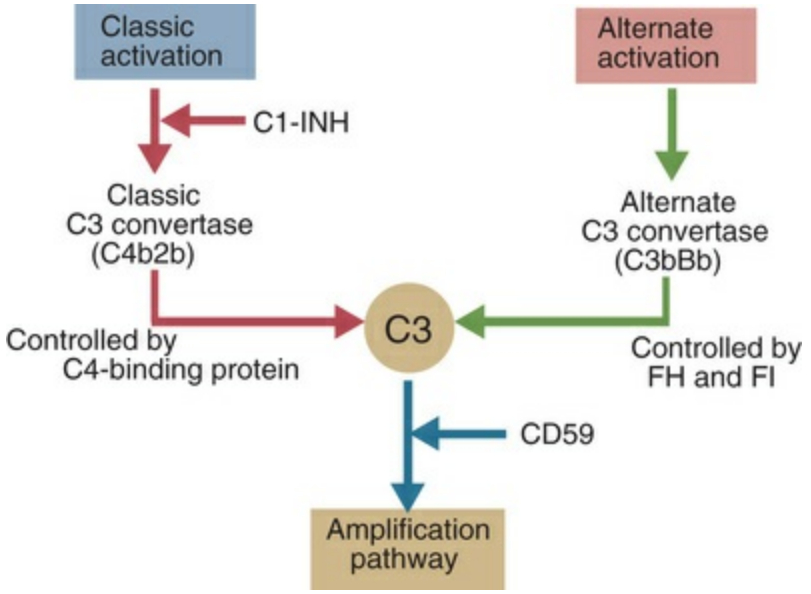


FIG. 4.14 Basic control mechanisms of the complement system.

The most important regulator of the classical pathway is C1-inhibitor (C1-INH). C1-INH blocks active C1r and C1s. Other regulatory proteins control the activities of the C3 and C5 convertases. Some compete with the MASPs for binding sites on MBL and ficolins. CD55, or decay accelerating factor, is a glycoprotein expressed on red blood cells, neutrophils, lymphocytes, monocytes, platelets, and endothelial cells. CD55 binds to the convertases and accelerates their decay. Its function is to protect normal cells from complement attack. Other proteins that accelerate degradation of the convertases include FH and C4-binding protein (C4BP) found in plasma, and CD35 (CR1) and CD46 found on cell membranes. The amplification pathway is controlled by three glycoproteins: vitronectin, clusterin, and, most important, protectin (CD59). They all inhibit assembly of the TCC

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by blocking C5b678 insertion and C9 polymerization.

Complement Receptors (CR)

Cells express five receptors for C3 or its fragments. These are called CR1 (CD35), CR2 (CD21), CR3 (CD11a/CD18), CR4 (CD11c/CD18), and CRIg.

CR1 is found on primate red cells, neutrophils, eosinophils, monocytes, macrophages, B cells, and some T cells. It is a receptor for C3b and C4b as well as for the C3b breakdown product, iC3b. Red cell CR1 accounts for 90% of all CR1 in the blood. In primates, CR1 removes immune complexes (antigen-antibody-complement complexes) from the circulation. These immune complexes bind to CR1 on red cells, and the coated red cells are then removed in the liver and spleen (Chapter 32). Deficiencies of complement components or their receptors may allow circulating immune complexes to accumulate in the kidney and cause damage. For example, some patients with the autoimmune disease systemic lupus erythematosus have a CR1 deficiency and are thus unable to remove these immune complexes effectively. C3-deficient dogs develop immune complex–mediated kidney lesions for the same reason (Chapter 32).

CR2 (CD21) is found on B cells. It is the receptor for C3d. CR2 associates with another B cell surface protein called CD19. This CD21/CD19 complex regulates B cell responses (see Fig. 15.11). In order to respond optimally to foreign antigens, B cells must be stimulated by C3d acting through CR2. When C3d binds to CR2 on B cells, it reduces their activation threshold 1000-fold. As always, mice are not a good guide to the situation in other mammals. Bovine CR2 consists of four distinct receptors that are generated by alternative splicing. They include two variants that are homologs of mouse CR1 and CR2 and each is expressed in a short and long form.

CR3 (CD11a/CD18) is an integrin that binds iC3b. It is found on macrophages, neutrophils, and natural killer cells. An inherited deficiency of CR3 (leukocyte adherence deficiency, LAD) occurs in humans, cattle, and dogs. Affected individuals die from overwhelming infections (Chapter 39).

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CR4 (CD11c/CD18) is an integrin found on neutrophils, T cells, natural killer cells, a few platelets, and macrophages. It is also a receptor for breakdown fragments of C3.

CRIg (complement receptor of the Ig family) is expressed on tissue macrophages. It is a receptor for the C3-dependent opsonization of blood-borne pathogens.

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Other Consequences of Complement Activation

Although microbial destruction mediated by terminal complement complexes is the most obvious beneficial activity of the complement system, its protective effects go far beyond this.

Opsonization

Bacteria normally lack complement regulators so that uncontrolled complement activation occurs on their surface. This leads to proinflammatory signaling, opsonization, phagocytosis, and in some organisms, especially Gram-negative bacteria and some parasites, TCC assembly and bacterial lysis. C3b and C4b are very effective opsonins (Chapter 5). Phagocytic cells express CR1, and tissue macrophages also express CRIg. C3b-coated organisms will be bound to these cells and undergo type II phagocytosis (Chapter 5). If for some reason these organisms cannot be ingested, neutrophils may secrete their lysosomal enzymes and oxidants into the surrounding tissue fluid. This can cause inflammation and tissue damage, a reaction classified as type III hypersensitivity (Chapter 32). Given the long evolutionary history of the complement system, it is not surprising that many bacteria have evolved mechanisms to neutralize the effects of complement (Chapter 26)

Removal of Apoptotic Cells

Complement contributes to recovery from inflammation by promoting the removal of apoptotic cells and immune complexes. Apoptotic cells lose their complement inhibitors CD46 and CD59. As a result, they are opsonized by C3b and C4b and removed by phagocytosis. Apoptotic cells also bind CRP that can then bind C1q, leading to classical pathway activation. Properdin (FP) binds to apoptotic T cells, resulting in C3b-mediated opsonization and destruction.

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Inflammation

Complement activation results in inflammation and possibly tissue damage. The anaphylatoxin C5a enhances TLR-induced production of TNF-α, IL-1β, and IL-6. After binding to its receptor, it also interacts with toll-like receptor 2 (TLR2), TLR4, and TLR9. This TLR stimulation enhances cellular expression of C3aR and C5aR.

Blood Coagulation

The complement system enhances blood coagulation and inhibits fibrinolysis. Thus C5a induces the expression of tissue factor and plasminogen activator inhibitor I. Likewise, components of the clotting system amplify the complement system. Activated clotting factor XII can cleave C1, and so activate the classical pathway. Thrombin acts on C5 to generate C5a.

Chemotaxis

Activation of the complement system generates chemotactic peptides, including C5a and C5b67 (Table 4.2). C5b67 attracts neutrophils and eosinophils, whereas C5a attracts not only neutrophils and eosinophils but also macrophages and basophils. C5a also stimulates the neutrophil respiratory burst and upregulates CR1 and integrin expression.

TABLE 4.2

Complement-Derived Chemotactic Factors

Factor	Target
C3a	Eosinophils
C5a	Neutrophils, eosinophils, macrophages
C567	Neutrophils, eosinophils
FBb	Neutrophils
C3e	Promotes leukocytosis

Immune Regulation

The complement system regulates both humoral and cell-mediated adaptive immunity. Thus C3d binds to antigen. When an antigen molecule binds to a B cell antigen receptor, any attached C3d will

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also bind to CR2/CD19 complexes on the B cell. (Remember that several hundred C3 molecules may attach to an antigen molecule as a result of C3 convertase activity.) Binding to CR2/CD19 enhances B cell antigen receptor signaling (Chapter15). Conversely, depletion of C3 is associated with reduced B cell responses. Coating of antigens with C3d also enables them to bind to CR2 on dendritic cells, and so permits memory B cells to sample the antigen over long periods. In the absence of C3, immune complexes will not bind to follicular dendritic cells in germinal centers (Chapter 12).

Many complement proteins such as C1q, C3b, and C5a also influence T cell activation. Thus C3 is present within CD4+ T cells (Chapter 14). This C3 is cleaved when T cells are activated and the C3a moves to the cell surface. Here it binds to its receptor and drives T cell cytokine production.

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Complement Genes

The genes coding for the complement proteins are scattered throughout the genome. However, two major gene clusters have been identified. The genes for C4, C2, and FB are located within the major histocompatibility complex class III region (Chapter 11). Likewise, the genes for C4BP, CD55, CD35, CD21, CD46, and FH are linked within the regulation of complement activation (RCA) cluster.

Complement components, like other proteins, show multiple genetic variations, and collectively these variants form an animal's "complotype." These genetic variations may influence an animal's susceptibility to infectious and inflammatory diseases. The precise number of variants varies between components and species. For example, bovine FH has three alleles, equine C3 has six, and canine C3 has two. Canine C6 has seven alleles, and porcine C6 has 14. Eleven alleles of canine C7 have been identified, whereas canine C4 has at least five. There is an association among C4-4 allele expression, low serum C4 levels, and the development of autoimmune polyarthritis in dogs. Feline and equine C4 each have at least four alleles.

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Complement Deficiencies

Canine C3 Deficiency

Because the complement system is an essential innate defensive mechanism, any complement deficiency will increase susceptibility to infections. The most severe of these diseases occurs in animals deficient in C3. For example, some Brittany Spaniels may have an autosomal recessive C3 deficiency (Fig. 4.15). Dogs that are homozygous for this trait have no detectable C3, whereas heterozygous animals have C3 levels that are approximately half normal. Heterozygous animals are clinically normal. The homozygous-deficient animals have lower IgG levels than normal, and their ability to make antibodies against defined antigens is reduced. These dogs tend to make more IgM and less IgG. They experience recurrent sepsis, pneumonia, pyometra, and wound infections. The organisms involved include *Clostridium* species, *Pseudomonas* species, *E. coli,* and *Klebsiella* species. Some affected dogs develop amyloidosis, and many develop immune complex-mediated kidney disease (Chapter 32). The mutation responsible for this deficiency (deletion of a single cytosine) shortens the C3 chain as a result of a frameshift and the generation of a premature stop codon (Fig. 4.16).

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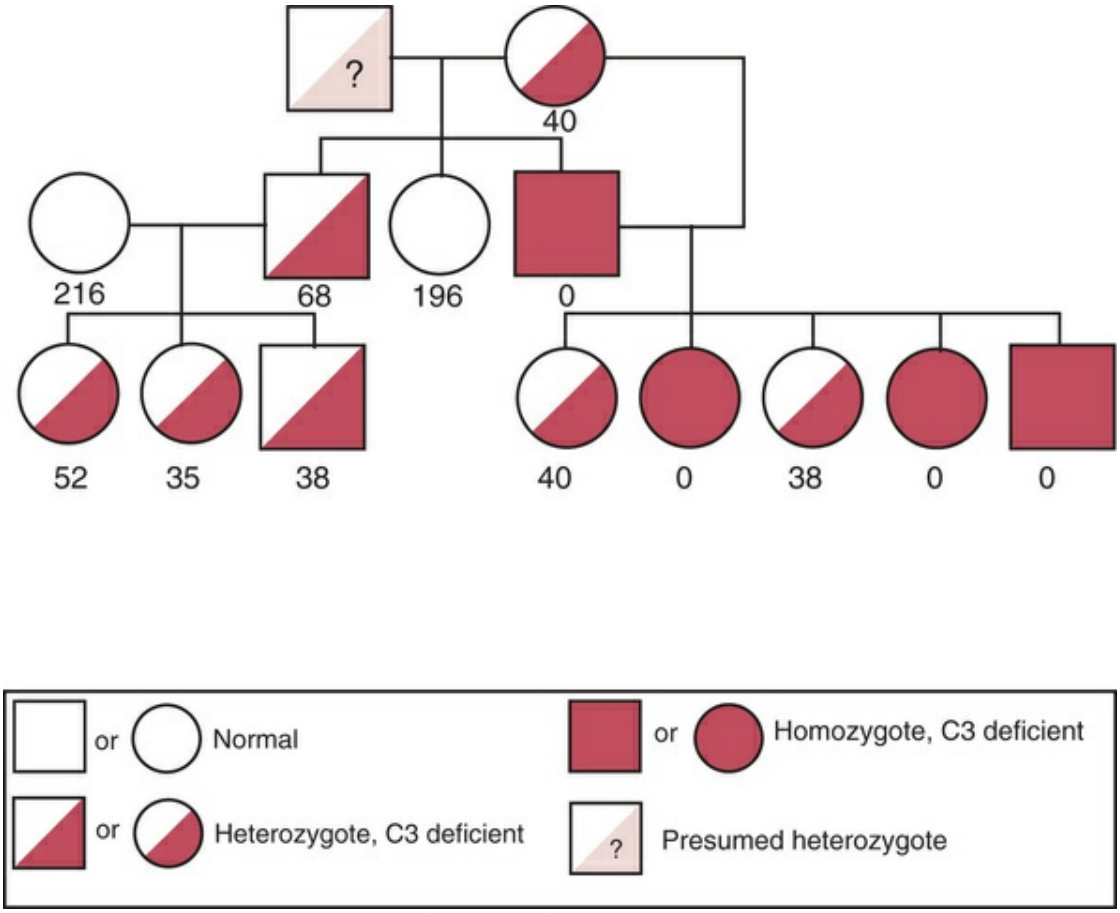


FIG. 4.15 Inheritance of a C3 deficiency in a colony of Brittany Spaniels. The number below each circle or square represents the animal's C3 level as a percentage of a standard reference serum. The mean level in healthy spaniels was 126. (*Squares* denote males, *circles* denote females.) (From Winkelstein JA, Cork LC, Griffin DE, et al: Genetically determined deficiency of the third component of complement in the dog, *Science* 212:1169-1170, 1981.)

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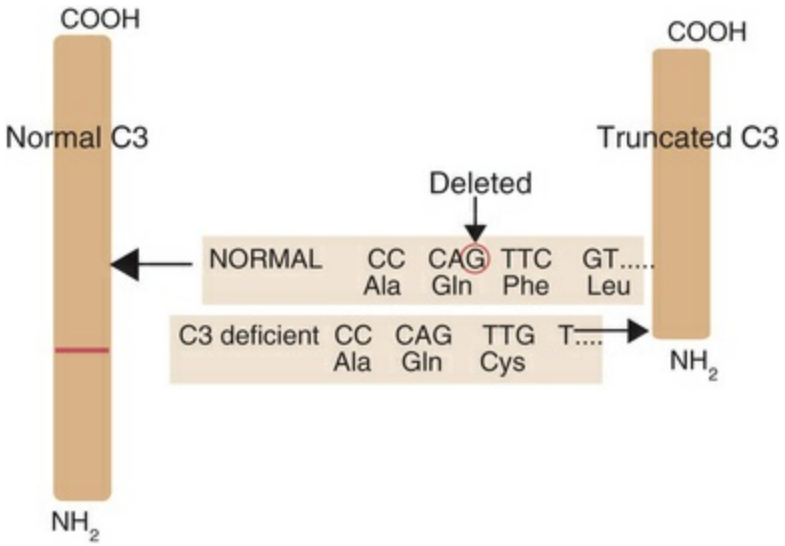


FIG. 4.16 The mutation that results in canine C3 deficiency. Deletion of a cytosine results in a frameshift and premature termination of transcription of the C3 gene.

Porcine Factor H Deficiency

FH is a critical component of the alternative complement pathway. It normally inactivates C3b as soon as it is generated and so prevents excessive complement activation. If an animal fails to make FH, C3b will be generated in an uncontrolled fashion. FH deficiency has been identified as an autosomal recessive trait in Yorkshire pigs. Affected piglets are healthy at birth and develop normally for a few weeks. However, eventually they fail to thrive, stop growing, become anemic, and die of renal failure.

On necropsy, there are multiple petechial hemorrhages on the surface of the kidneys, accompanied by atrophy of the renal papillae. Mesangial cell proliferation and capillary basement membrane thickening occur within glomeruli (Fig. 4.17). On electron microscopy, extensive intramembranous electron-dense deposits are found in the glomerular basement membranes (Fig. 4.18). This is typical of type II membranoproliferative glomerulonephritis (Chapter 32). Indirect immunofluorescence tests demonstrate massive deposits of C3, but no immunoglobulins in the basement membranes. C3 can be found in the glomeruli before birth, but mesangial proliferation and intramembranous dense deposits develop after 5 days of age.

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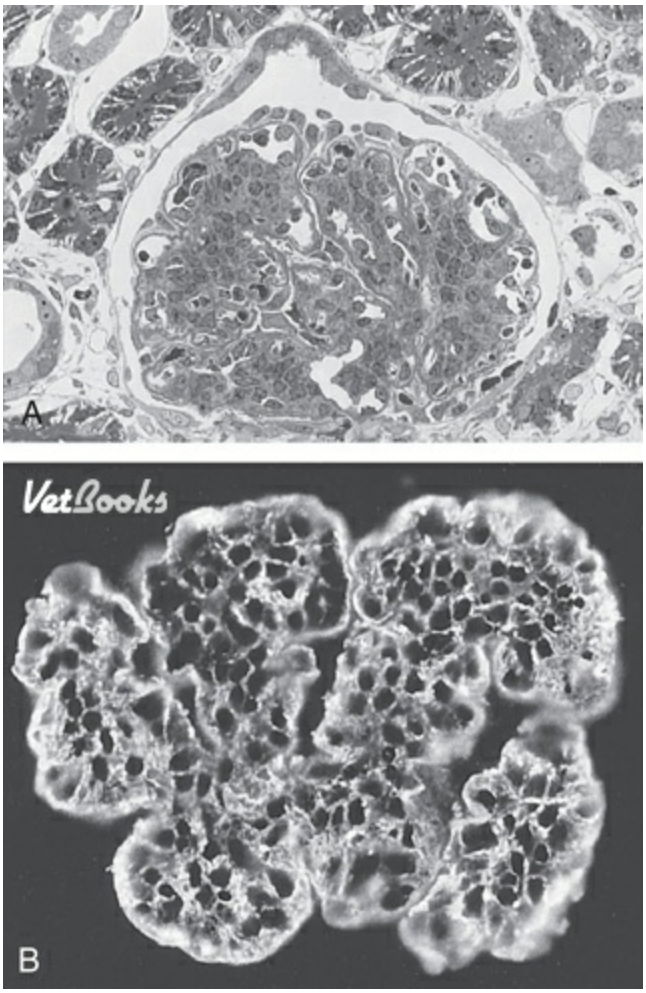


FIG. 4.17 **A,** A thin section of the glomerulus of a piglet with factor H deficiency. Note the thickened basement membrane and increased numbers of mesangial cells, hence the name membranoproliferative glomerulonephritis. **B,** An immunofluorescence photomicrograph of another glomerulus from a factor H–deficient piglet. This is stained with fluorescent anti-C3. The bright fluorescence indicates the presence of C3 deposited in this glomerulus. Compare this figure with Fig. 32.11. (**A,** Courtesy J.H. Jansen; **B,** from Jansen JH, Hogasen K, Mollnes TE: Extensive complement activation in hereditary porcine membranoproliferative glomerulonephritis type II [porcine dense deposit disease], *Am J Pathol* 143:1356-1365, 1993.)

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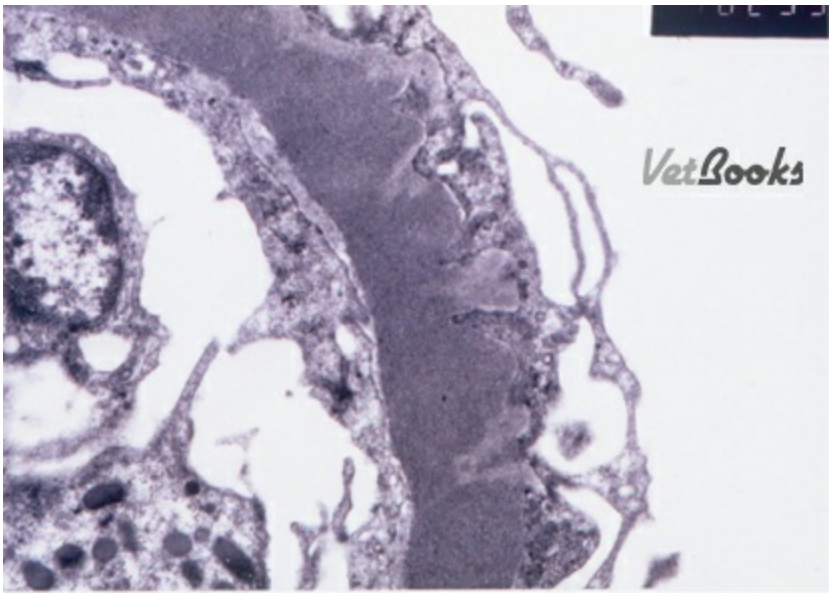


FIG. 4.18 Electron micrograph showing dense intramembranous deposits in the glomerulus of a piglet with factor H deficiency. (From Jansen JH: Porcine membranoproliferative glomerulonephritis with intramembranous dense deposits [porcine dense deposit disease], *APMIS* 101:281-289, 1993.)

Nephritic piglets are almost totally deficient in FH (2% of normal levels), whereas heterozygotes have half the normal levels. If FH is replaced by plasma transfusions, the progress of the disease can be slowed and piglets survive longer. Affected pigs have no plasma C3. Since heterozygotes are readily detected by measurement of plasma C3, this disease can be eradicated from affected herds.

Other Complement Deficiencies

Mannose-binding lectin deficiency has been described in children, where it causes increased susceptibility to infection. It has not yet been described in domestic animals. In contrast to the severe effects of a C3 deficiency, congenital deficiencies of other complement components in laboratory animals or humans are not necessarily lethal. Thus individuals with C6 or C7 deficiencies have been described who are quite healthy. Apparently healthy C6-deficient pigs have been described. The lack of discernible effect of these deficiencies suggests that the terminal portion of the complement pathway leading to terminal complex formation may not be biologically essential.

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Cellular Innate Immunity

Neutrophils and Phagocytosis

CHAPTER OUTLINE

Leukocyte Classification, 39
Neutrophils, 39
Structure, 40
Emigration From the Bloodstream, 41
Changes in Endothelial Cells, 41
Changes in Neutrophils, 41
Integrins, 41
Emigration and Swarming, 42
Phagocytosis, 42
Activation, 42
Chemotaxis, 42
Adherence and Opsonization, 43
Extracellular Traps, 43
Ingestion, 44
Destruction, 45
The Respiratory Burst, 45
Lytic Enzymes, 46

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Cytokines, 46
Surface Receptors, 47
Fate, 47

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Understand that many cell types participate in innate immunity. These include neutrophils, macrophages, dendritic cells, eosinophils, mast cells, and innate lymphoid cells.
- Be able to identify a neutrophil on sight.
- Outline the life history and fate of neutrophils.
- Describe how the first cells attracted to sites of tissue damage or microbial invasion are neutrophils.
- Explain how neutrophils can leave blood vessels and outline the mechanisms involved in this emigration.
- Explain how neutrophils bind, ingest, and kill invading microorganisms—the process of phagocytosis.
- List the mechanisms by which neutrophils kill invaders.
- Describe the importance of opsonization and the role of antibodies and complement in the phagocytic process.
- Explain the mechanisms of the respiratory burst and its importance both as a killing mechanism of bacteria and a cause of tissue damage.
- Explain how neutrophils alone cannot provide a long-term solution to the body's defense needs.
- Define opsonin, chemotaxis, respiratory burst, and NETosis.

Although physical barriers such as the skin exclude many organisms, these barriers are not impenetrable, and microbes can gain access to body tissues through wounds, inhalation, or food. Once sentinel cells recognize the invaders, signals are generated that activate and attract defensive cells to the sites of invasion.

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Depending upon the nature of the invader, these cells may include dendritic cells, mast cells, eosinophils, neutrophils, macrophages, and innate lymphoid cells. The most significant of these, at least in the early stages of inflammation, are neutrophils, large numbers of which are attracted to sites of invasion where they kill and eat the invaders. This process is called phagocytosis (Greek for "eating by cells"). The prime purpose of the inflammatory process is to ensure that phagocytic cells intercept and destroy invading microbes as rapidly and efficiently as possible.

Defensive cells circulate in the bloodstream where they are collectively called leukocytes (white blood cells) (Fig. 5.1). All leukocytes originate from bone marrow (myeloid) stem cells, and all help defend the body. Two types of leukocytes specialize in killing and eating invading microorganisms. These, called neutrophils and macrophages, originate from a common stem cell but look very different and have different, but complementary, functions. Neutrophils respond and eat invading organisms rapidly but are incapable of sustained phagocytic effort. Macrophages, in contrast, move more slowly but are highly effective phagocytes and are capable of repeated phagocytosis. In this chapter, we review the properties of neutrophils and their role in inflammation and innate immunity. We will look at macrophages in the next chapter.

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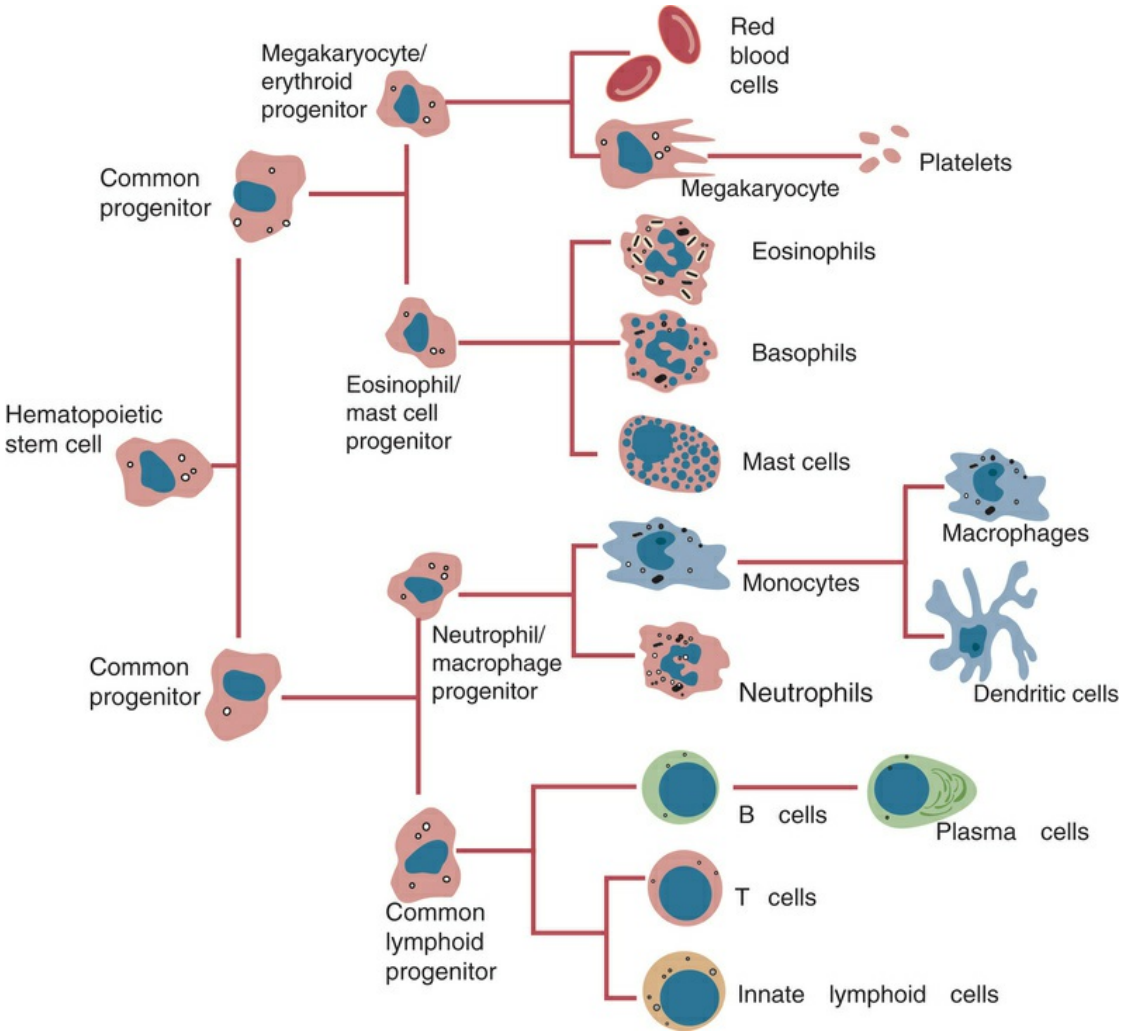


FIG. 5.1 The origin of leukocytes from the bone marrow. Note that lymphoid cells originate from different stem cells than the cells of the myeloid system. Note too that cells such as eosinophils and basophils are probably closely related despite significant morphological differences.

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Leukocyte Classification

Examination of a stained blood smear reveals many types of leukocyte. Those with a cytoplasm filled with granules are called granulocytes (Fig. 5.2). Granulocytes also have a characteristic lobulated, irregular nucleus, so they are described as "polymorphonuclear" (as opposed to the single, rounded nucleus of "mononuclear" cells such as macrophages). Granulocytes are classified based on the staining properties of their granules. Cells whose granules take up basic dyes such as hematoxylin are called basophils; those that take up acidic dyes such as eosin are called eosinophils; and those that take up neither are called neutrophils. All three populations play important defensive roles.

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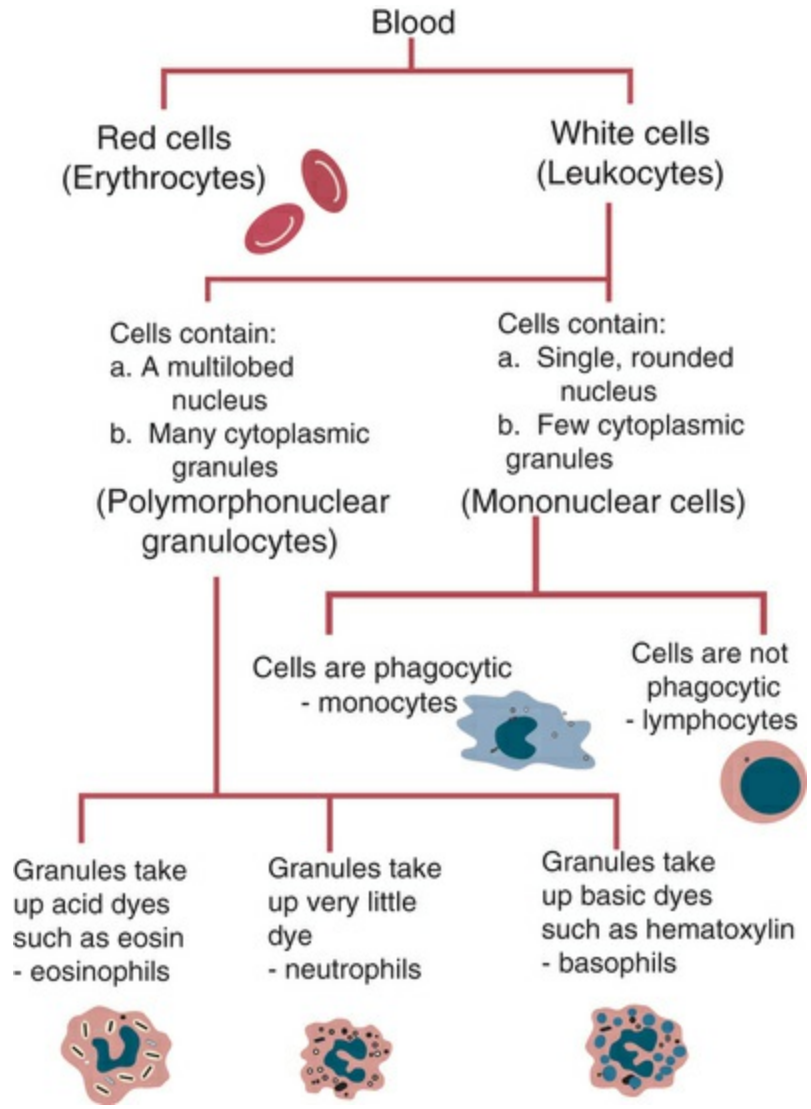


FIG. 5.2 Differentiation and nomenclature of the cells found in blood. Leukocytes are first classified on the basis of their nuclear shape. Polymorphonuclear cells are then differentiated on the basis of their granule staining. Lymphocytes and macrophages are classified on the basis of nuclear shape and extent of cytoplasm. Note that the different subpopulations of lymphocytes cannot be distinguished on the basis of their morphology.

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Neutrophils

Polymorphonuclear neutrophil granulocytes, otherwise called neutrophils, predominate in the blood of many mammals (Fig. 5.3). As a result, about two thirds of the hematopoietic activity of the bone marrow is devoted to neutrophil production. Neutrophils are formed by stem cells at a rate of about 8 million per minute in humans; they migrate to the bloodstream and about 12 hours later move into the tissues. They live for only a few days unless activated by inflammation and must therefore be constantly replaced. Neutrophils constitute about 60% to 75% of the blood leukocytes in most carnivores, about 50% in the horse, and 20% to 30% in cattle, sheep, and laboratory rodents. However, blood neutrophils account for only 1% to 2% of their total population. The vast majority of neutrophils are sequestered in capillaries within the liver, spleen, lungs, and bone marrow. During bacterial infections, the numbers of circulating neutrophils may increase 10-fold as these stored cells are released from these organs. These neutrophils migrate to the bloodstream and eventually the tissues, where they die by apoptosis.

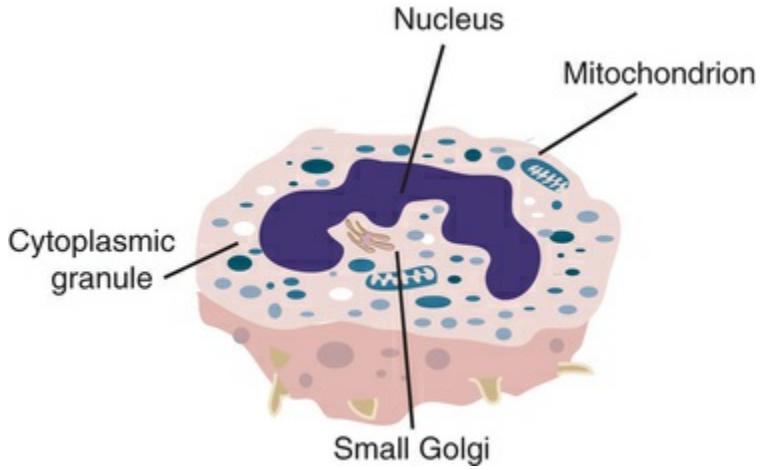


FIG. 5.3 The major structural features of a neutrophil. Note the characteristic nucleus and the plentiful cytoplasmic granules.

The production of neutrophils by stem cells is regulated by a cytokine called granulocyte colony-stimulating factor (G-CSF). The

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production of G-CSF is coordinated with the rate of neutrophil apoptosis. Thus apoptotic neutrophils are removed by macrophages. These macrophages then produce interleukin-23 (IL-23) so that, as neutrophils die, IL-23 production increases. IL-23 in turn promotes IL-17 production by lymphocytes and the IL-17 in turn stimulates G-CSF production and stem cell activity. As a result, the rate of neutrophil production matches the rate of their removal. Toll-like receptors (TLRs) are also expressed on myeloid stem cells. During microbial infections, pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides bind to these TLRs and trigger production of more neutrophils. TLRs thus provide a mechanism whereby neutrophil availability increases rapidly in response to infection. Administration of G-CSF stimulates neutrophil production. If administered to cattle around the time they are calving, it will increase neutrophil numbers and as a result reduce the prevalence of mastitis. A modified form of G-CSF is currently available for this purpose in dairy cattle (Chapter 41).

Structure

Neutrophils are about 10 to 20 μm in diameter. They have a finely granular cytosol at the center of which is an irregular sausage-like or segmented nucleus (Fig. 5.4). The chromatin within the nucleus is condensed so that neutrophils cannot divide. Electron microscopy shows three major types of enzyme-rich granules in their cytosol (Fig. 5.5). Primary (azurophil) granules contain enzymes such as myeloperoxidase, lysozyme, elastase, β-glucuronidase, and cathepsin B. Secondary (specific) granules lack myeloperoxidase but contain lysozyme and collagenase and the iron-binding protein lactoferrin. Tertiary granules contain gelatinase. Mature neutrophils have a small Golgi apparatus, some mitochondria, a few ribosomes, and a little rough endoplasmic reticulum. Although neutrophil DNA is tightly condensed and they have a very short life span, they produce many proteins. Neutrophils express a broad repertoire of PRRs and can respond dynamically to PAMPs by producing proinflammatory cytokines. As a result, they are major mediators of innate immunity.

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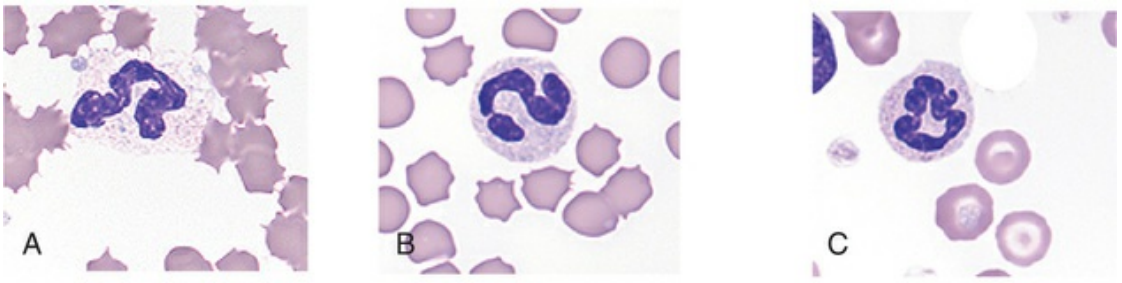


FIG. 5.4 Neutrophils in peripheral blood smears. **A,** Horse. **B,** Cat. **C,** Dog. These cells are about 10 µm in diameter. Giemsa stain.
(Courtesy Dr. M.C. Johnson.)

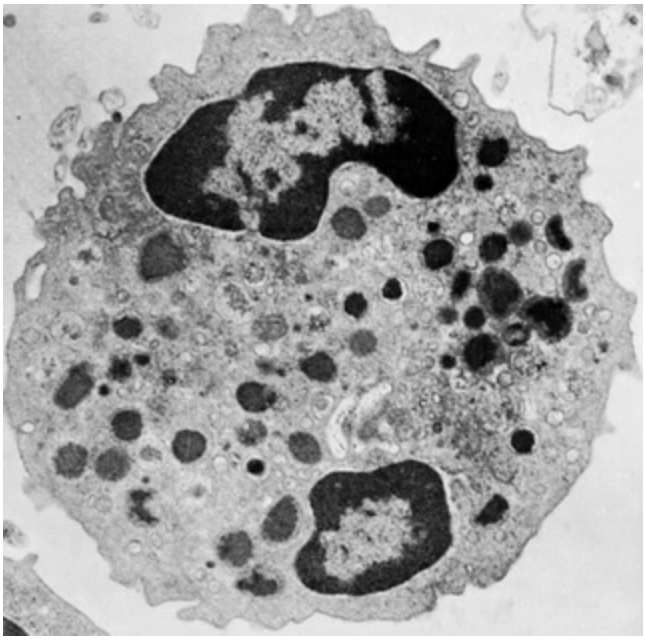


FIG. 5.5 Transmission electron micrograph of a rabbit neutrophil. Note the two lobes of the nucleus and the granule-filled cytoplasm.
(Courtesy Dr. S. Linthicum.)

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Emigration From the Bloodstream

Neutrophils in the bloodstream are simply carried along by the flow. In inflamed tissues, however, these fast-moving cells slow down, stop, bind to blood vessel walls, and emigrate into the tissues. This emigration is triggered by changes in the endothelial cells that line blood vessel walls.

Changes in Endothelial Cells

In aggregate, the endothelial cells that line blood vessels collectively have a huge surface area (estimated at 4000 square meters in humans) and thus serve as a broad sensor of microbial invasion. When PAMPs and DAMPs such as lipopolysaccharides or histamine and platelet activating factor (PAF) from damaged tissues reach blood vessels, they stimulate the endothelial cells to express a sticky glycoprotein called P-selectin (CD62P). P-selectin is stored in cytoplasmic granules but moves to the cell surface within minutes after stimulation. The P-selectin can bind a protein called L-selectin (CD62L) on passing neutrophils. At first, this binding is weak and transient because the neutrophils shed their L-selectin, but these neutrophils express more selectins so that they gradually slow, roll along the endothelial cell surface, and eventually stop (Fig. 5.6). This mainly happens in venules where the vessel wall is thin and its diameter sufficiently small to permit the neutrophils to make firm contact with the endothelium.

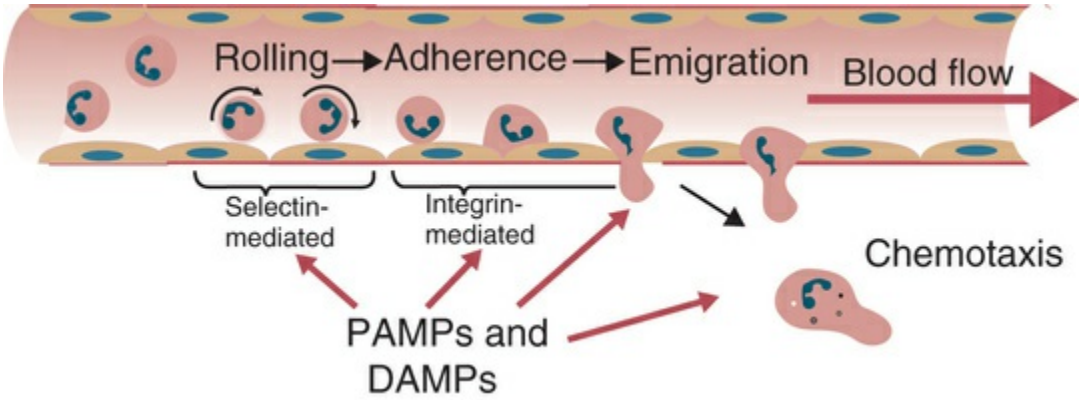


FIG. 5.6 The stages of neutrophil adhesion and emigration from

blood vessels. Changes in vascular endothelial cells are triggered by tissue damage and microbial invasion. Selectins on endothelial cells tether neutrophils and stimulate them to roll. When they come to a halt, integrins bind them firmly to vascular endothelial cells and signal the neutrophils to emigrate into tissues by passing through the blood vessel walls.

Changes in Neutrophils

As neutrophils roll along the endothelial surface, a second set of changes occurs. Platelet activating factor, chemokines, and leukotrienes from the endothelial cells trigger the rolling neutrophils to express the adhesive protein, leukocyte function–associated antigen-1 (LFA-1). LFA-1 is an integrin that binds to an intercellular adhesion molecule-1 (ICAM-1 or CD54) on endothelial cells (Fig. 5.7). Their strong binding brings neutrophils to a complete stop and attaches them firmly to the vessel wall, despite the shearing force of the blood flow. After several hours, endothelial cells activated by cytokines such as tumor necrosis factor-α (TNF-α) express the strongly adhesive E-selectin (CD62E). IL-1 and IL-23 also induce endothelial cells to produce chemokines that attract still more neutrophils.

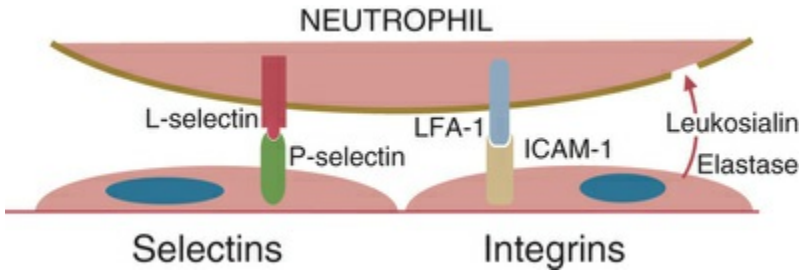


FIG. 5.7 A simplified view of the proteins and their ligands engaged in neutrophil-vascular endothelial cell binding. Selectins are carbohydrate-binding proteins that bind other glycoproteins. This selectin-mediated binding is weak and temporary. Subsequently, integrins on leukocytes, especially LFA-1, bind strongly to their ligand ICAM-1 on vascular endothelial cells. Elastase secreted by endothelial cells removes leukosialin, thus permitting the neutrophil to bind strongly to the endothelial cell.

Integrins

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Many cell surface proteins bind cells together, but the most important of these are the integrins. Integrins consists of paired protein chains (heterodimers) that use a unique α-chain paired with a common β-chain. For example, three β_2-integrins are found on neutrophils. Their α-chain, called CD11a, b, or c, is associated with common β_2-chain called CD18. Therefore these three integrins are CD11a/CD18 (LFA-1), CD11b/CD18, and CD11c/CD18. As described previously, LFA-1 on activated neutrophils binds to ICAM-1 on capillary endothelial cells. CD11b/CD18 also binds leukocytes to endothelial cells and acts as a complement receptor (complement receptor 3, CR3) (Chapter 4).

Emigration and Swarming

After binding to blood vessel walls, neutrophils swarm into the surrounding tissues under the influence of chemokines, lipids, and other chemoattractants (Fig. 5.8). Most migrating neutrophils squeeze between the endothelial cells, but about 20% actually pass through these cells and produce proteases to get through the basement membrane. They then crawl toward any invading microbes becoming activated in the process. Since neutrophils are the most mobile of all the blood leukocytes (they can move at up to 12 μm/min), they are the first to arrive at damaged tissues. The first cells to arrive sense short-range chemotactic factors. Some of these neutrophils die and release more chemoattractants. The newly arrived cells in turn synthesize leukotriene B_4 that is even more attractive. Within hours neutrophil swarms gather in enormous numbers. Under some conditions the numbers of neutrophils in these swarms may be sufficient to generate pus. This whitish-yellow liquid consists of accumulated dead neutrophils and tissue debris. It is produced in especially large amounts in the presence of bacteria that produce leukotoxins—toxins that kill leukocytes. Such bacteria are said to be pyogenic (Chapter 26).

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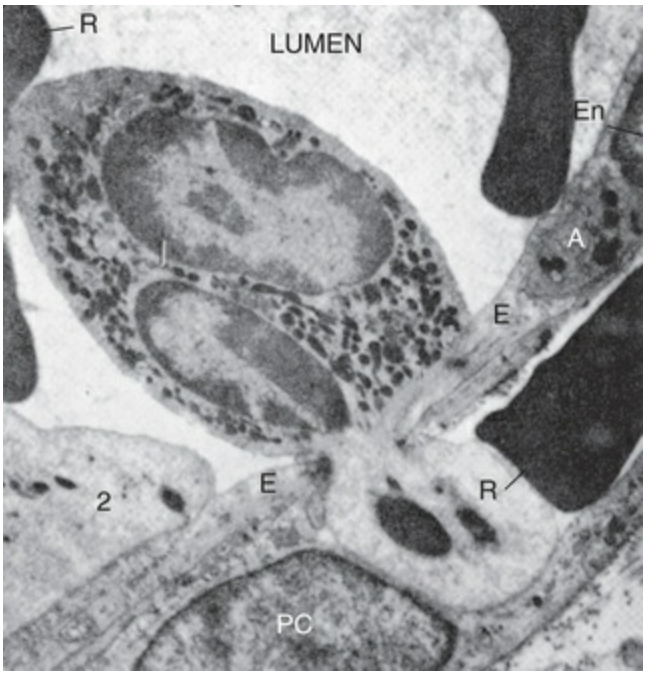


FIG. 5.8 Inflamed venule of a rat. Cell **1** is a neutrophil pushing its way through a capillary wall to reach the surrounding tissues. **R,** red blood cells; **E,** endothelium; **PC,** periendothelial cell; cell **2** is also a neutrophil. (From Marchesi VT, Florey HW: Electron micrographic observations on the emigration of leucocytes. *Q J Exp Physiol* 45:343, 1960.)

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Phagocytosis

Once they reach sites of microbial invasion, neutrophils eat and destroy the invading bacteria through phagocytosis. Although a continuous process, phagocytosis can be divided into discrete stages: activation, chemotaxis, adherence, ingestion, and destruction (Fig. 5.9).

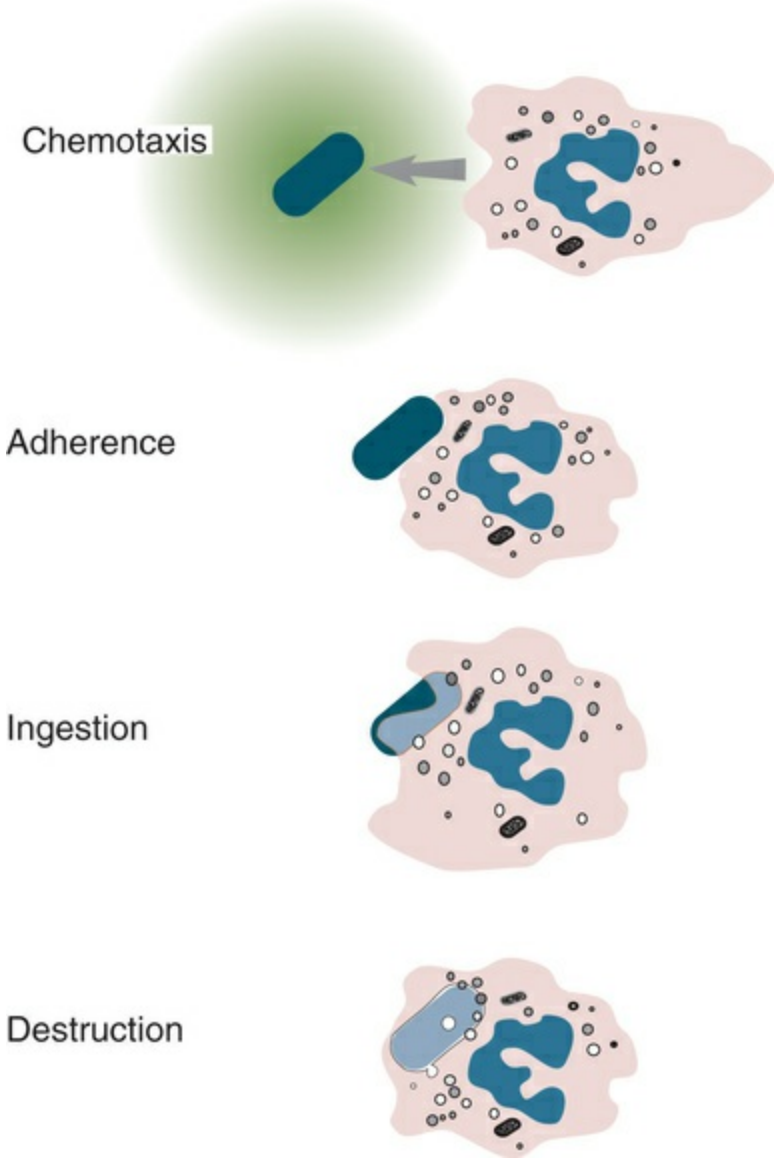


FIG. 5.9 The stages in the process of phagocytosis. While in fact a continuous process, this division into stages provides a useful method of analyzing the process.

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Activation

Neutrophils must be activated before they attack and destroy invaders. Thus, when neutrophils bind to endothelial cell integrins and are stimulated by CXCL8 or C5a, they secrete elastase, defensins, and oxidants. The elastase promotes their adhesiveness. The oxidants activate tissue proteases, which in turn release TNF-α from macrophages. The TNF-α, in turn, attracts more neutrophils, thus providing feedback amplification.

Chemotaxis

Neutrophils crawl directly toward invading organisms and damaged tissues attracted by chemotactic molecules. These chemoattractants diffuse from sites of microbial invasion and form a gradient. Neutrophils crawl toward the area of highest concentration—the source of the material. The moving cells generate projections (lamellipodia) at their leading edge. Chemoattractant receptors are distributed over the neutrophil surface, but the formation of lamellipodia is driven by the higher concentration of attractants at the cell's leading edge.

Microbial invasion and tissue damage generate many chemoattractants. These include the complement peptide C5a (Chapter 4); a peptide called fibrinopeptide B, derived from fibrinogen; and hydrogen peroxide. A damage-triggered gradient of H_2O_2 is established within 5 minutes of wounding, just preceding the movement of the first neutrophils toward a wound. Other attractants include chemokines, cathelicidins, and lipids such as leukotriene B_4 (Chapter 3). Invading bacteria release peptides with formylated methionine groups that are very attractive to the neutrophils of some mammals. Thus migrating neutrophils receive a multitude of signals, causing them to swarm toward sites of invasion and tissue damage in large numbers.

Some cattle with a specific genotype of the chemokine receptor CXCR2 have reduced neutrophil migration compared with normal cattle. Cows with this genotype also have reduced expression of the integrin chains CD18 and CD11b and as a result have decreased resistance to bacterial mastitis.

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Adherence and Opsonization

Once a neutrophil encounters a bacterium, it must "catch" it. This does not happen spontaneously because both cells and bacteria suspended in body fluids usually have a negative charge (zeta potential) and repel each other. The electrostatic charge on the bacteria must be neutralized by coating them with positively charged molecules. Molecules that coat bacteria in this way and promote phagocytosis are called opsonins. This word is derived from the Greek word for "sauce," implying that they make the bacterium more attractive to neutrophils. Examples of such opsonins include mannose-binding lectin, fibronectin, some complement components, and most importantly, antibodies (Chapter 26).

Antibodies, the major proteins of the adaptive immune system, are by far the most effective opsonins. They coat bacteria, link them to receptors on phagocytic cells, and trigger their ingestion. Antibody receptor–mediated phagocytosis (or type 1 phagocytosis) is triggered when antibody-coated bacteria attach to receptors on the neutrophil (Fig. 5.10). CD32 is an example of such an antibody receptor. It binds to the Fc region of antibody molecules (Chapter 15). CD32 is therefore an example of an Fc receptor (FcR). (There are several different Fc receptors; CD32 is classified as FcγRII.) However, as pointed out previously, antibodies are not produced until several days after the onset of an infection, and the body must rely on innate opsonins for immediate protection. CD35 (or complement receptor-1, CR1) binds the complement component C3b. C3b-coated bacteria bind to neutrophil CD35, but this may not necessarily trigger ingestion. The surface of phagocytic cells is also covered with many pattern-recognition receptors (PRRs) that can bind their ligands on bacteria.

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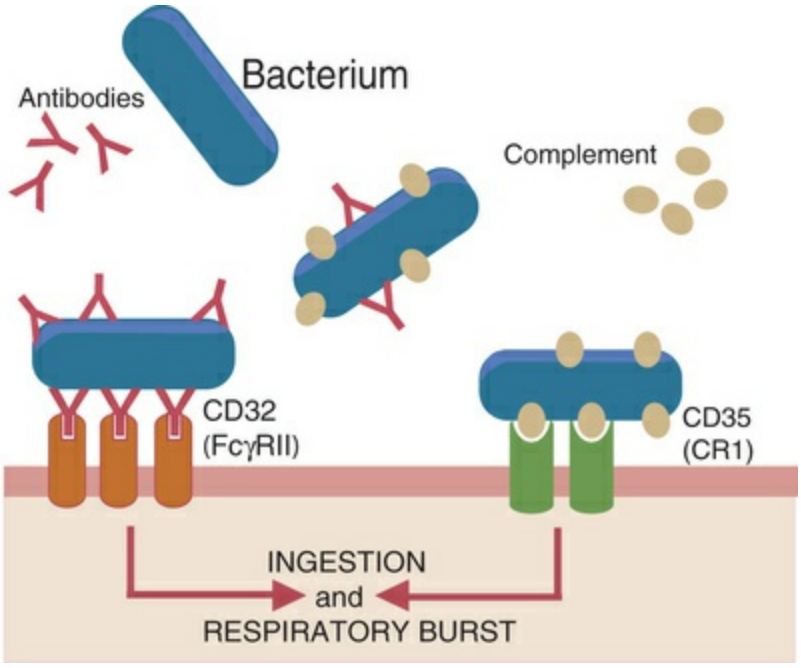


FIG. 5.10 The opsonization of a bacterium by antibodies and complement. Binding of these ligands to their receptors triggers ingestion and the respiratory burst. The antibody receptor is called CD32, and the complement receptor is called CD35. Type 1 phagocytosis is mediated by antibodies through CD32. Type 2 phagocytosis is mediated by complement through CD35.

Extracellular Traps

Neutrophils trap and kill extracellular bacteria. Some neutrophils undergo a form of cell death termed *NETosis* as an alternative to apoptosis or necrosis. After activation by CXCL8 or lipopolysaccharides, neutrophil oxidants release of the contents of the azurophil granules. These enzymes cause chromatin condensation and release strands of nuclear DNA and its associated proteins into the extracellular fluid. These strands form a network of extracellular fibers called neutrophil extracellular traps (NETs) (Fig. 5.11). These NETs are coated with antimicrobial proteins, including histones and granule components such as elastase, myeloperoxidase, lactoferrin, and gelatinase. As a result, NETs not only physically capture bacteria but also kill them. NETs are abundant in sites of acute inflammation including mastitic milk. It is believed that neutrophils can sense the size of their microbial targets and resort to NETosis when their targets are "too large to eat." They also use NETosis to trap and kill fungi such as *Candida*

albicans and protozoa such as *Leishmania amazonensis* and *Eimeria bovis* (Fig. 5.12). NETs may be very important in containing microbial invaders by acting as physical barriers and so preventing their spread.

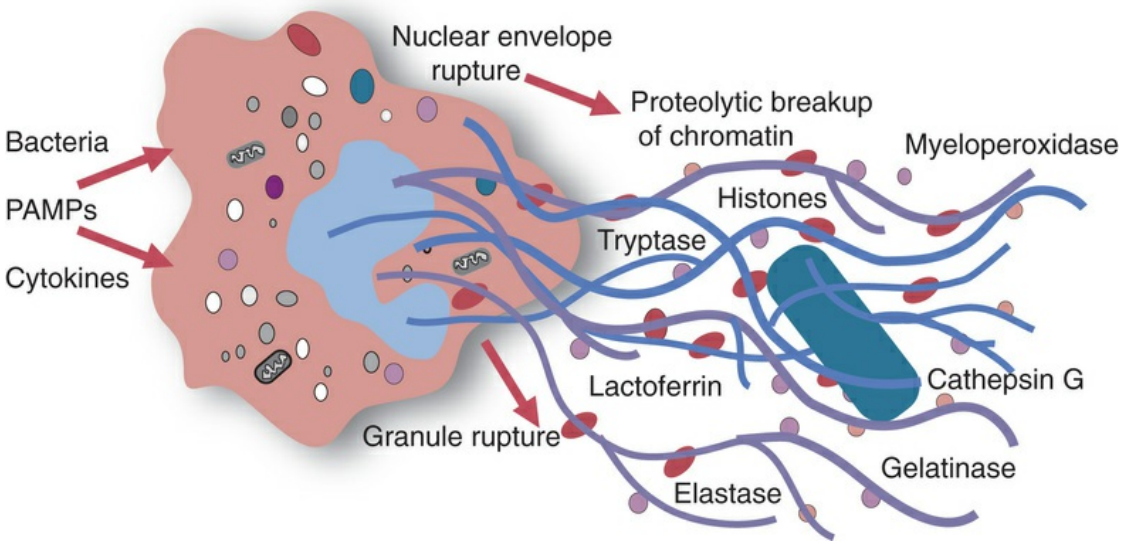


FIG. 5.11 The structure of neutrophil extracellular traps (NETs). NETs are composed of a network of DNA strands to which are attached neutrophil lysosomal enzymes such as myeloperoxidase, cathepsins, and elastases.

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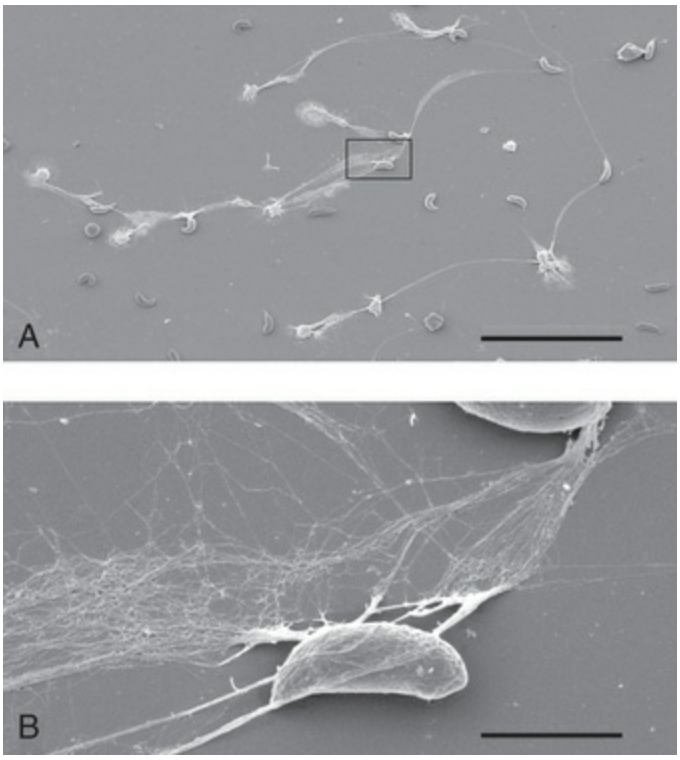


FIG. 5.12 NETs formed by bovine neutrophils cocultured with sporozoites of the protozoan parasite, *Eimeria bovis.* **A,** Several sporozoites can be seen sticking to a network of fibers originating from dead and disrupted neutrophils (scale bar, 50 μm). **B,** At higher magnification, it can be seen that the NETs consist of a meshwork of filaments, many of which are attached to a sporozoite (scale bar, 5 μm). (From Behrendt JH, Ruiz A, Zahner H, et al: Neutrophil extracellular trap formation as innate immune reactions against the apicomplexan parasite *Eimeria bovis, Vet Immunol Immunopathol* 133:1-8, 2010.)

Ingestion

As a neutrophil crawls toward a chemotactic source, a lamellipod advances first, followed by the main portion of the cell. The cytosol of the lamellipod contains a filamentous network of actin and myosin, whose state determines the fluidity of the cytoplasm. When a neutrophil meets a bacterium, the lamellipod flows over and around the organism, and binding occurs between opsonins on the organism and receptors on the neutrophil surface (Fig. 5.13). When antibody-coated microbes are bound by neutrophil CD32, they trigger polymerization of actin. As a result, actin-rich lamellipods extend from the cell to engulf the particle (type 1 phagocytosis).

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FIG. 5.13 A scanning electron micrograph of a bovine milk neutrophil ingesting *Streptococcus agalactiae.* Note how a film of neutrophil cytoplasm appears to flow over the surface of the bacterium. Original magnification ×5000.

In complement-mediated phagocytosis, particles sink into the neutrophil without lamellipod formation, suggesting that the ingestion process is fundamentally different from that mediated by antibodies (type 2 phagocytosis). The bacterium is eventually drawn into the cell, and as it is engulfed, it is enclosed in a vacuole called a phagosome. The ease of ingestion depends on the properties of the bacterial surface. Neutrophils readily flow over lipid surfaces so that hydrophobic bacteria, such as *Mycobacterium tuberculosis,* are readily ingested. In contrast, *Streptococcus pneumonia* has a hydrophilic capsule. It is poorly phagocytosed unless made hydrophobic by opsonization. A third type of ingestion occurs with bacteria such as *Legionella pneumophila* and *Borrelia burgdorferi,* where a single lamellipod may wrap itself several times around the organism. This is called coiling phagocytosis.

Destruction

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Neutrophils kill ingested bacteria through two distinct processes. One involves the generation of potent oxidants by a respiratory burst. The other involves release of lytic enzymes and antimicrobial peptides from intracellular granules (Box 5.1).

Box 5.1

Autophagy

Phagocytosis, as described in this chapter, involves the ingestion, killing, and digestion of extracellular particles such as invading bacteria. Cells can also destroy particles within the cytosol by autophagy (see Fig. 5.16). Autophagy is a form of cellular waste disposal. The structure to be destroyed, such as an intracellular microbe or a damaged cytoplasmic organelle, is first enclosed within a double membrane to form a cytosolic vesicle called an autophagosome. This then fuses with lysosomes, whose enzymes digest the contents of the autophagosome. Their macromolecules are then released back into the cytosol, where they can be recycled. Autophagy can be triggered by starvation to provide more amino acids for protein synthesis, but it can also be used to selectively remove organelles such as mitochondria, misfolded and aggregated proteins, and intracellular infectious agents. Thus TLR7 or FcγR signaling from phagosomes can initiate their targeting by the autophagy system, possibly by acting through the NOX complex. Autophagy plays important roles in elimination of intracellular pathogens, activation of intracellular pattern-recognition receptors, regulation of inflammasome activation, and intracellular antigen processing. Disorders of autophagy are associated with cancer, neurodegeneration, microbial infections, and aging.

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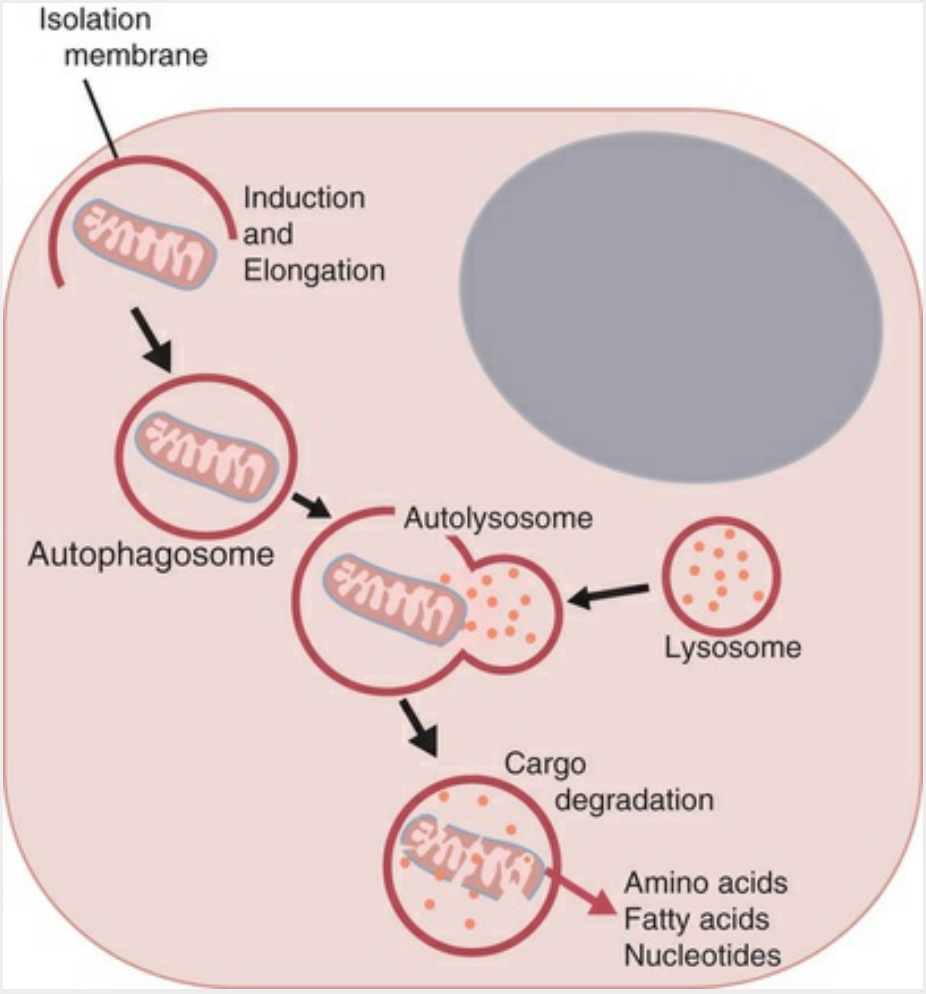


FIG. 5.16 The process of autophagy, a method of cellular waste disposal. An autophagosome forms within the cytoplasm and encloses the organelle or microbe to be destroyed. Lysosomes fuse with the autophagosome to form an autolysosome. The contents are degraded and recycled. This is a way to get rid of old, damaged organelles as well as intracellular bacteria.

The Respiratory Burst

Within seconds after binding a bacterium, neutrophils increase their oxygen consumption nearly 100-fold by activating a cell surface enzyme complex called NADPH oxidase (NOX). The NOX components are separate in resting cells, but when a neutrophil encounters TNF or is exposed to other inflammatory stimuli, they assemble to form a complete complex (Fig. 5.14). Activated NOX converts NADPH (the reduced form of NADP, nicotinamide adenine dinucleotide phosphate) to $NADP^+$ with the release of electrons. A molecule of oxygen accepts a donated electron, generating a superoxide anion (the dot in $^{\bullet}O_2^-$ denotes the presence

of an unpaired electron):

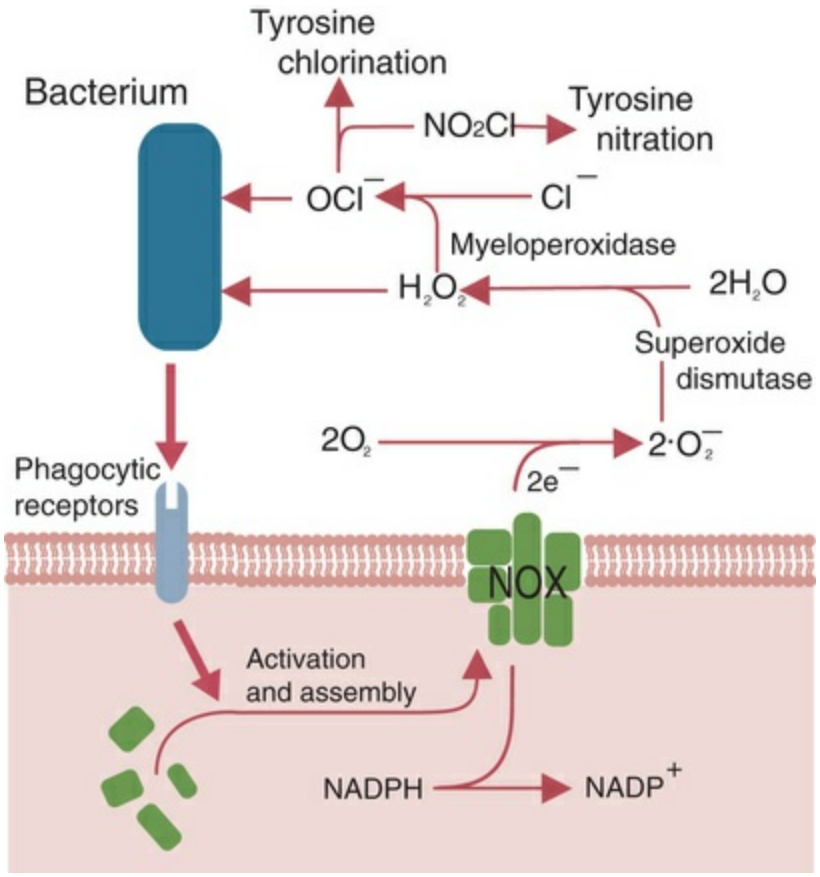


FIG. 5.14 The major features of the respiratory burst pathway in neutrophils. Binding of opsonized bacteria to phagocytic receptors such as CD32 triggers the process. It results in the assembly of the multicomponent enzyme NADPH oxidase (NOX) in the membrane of the phagosome. Once assembled, NOX catalyzes the generation of singlet oxygen. In association with other enzymes such as superoxide dismutase and myeloperoxidase, bactericidal products such as hydrogen peroxide (H_2O_2) and hypochlorite ions (OCl^-) are then generated.

$$NADPH + 2O_2 \xrightarrow{NOX} NADP^+ + H^+ + 2^{\bullet}O_2^-$$

Two superoxide anions interact spontaneously (dismutate) to generate one molecule of H_2O_2 under the influence of the enzyme superoxide dismutase:

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$$2^{\bullet}O_2^- + 2H^+ \xrightarrow{\text{superoxide dismutase}} H_2O_2 + O_2$$

Myeloperoxidase then catalyzes the reaction between hydrogen peroxide and chloride ions to produce hypochlorite:

$$H_2O_2 + Cl^- \xrightarrow{\text{myeloperoxidase}} H_2O + OCl^-$$

Plasma Cl^- is used in most inflammatory sites except in milk and saliva, where SCN^- is also employed. Hypochlorous acid (HOCl) is the major product of neutrophil oxidative metabolism. Because of its reactivity, HOCl is rapidly consumed in multiple reactions. As long as H_2O_2 is supplied (neutrophils can generate H_2O_2 for up to 3 hours after triggering), myeloperoxidase will generate HOCl. HOCl kills bacteria by unfolding and aggregating their proteins and oxidizing their lipids, and it enhances the bactericidal activities of the lysosomal enzymes. (Remember that HOCl is the active ingredient of household bleach and is commonly used to prevent bacterial growth in swimming pools.)

There are quantitative differences in neutrophil activity between the domestic species, especially in the intensity of the respiratory burst. For example, sheep neutrophils produce less superoxide than human or bovine neutrophils. Neutrophils also have safety mechanisms to detoxify oxidants and minimize collateral damage. Thus they contain large amounts of glutathione, which reduces the oxidants. Redox-active metals such as iron can be bound to lactoferrin to minimize -OH formation, and antioxidants such as ascorbate and vitamin E interrupt these reactions.

Superoxide, hydrogen peroxide, singlet oxygen, hypohalides and organic peroxides are collectively known by the term *reactive oxygen species (ROS)*. These molecules act at the atomic level, binding to sulfur atoms in the side chains of cysteine and methionine. They inhibit serine/threonine kinases and phosphatases, many transcription factors, signal-regulating proteins and ion channels. They also oxidize bases in DNA and so influence transcription. ROS activate inflammasomes and promote B and T cell activation.

Lytic Enzymes

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Once a bacterium is ingested by a neutrophil, the cell's granules (or lysosomes) migrate through the cytoplasm, fuse with the maturing phagosome, and release their enzymes as the pH drops. (The complete vacuole is then called a phagolysosome.) The rise in ionic strength within phagosomes releases elastase and cathepsin G from their sulfated proteoglycan matrix (Fig. 5.15). Other lysosomal enzymes include lysozyme, proteases, acid hydrolases, and myeloperoxidase. They also contain high concentrations of antimicrobial defensins and cathelicidins. The enzymes that accumulate in phagosomes can digest bacterial walls and kill most microorganisms, but, as might be expected, variations in susceptibility are observed. Gram-positive bacteria susceptible to lysozyme are rapidly destroyed. Gram-negative bacteria such as *Escherichia coli* survive somewhat longer since their outer wall is resistant to digestion. Lactoferrin, by binding iron, may deprive bacteria of this essential nutrient and limit bacterial growth (Chapter 7). Some organisms such as *Brucella abortus* and *Listeria monocytogenes* can interfere with phagosomal maturation in such a way that they do not come into contact with the lysosomal enzymes and can therefore grow inside phagocytic cells. Neutrophil enzymes released into tissues cleave membrane-bound TNF-α from macrophages. The TNF-α attracts and activates yet more neutrophils.

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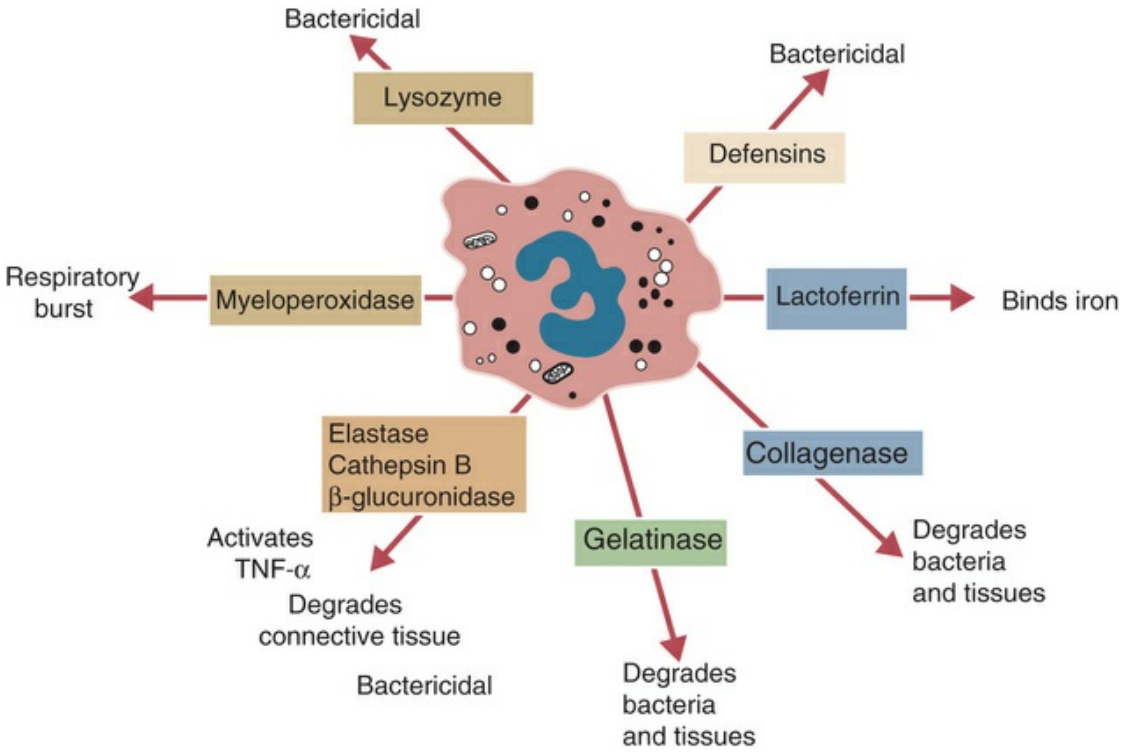


FIG. 5.15 Some of the enzymes and other antibacterial molecules found in the cytoplasmic granules of neutrophils.

Cytokines

Under the influence of bacterial products such as lipopolysaccharides, neutrophils produce many different cytokines such as IL-1, TNF-α, IL-6, CXCL8, IL-10, and transforming growth factor-β (TGF-β). Although individual neutrophils produce only small amounts of these cytokines, they invade inflammatory sites in enormous numbers, so their total contribution may be significant.

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Surface Receptors

Cells must interact with many molecules and other cells in their environment. To this end, they express many cell surface receptors. As mentioned in Box 2.4, cell surface glycoproteins are classified by the cluster of differentiation (CD) system. Neutrophils carry many different CD molecules on their surface (Fig. 5.17). The most relevant of these are the receptors for opsonins and those that attach neutrophils to blood vessel walls. Other neutrophil surface molecules include receptors for inflammatory mediators such as leukotrienes, complement components such as C5a, chemokines, and cytokines.

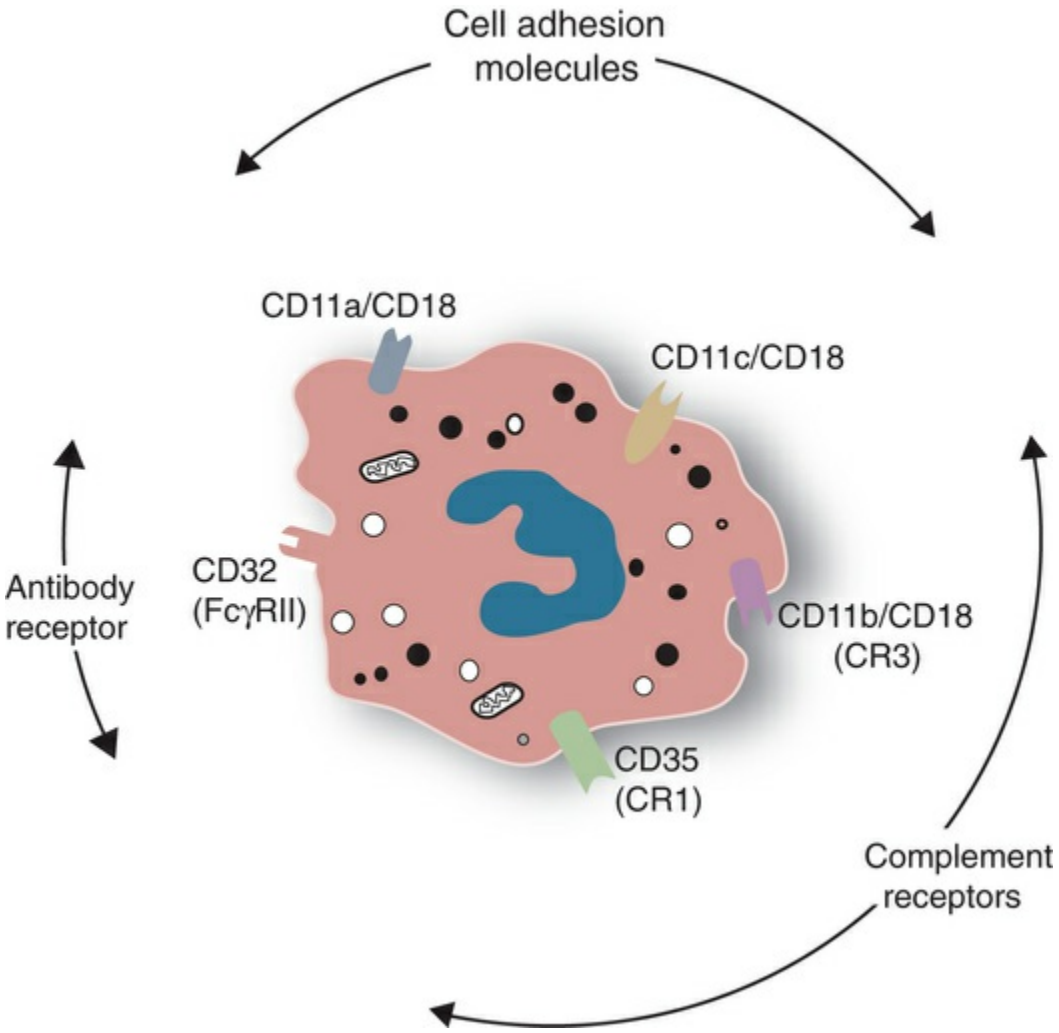


FIG. 5.17 Some of the major surface receptors on neutrophils and their functions.

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Fate

Neutrophils are short-lived terminally differentiated cells that have a limited reserve of energy that cannot be replenished. They are therefore most active immediately after release from the bone marrow but are rapidly exhausted and can undertake only a limited number of phagocytic events. Most neutrophils survive for only a few days. They die as a result of apoptosis and mononuclear phagocytes remove the cell corpses. Most such cell death is physiological, simply removing unwanted, unused cells. As neutrophils age they express changes in their surface that send an "eat-me" message to monocytes. For example, the phospholipid phosphatidyl serine is normally found only on the inner side of the plasma membrane. As neutrophils age the membrane flips, and phosphatidyl serine is exposed and recognized by macrophages that promptly eat the affected cell, a form of cell death called efferocytosis.

Neutrophil apoptosis also occurs in the presence of inflammatory stimuli, especially ROS. This may also involve the formation of NETs of exocytosed DNA. When dendritic cells ingest apoptotic neutrophils containing bacteria, they secrete TGF-β, IL-6, and IL-23. As described previously, this IL-23 stimulates the differentiation of Th17 cells that attract even more neutrophils (Chapter 20). Conversely, ingestion of uninfected apoptotic neutrophils triggers the secretion of IL-10 and TGF-β, promoting the production of regulatory T cells and suppressing inflammation (Chapter 20).

Thus neutrophils may be considered a first line of defense, converging rapidly on invading organisms and destroying them promptly but being incapable of sustained effort. The second line of defense is the mononuclear phagocyte system. DAMPs released by neutrophil degranulation or death promote the recruitment and activation of both macrophages and dendritic cells, augmenting both the innate and adaptive immune responses (see Fig. 2.8).

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Cellular Innate Immunity

Macrophages and Recovery From Inflammation

CHAPTER OUTLINE

Macrophages, 49
- Structure, 50
- Life History, 50

Functions, 51
- Sentinel Cells, 51
- Inflammation, 51
- Phagocytosis, 51
- *Generation of Reactive Nitrogen Species, 52*
- Macrophage Polarization, 52
- Activation, 53
- Receptors, 54

Fate of Foreign Material, 55
- Soluble Proteins Given Intravenously, 56
- Fate of Material Administered by Other Routes, 56

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Respiratory Tract, 56
Resolution of Inflammation, 57

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Explain the origins, relationships, and life history of monocytes and macrophages.
- Identify a macrophage based on its morphology.
- Explain the importance of macrophages in inflammation.
- Describe the importance of macrophages in repairing damaged tissues and initiating the wound healing process.
- Explain how macrophages generate the powerful oxidizing agent, nitric oxide.
- Understand the important role that macrophages play as antigen-presenting cells for the adaptive immune system.
- Describe the two major functional subclasses of macrophages and the significance of this polarization in combating infection and restoring tissue integrity.
- Explain the role of macrophages in granuloma formation.
- Define monocyte, macrophage, reactive nitrogen species, macrophage activation, granuloma, and pulmonary intravascular macrophage.
- Explain how the respiratory tract is cleared of foreign particles.
- Explain the role of macrophages in removing foreign material from the bloodstream.

Although neutrophils act as a first line of defense, mobilizing rapidly, converging, and eating and killing invading microorganisms with enthusiasm, they cannot, by themselves, ensure that all invaders are killed. The body therefore employs a "backup" system employing multipurpose phagocytic cells collectively known as macrophages. Macrophages differ from neutrophils in their speed of response, which is slower; in their antimicrobial abilities, which are greater; and in their ability to

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initiate adaptive immunity by processing antigens. They also act as sentinel cells and initiate tissue repair. Unlike neutrophils, which are specialized for a single task—the killing of invading organisms—macrophages are much more flexible. Depending on their location and environmental signals, macrophages can respond in many different ways. The use of two phagocytic cell systems also permits cooperation between neutrophils and macrophages to enhance many aspects of innate immunity. For example, neutrophils tend to be of greater importance in killing extracellular pathogens, whereas macrophages dominate in the fight against intracellular pathogens.

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Macrophages

Macrophages not only detect and kill invading microbes, but they also produce a mixture of cytokines that promotes both innate and adaptive immune responses; they can control inflammation; and they contribute directly to the repair of damaged tissues by removing dead, dying, and damaged cells and assist the healing process. Their name is derived from the fact that they are "large-eating" cells (Greek *macro, phage*).

Macrophages are a diverse family of cells that reside in different tissues, and as a result differ greatly in their transcriptional profiles and in their functions. They can change their activation state and phenotype in response to signals, especially cytokines, from other cells and from their local environment. This adaptability and resulting phenotypic diversity has given rise to a confusing nomenclature.

One population of bone marrow-derived macrophages circulates in the bloodstream, where they are called monocytes. It was long believed that all tissue macrophages were derived from these blood monocytes. Recent studies, however, have shown that some tissue macrophages such as those in the brain develop directly from embryonic precursors. Their stem cells enter tissues in embryonic life and persist in adults by self-renewal. Macrophages are found throughout the body in connective tissue, where they are called histiocytes; those found lining the sinusoids of the liver are called Kupffer cells; those in the brain are microglia. The macrophages in the alveoli of the lungs are called alveolar macrophages, whereas those in the capillaries of the lung are called pulmonary intravascular macrophages. Some macrophages can develop into dendritic cells. Large numbers are found in the sinusoids of the spleen, bone marrow, and lymph nodes. Irrespective of their name, origin, or location, they are all considered macrophages and collectively form the mononuclear phagocyte system (Fig. 6.1).

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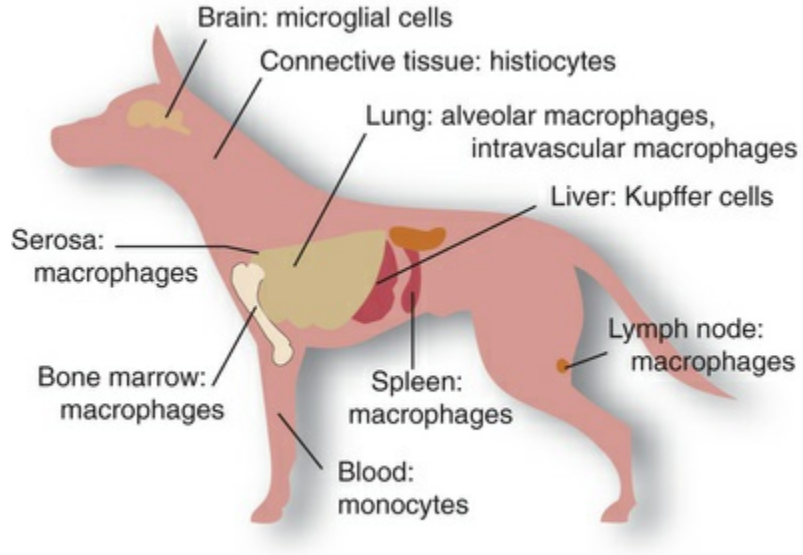


FIG. 6.1 The location of the major populations of cells of the mononuclear phagocyte system.

Structure

In suspension, monocytes are round cells about 15 to 20 μm in diameter. They possess abundant cytoplasm, at the center of which is a single large rounded nucleus (Fig. 6.2). Their central cytoplasm contains mitochondria, large numbers of lysosomes, some rough endoplasmic reticulum, and a Golgi apparatus, indicating that they can synthesize and secrete proteins (Figs. 6.3 and 6.4). In living cells, the peripheral cytoplasm is in continuous movement, forming and reforming veil-like ruffles. Many macrophages show variations in this basic structure. For example, blood monocytes have round nuclei that elongate as the cells mature. Alveolar macrophages contain very little rough endoplasmic reticulum, but their cytosol is full of granules. The microglia of the central nervous system have rod-shaped nuclei and very long cytoplasmic processes (dendrites) that are lost when the cell responds to tissue damage.

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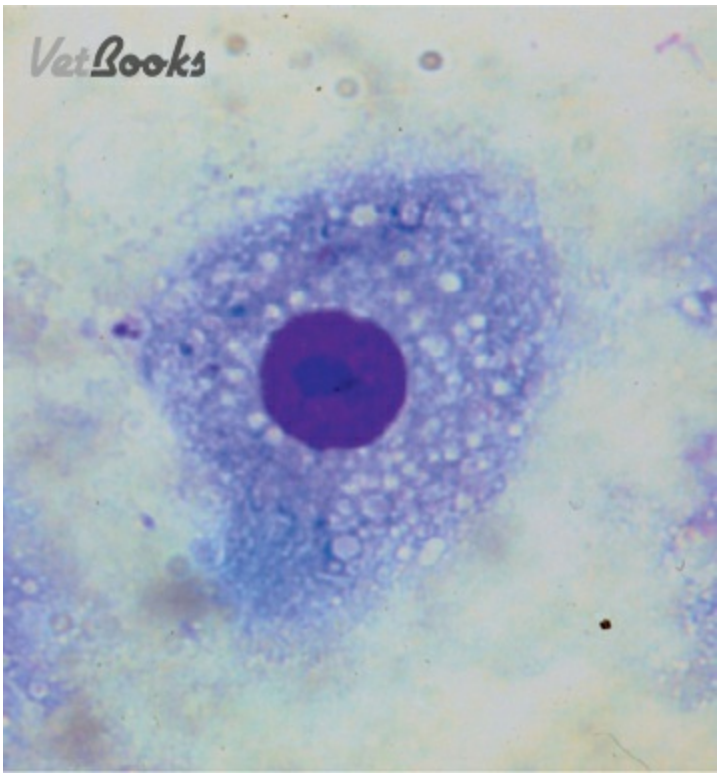


FIG. 6.2 A typical bovine macrophage. Original magnification ×500.

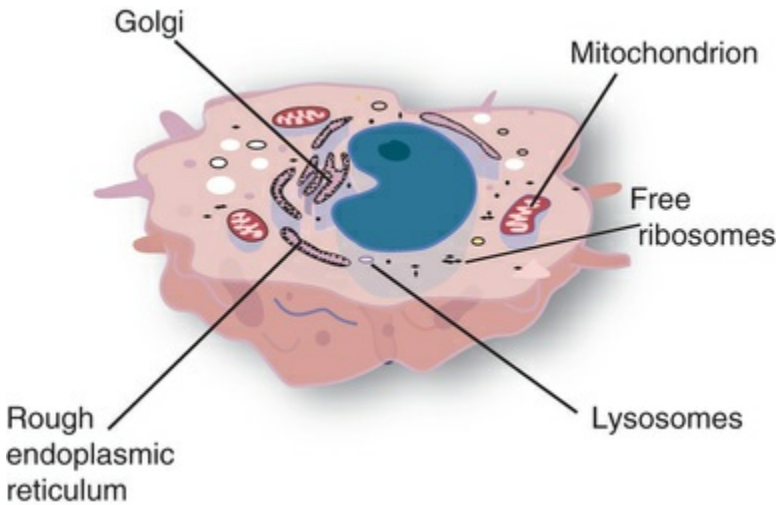


FIG. 6.3 The major structural features of a macrophage. The presence of a rough endoplasmic reticulum demonstrates that these cells can synthesize significant amounts of proteins.

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FIG. 6.4 Transmission electron micrograph of a normal rabbit macrophage. The nature of the large inclusion is unknown. (Courtesy Dr. S. Linthicum.)

Life History

Macrophages arise from multiple sources. Thus monocytes and intestinal macrophages develop from myeloid stem cells in the bone marrow (Fig. 6.5). On the other hand, tissue macrophages such as Kupffer cells and microglia arise from yolk sac or fetal liver stem cells. During development, the myeloid stem cells give rise in sequence to monoblasts, promonocytes, and eventually to monocytes, all under the influence of cytokines called colony-stimulating factors. Monocytes enter the bloodstream and circulate for about 3 days before entering tissues and developing into macrophages. They account for about 5% of the total leukocyte population in mammalian blood. Tissue macrophages either originate from monocytes or arise by division of precursor stem cells within tissues. They are usually long-lived cells, replacing themselves at a rate of about 1% per day unless activated by inflammation or tissue damage. Macrophages may live for a long time after ingesting inert particles, such as the carbon in tattoo ink, although they may fuse together to form multinucleated giant cells in response to DNA damage and mitotic defects. At least some

dendritic cell populations appear to be specialized macrophages optimized for antigen processing and presentation (Chapter 10).

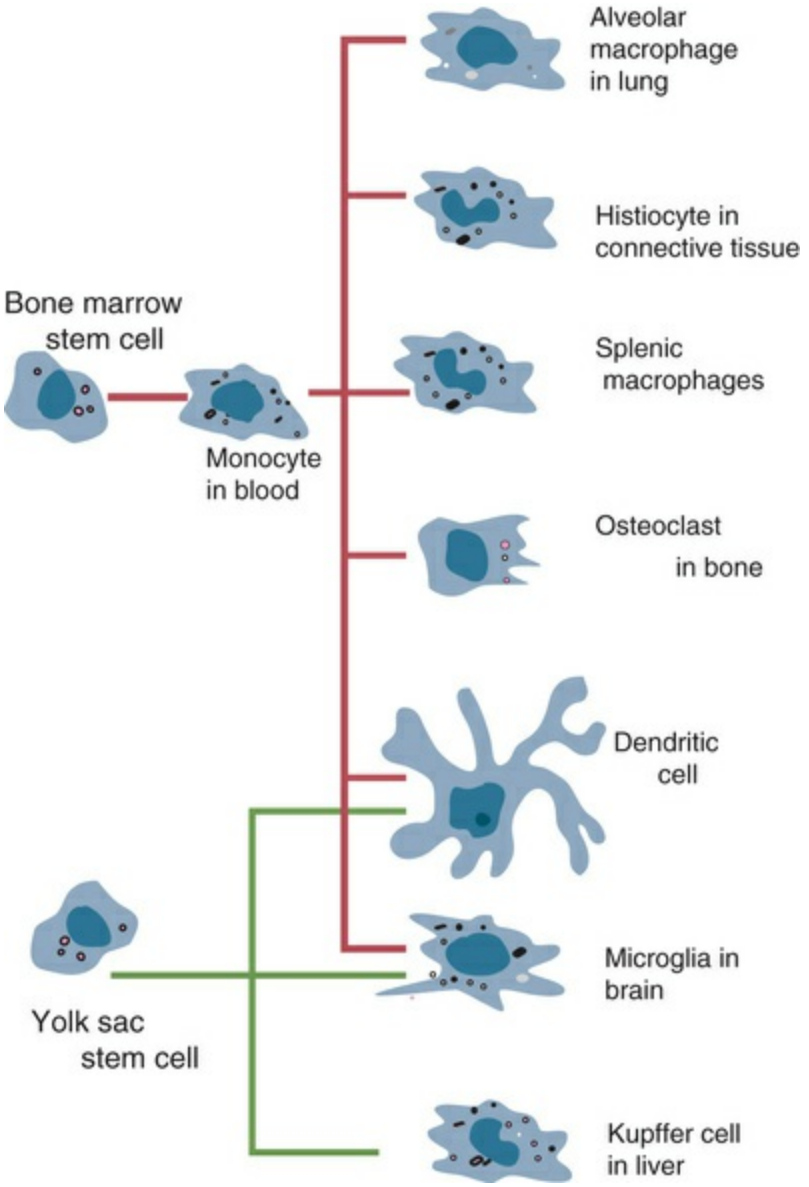


FIG. 6.5 The origin and development of macrophages. Monocytes in blood can differentiate into many different types of macrophage. They can also differentiate into dendritic cells. Some macrophage populations such as the microglia and Kupffer cells may originate from stem cells in the fetal yolk sac.

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Functions

Sentinel Cells

As described in Chapter 2, macrophages act as sentinel cells. They are widely distributed through the body and express many different pattern-recognition receptors (PRRs). They detect and respond to invading bacteria and viruses as well as tissue damage. In addition to triggering effective phagocytosis, macrophages produce many cytokines. The most important of these are interleukin-1 (IL-1), IL-6, IL-12, IL-18, and tumor necrosis factor-α (TNF-α) as well as high-mobility group box protein-1 (HMGB1) (Fig. 6.6). They also produce chemokines, such as CXCL8 (IL-8), that recruit and attract neutrophils.

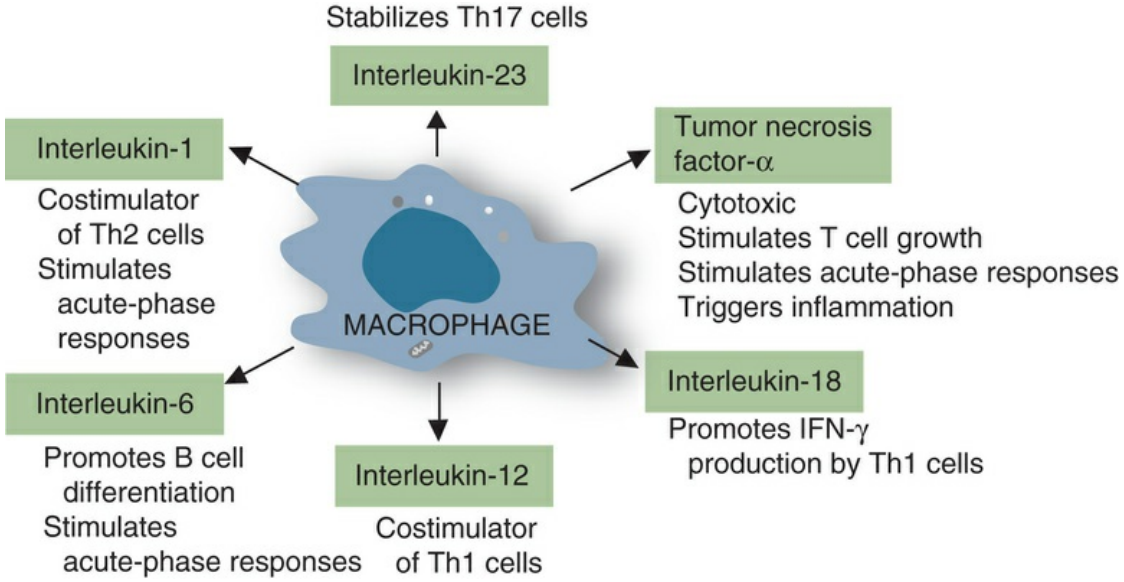


FIG. 6.6 Some of the most important cytokines produced by macrophages and their functions.

Inflammation

Macrophages recognize tissue damage, promote the recruitment of neutrophils, and regulate the processes by which neutrophils recruit monocytes. As sentinel cells, macrophages trigger

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neutrophil emigration from blood vessels. The release of HMGB1 and other damage-associated molecular patterns (DAMPs) from damaged tissues stimulates macrophages to produce TNF-α and IL-6 as well as neutrophil chemotactic chemokines and reactive oxygen species (ROS).

Exosomes are small cytoplasmic vesicles, about 50 to 100 nm in diameter, that can transmit signals between cells. They are released by stimulated macrophages, dendritic cells, and B cells. These exosomes carry with them a mixture of immunostimulatory and proinflammatory molecules. They can spread through the extracellular fluid and interact with nearby cells. Thus exosomes from macrophages containing ingested bacteria can express bacterial cell wall components such as glycopeptidolipids and other pathogen-associated molecular patterns (PAMPs) on their surfaces. These exosomes can bind to PRRs on nearby neutrophils and macrophages, triggering the release of TNF-α and iNOS, and promoting more inflammation.

Phagocytosis

When microbial invasion occurs and inflammation develops, blood monocytes respond to PAMPs and DAMPs by binding to vascular endothelial cells in a manner similar to their partners, the neutrophils. Thus adherence and rolling are triggered by selectin binding, and the cells are brought to a gradual halt by integrins binding to ligands on vascular endothelial cells. The monocytes bind to endothelial cell intracellular adhesion molecule-1 (ICAM-1) by using their β_2-integrins and then emigrate into the tissues (and change their name to macrophages). Several hours after neutrophils have entered an inflammatory site, the macrophages arrive. Neutrophils can reach targets in damaged tissues within 3 to 4 hours. Macrophages require at least 12 hours. These macrophages are attracted not only by bacterial products and complement components such as C5a but also by DAMPs from damaged cells and tissues. Once neutrophils have emigrated into tissues, they too attract macrophages. Thus neutrophil granules contain macrophage chemoattractants such as azurocidin and cathelicidins. Activated neutrophils and endothelial cells also produce monocyte

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chemoattractant protein-1 (CCL2) under the influence of IL-6. Neutrophils are the martyrs of the immune system: they reach and attack foreign material first, and in undergoing apoptosis, they attract macrophages to the site of invasion. They also release defensins that augment the antimicrobial activities of macrophages.

Phagocytosis by macrophages differs from the process in neutrophils in that macrophages have few cytoplasmic granules. Instead, they generate oxidants using a respiratory burst or inducing nitric oxide synthesis. They also synthesize many new proteins including pro- and antiinflammatory cytokines, antimicrobial peptides and proteins, and enzymes. Macrophages destroy bacteria by both oxidative and nonoxidative mechanisms. In contrast to neutrophils, however, macrophages undertake sustained, repeated phagocytic activity. In addition, macrophages produce proteases such as collagenases and elastases that destroy nearby connective tissue. They produce plasminogen activator that generates plasmin, another potent protease. Thus macrophages can "soften up" the local connective tissue matrix and permit more effective penetration of the damaged tissue. Macrophages phagocytose both apoptotic neutrophils and their exosomes. The contents of neutrophil granules are not always destroyed but may be carried to macrophage endosomes where they can continue to inhibit the growth of bacteria. Thus neutrophils can enhance the effectiveness of macrophages in host defense. Macrophages can also release nuclear DNA and histones to form macrophage extracellular traps (METs) in response to bacterial pathogens and their exotoxins. Bovine monocyte-derived and alveolar macrophages exposed to *Mannheimia. haemolytica* have also been shown to produce METs. These METs, together with any captured bacteria, can then be endocytosed and destroyed by other macrophages.

Generation of Reactive Nitrogen Species

Major species differences emerge between macrophages in relation to the production of reactive nitrogen species (RNS), especially nitric oxide. Thus in some mammals, especially laboratory rodents, cattle, sheep, and horses (but not in humans, pigs, goats, or rabbits), microbial PAMPs trigger macrophages to express type 2 nitric oxide synthase (NOS2). (The overall gene expression profile of pig

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macrophages is much closer to humans than to mice.) NOS2 converts L-arginine and oxygen to citrulline and nitric oxide (NO) (Fig. 6.7). Nitric oxide alone is not highly toxic, but it can react with superoxide anion to produce potent RNS such as peroxynitrite and nitrogen dioxide radical.

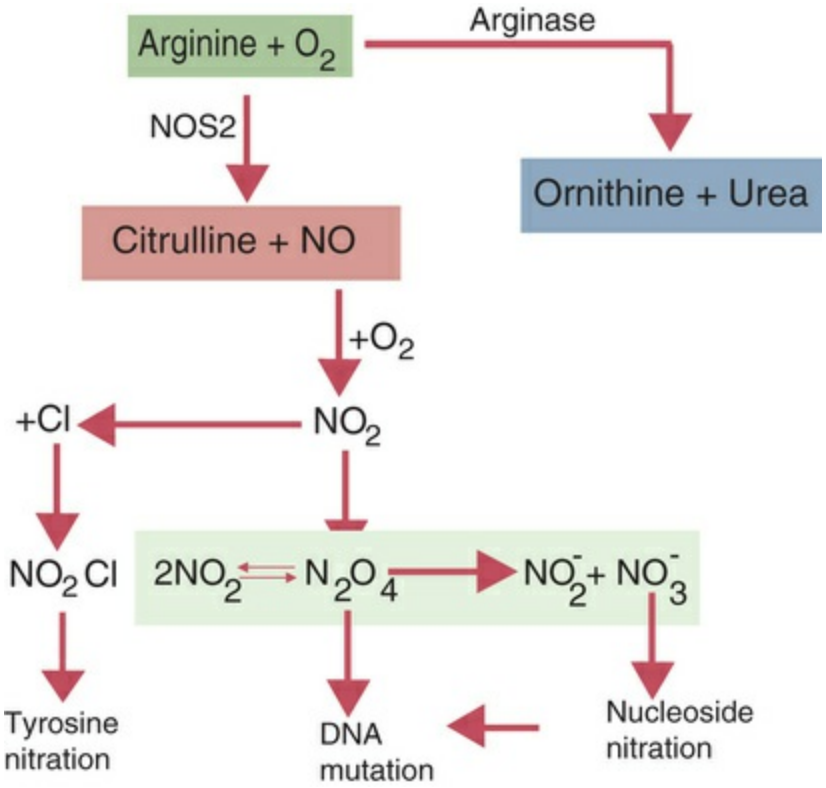


FIG. 6.7 The two pathways of arginine metabolism in macrophages. The production of nitric oxide through the use of nitric oxide synthase 2 is a major antimicrobial pathway and the key feature of M1 macrophages. The use of arginase to produce ornithine, however, reduces the antimicrobial activities of M2 cells.

$$NO + O_2^- \rightarrow OONO^- \rightarrow HOONO \rightarrow OH + NO_2^-$$

RNS inactivate iron and sulfur-containing enzymes such as the heme-containing respiratory enzymes. They nitrosylate proteins, oxidize lipids, and damage DNA.

Macrophage Polarization

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Macrophages are divided into two subsets based on their activation states and functions. M1 cells promote host defense through inflammation. M2 cells suppress inflammation and promote tissue repair. This polarization is not necessarily permanent. Macrophages can change their phenotype under the influence of other cytokines and microbial products. M1 and M2 cells should perhaps be considered a spectrum of phenotypes rather than distinct subpopulations.

M1 cells generate large amounts of RNS, and their function is enhanced by exposure to granulocyte-macrophage colony stimulating factor (GM-CSF), type I interferons, and IFN-γ as well as microbial pathogens and their products. The sustained production of RNS permits M1 macrophages to kill bacteria, fungi, protozoa, and some helminths, but at the cost of some tissue damage.

The second macrophage subset, called M2 cells, is generated by exposure to macrophage colony stimulating factor (M-CSF) and IL-4. M2 cells do not produce NO but instead convert arginine to ornithine using the enzyme arginase. These two macrophage populations play different roles in defending the body. M1 cells defend against microbial invaders and produce proinflammatory cytokines. M2 cells have opposite effects: they reduce inflammation and produce cytokines that suppress immune responses. M2 cells thus promote blood vessel formation, tissue remodeling, and tissue repair. M1 cells are produced early in the inflammatory process when inflammation is required. M2 cells, on the other hand, tend to appear late in the process when healing is required. M1 and M2 cells are attracted by different chemokine mixtures.

A third subset of macrophages, regulatory macrophages, are generated by exposure to the cytokine, interleukin-10 (IL-10). They have potent antiinflammatory activity and are discussed in Chapter 20.

Activation

Although macrophages are competent phagocytes, their activities are greatly enhanced by several activating pathways. Neutrophil granule proteins enhance monocyte adherence to vascular

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endothelium, trigger macrophages to secrete cytokines, and activate dendritic cells, thus promoting antigen presentation. Molecules that promote M1 macrophage polarization include PAMPs such as lipopolysaccharides, CpG di-nucleotides DNA, microbial carbohydrates, and heat-shock proteins, as well as many DAMPs. Different levels of M1 activation are recognized, depending on the triggering agent, and some bacteria, such as *Mycobacterium tuberculosis,* are better able to activate macrophages than others. Thus when monocytes first move into inflamed tissues, they produce increased amounts of lysosomal enzymes, increase phagocytic activity, increase the expression of antibody and complement receptors, and secrete more proteases (Fig. 6.8). The cytokines produced by these macrophages, especially TNF-α and IL-12, activate a population of lymphocytes called natural killer (NK) cells (Chapter 19). NK cells in turn secrete interferon-γ (IFN-γ), which activates macrophages still further. IFN-γ upregulates many different genes, especially that for NOS2. The NOS2 gene can be upregulated 400-fold by a combination of IFN-γ and mycobacteria. This increases NO production so that activated M1 macrophages become even more bactericidal (Box 6.1) (Chapter 18).

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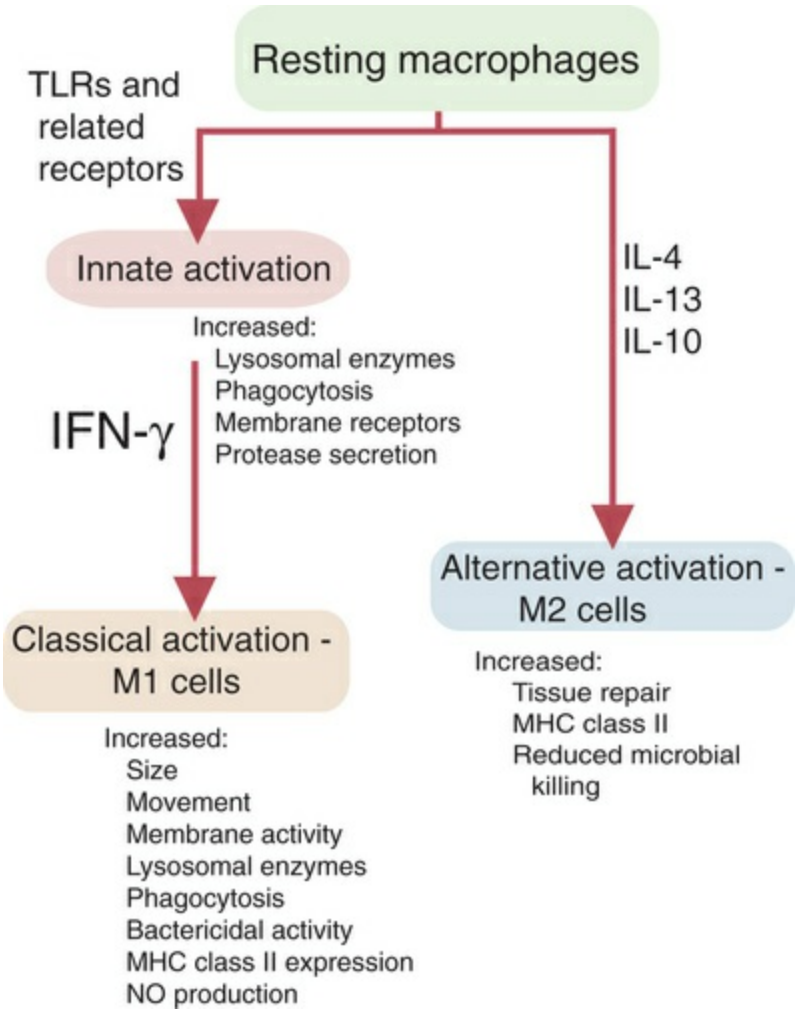


FIG. 6.8 The progressive activation of macrophages can involve three pathways. Innate activation occurs in response to TLR ligation. Additionally, macrophages can become classically activated M1 cells by exposure to microbial products and subsequent exposure to Th1 cytokines such as IFN-γ. Alternatively, they may undergo "alternative activation" on exposure to Th2 cytokines and become M2 cells.

Box 6.1

Circadian Rhythm and Monocytes

There is a daily oscillation of the numbers of monocytes present in mouse tissues. The numbers tend to increase while an animal is resting and decrease when the animal is active. Monocytes also express an intrinsic circadian clock where their gene expression pattern varies over 24 hours. The monocytes that exhibit the diurnal rhythm in tissues are "inflammatory" monocytes. At the beginning of the animal's resting time (daytime for a mouse), the

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circulating inflammatory monocyte numbers are low as is their tissue infiltration. At nighttime, when the animal is active, the number of circulating monocytes and their tissue infiltration increase. Similar rhythms have been observed in neutrophil homing to bone marrow and in the expression of TLR9 by B cells and splenic macrophages. One practical consequence of this is that an animal's response to a vaccine may also depend on the time of day when it receives the vaccine.

Druzd D, Scheiermann C: Some monocytes got rhythm, *Science* 341:1462-1464, 2013.

Receptors

Macrophages express thousands of different receptors on their surface (Fig. 6.9). All are glycoproteins. Some are PRRs, such as the toll-like receptors (TLRs) and the mannose-binding receptor (CD206). CD206 can bind carbohydrate chains terminating in mannose, fucose, or glucose on bacteria, and permits macrophages to ingest nonopsonized bacteria.

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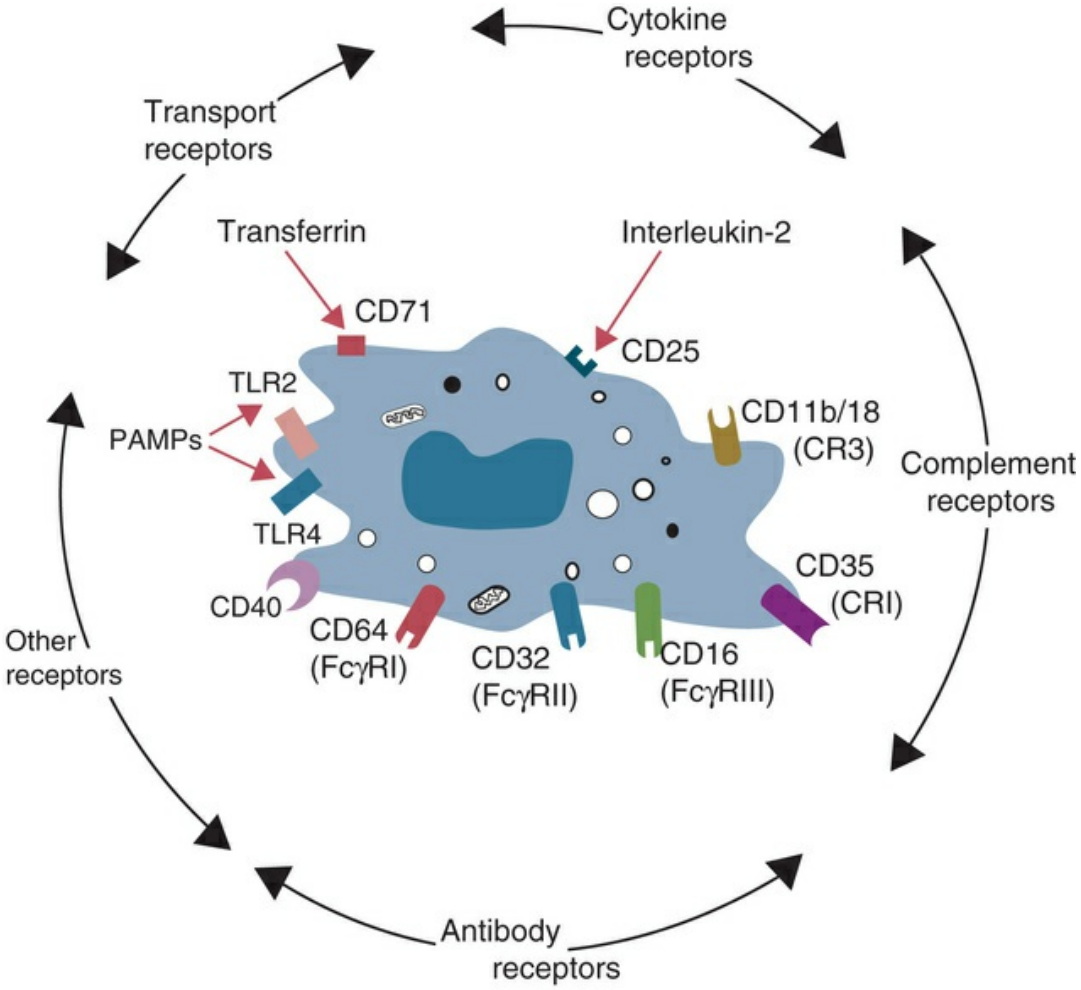


FIG. 6.9 Some of the major surface receptors expressed by macrophages and their functions.

CD64 is a high-affinity antibody receptor expressed on macrophages and, to a lesser extent, on neutrophils. Like other antibody receptors, CD64 binds the Fc region of antibody molecules and thus is called an Fc receptor (FcγRI). Its expression is enhanced by IFN-γ-induced activation so that a combination of antibody and IFN-γ are potent macrophage activators. Human macrophages also express two low-affinity antibody receptors, CD32 (FcγRII) and CD16 (FcγRIII). Cattle and sheep macrophages express a unique Fc receptor called Fcγ2R, which can bind particles coated with a specific type of antibody called immunoglobulin G2 (IgG2) (Chapter 16). Macrophages also have receptors for complement components. These include CD35 (CR1) and CD11b/CD18, both of which are C3b receptors.

Cell surface integrins bind macrophages to other cells, to connective tissue molecules such as collagen and fibronectin, and to

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some complement components. CD40 is used by macrophages to communicate with lymphocytes. Its ligand (CD154) is expressed on T cells. Thus T cells can activate macrophages via CD40.

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Fate of Foreign Material

Macrophages are located throughout the body and thus can detect and capture bacteria or fungi invading by many different routes. For example, bacteria injected intravenously are rapidly removed from the blood. Their precise fate depends on the species involved. In dogs, rodents, and humans, 80% to 90% are trapped and removed in the liver. The bacteria are removed by the macrophages (Kupffer cells) that line the sinusoids of the liver. The process occurs in two stages. Bacteria are first phagocytosed by blood neutrophils. These neutrophils are then ingested and destroyed by the Kupffer cells. These processes thus resemble acute inflammation in which neutrophils are primarily responsible for destruction of invaders, whereas the macrophages are responsible for preventing damage caused by apoptotic neutrophils (Table 6.1). In ruminants, pigs, horses, and cats, bacteria are mainly removed from the bloodstream by macrophages that line the endothelium of lung capillaries (pulmonary intravascular macrophages) (Figs. 6.10 and 6.11).

TABLE 6.1

Sites of Clearance of Particles From the Blood in Domestic Mammals

Species	LOCALIZATION (%)	
	Lung	Liver/Spleen
Calf	93	6
Sheep	94	6
Dog	6.5	80
Cat	86	14
Rabbit	0.6	83
Guinea pig	1.5	82
Rat	0.5	97
Mouse	1.0	94

Selected data from Winkler GC: Pulmonary intravascular macrophages in domestic animal species: review of structural and functional properties. *Am J Anat* 181:223, 1988; and Chitko-McKown CG, Blecha F: Pulmonary intravascular macrophages, a review of immune properties and functions, *Ann Rech Vet* 23:201-214, 1992.

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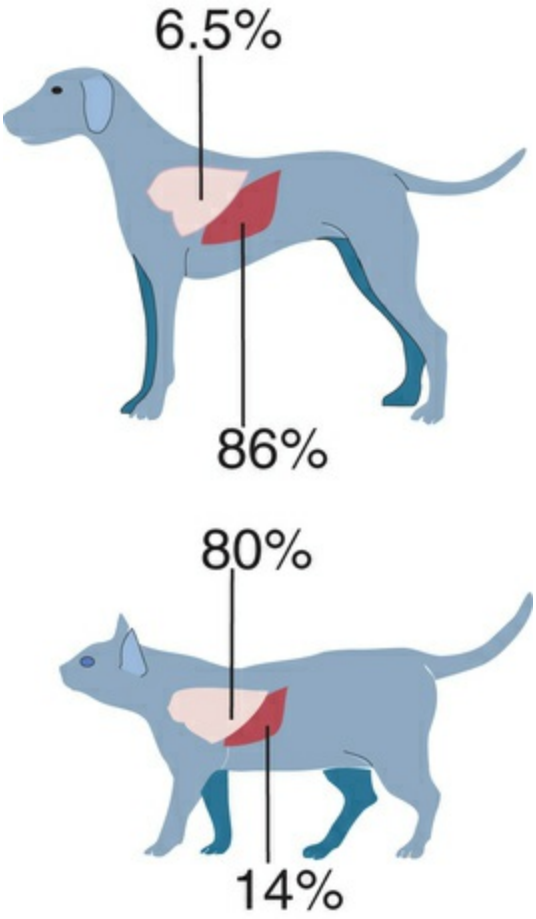


FIG. 6.10 The different routes by which bacteria are cleared from the bloodstream in the dog and cat expressed as percentages of an administered dose. Dogs mainly use Kupffer cells in the liver. Cats mainly employ pulmonary intravascular macrophages.



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FIG. 6.11 An intravascular macrophage *(M)* from the lung of a 7-day-old pig. The cell has numerous pseudopods, electron-dense siderosomes, phagosomes, and lipid droplets. It is closely attached to the thick portion of the air-blood tissue barrier that contains fibroblasts *(F)* and a pericyte *(P)* between basal laminae of the capillary endothelium *(E)* and the alveolar epithelium. At sites of close adherence, intercellular junctions with subplasmalemmal densities are seen *(arrow).* Bar = 2 μm. Original magnification ×8000. (From Winkler GC, Cheville NF: Postnatal colonization of porcine lung capillaries by intravascular macrophages: an ultrastructural morphometric analysis, *Microvasc Res* 33:224-232, 1987.)

In species where hepatic clearance is important, large viruses or bacteria may be cleared completely by a single passage through the liver (Fig. 6.12). The spleen also filters blood. It is a more effective filter than the liver, but since it is much smaller, it traps much less material. There are also differences in the type of particle removed by the liver and spleen. Splenic macrophages express antibody receptors (CD64) so that particles opsonized with antibody are preferentially removed in the spleen. In contrast, phagocytic cells in the liver express CD35, a receptor for complement C3, so that particles opsonized by C3 are preferentially removed in the liver. The clearance of particles from the blood is regulated by soluble opsonins such as fibronectin, or mannose-binding lectin. Experimentally, if an animal is injected intravenously with a very large dose of particles such as colloidal carbon, these opsonins are temporarily depleted, and particles such as bacteria will not be removed from the bloodstream. In this situation, the mononuclear-phagocytic system is said to be “blockaded.”

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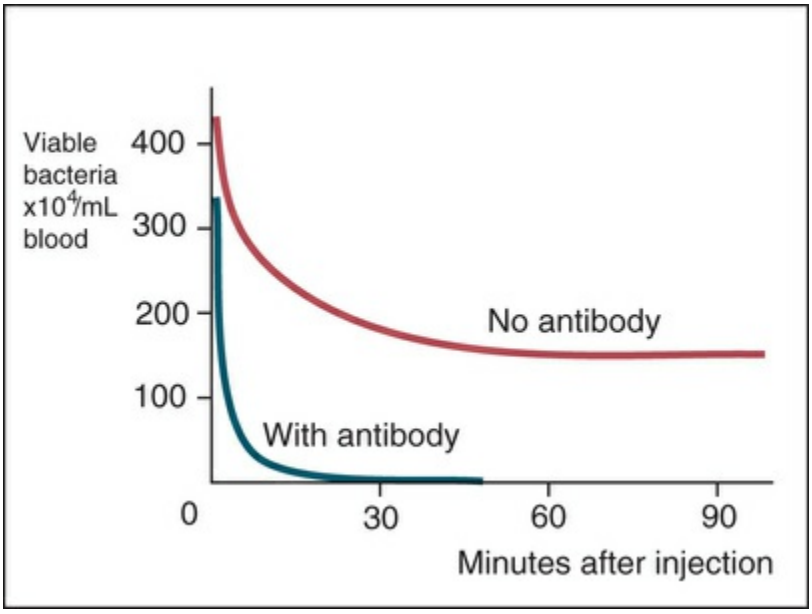


FIG. 6.12 The clearance of bacteria from the blood (in this case *Escherichia coli* from piglets). In the absence of antibodies, bacteria are slowly and incompletely removed.

Removal of bacteria from the bloodstream is greatly enhanced if they are opsonized by specific antibodies. If antibodies are absent or the bacteria possess an antiphagocytic polysaccharide capsule, the rate of clearance is decreased. Some molecules, such as bacterial endotoxins, estrogens, and simple lipids, stimulate macrophage activity and therefore increase the rate of bacterial clearance. Corticosteroids and other drugs that depress macrophage activity depress the clearance rate.

Soluble Proteins Given Intravenously

Unless carefully treated, protein molecules in solution tend to aggregate spontaneously. If such a protein solution is injected intravenously, neutrophils, monocytes, and macrophages rapidly remove these protein aggregates. Unaggregated protein molecules remain in solution and are distributed evenly through the animal's blood. Small proteins (<60 kDa) also spread throughout the extravascular tissue fluids. Once distributed, these proteins are catabolized, resulting in a slow but progressive decline in their concentration. Within a few days, however, the animal will mount an immune response against the foreign protein. Antibodies combine with the antigen; phagocytic cells remove these antigen-

antibody complexes from the blood; and the protein is rapidly eliminated (Fig. 6.13).

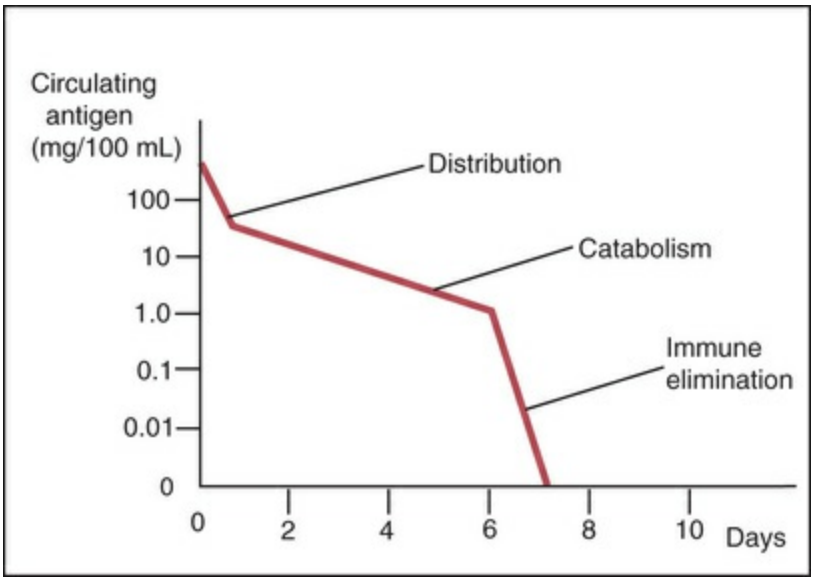


FIG. 6.13 The clearance of a soluble antigen from the bloodstream. Note the three phases of this clearance.

This triphasic clearance pattern of redistribution, catabolism, and immune elimination may change according to circumstances. For example, if the animal has not been previously exposed to a protein antigen, it takes between 5 and 10 days before immune elimination occurs. If, on the other hand, the animal has been primed by prior exposure to the protein, a secondary immune response will occur in 2 to 3 days, and the catabolic phase will be short. If antibodies are present at the time of antigen administration, immune elimination is immediate, and no catabolic phase is seen. If the injected material is not antigenic, or if an immune response does not occur, catabolism will continue until all the material is eliminated.

Fate of Material Administered by Other Routes

When foreign material is injected into a tissue, some local damage and inflammation are bound to occur, and DAMPs are released. As a result, neutrophils and macrophages migrate toward the injection site and phagocytose the injected material. Some will also be

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captured by dendritic cells. The material captured by macrophages and the dendritic cells can be processed and used to initiate adaptive immune responses. Antibodies and complement (Chapter 4) interact with the antigenic material, generating chemotactic factors that attract still more phagocytic cells and hastening its final elimination. In the skin, a web of antigen-trapping dendritic cells called Langerhans cells may trap foreign molecules and present them directly to lymphocytes. For this reason, intradermal injection of antigen may be most effective in stimulating an immune response.

Soluble macromolecules injected into a tissue are redistributed by the flow of tissue fluid through the lymphatic system. They eventually reach the bloodstream, so their final fate is similar to intravenously injected material. Any aggregated material present is phagocytosed by neutrophils, or tissue macrophages, or by the macrophages and dendritic cells of lymph nodes through which the tissue fluid flows.

Respiratory Tract

The fate of inhaled particles such as dust or aerosol droplets depends on their size. Large particles (>5 μm in diameter) stick to the mucous layer that covers the respiratory epithelium from the trachea to the terminal bronchioles (see Fig. 22.2). These particles are then removed by the flow of mucus toward the pharynx or by coughing. Very small particles that reach the lung alveoli are ingested by alveolar macrophages, which carry them back to the bronchoalveolar junction; from there, they are also removed by the flow of mucus. Nevertheless, some material may be absorbed from the alveoli. Small particles absorbed in this way are cleared to the draining lymph nodes, whereas soluble molecules enter the bloodstream and are distributed throughout the body. When large amounts of dust are inhaled, as occurs in workers exposed to industrial dusts or in cigarette smokers, the alveolar macrophage system may be "blockaded" and the lungs made more susceptible to microbial invasion.

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Resolution of Inflammation

It was once thought that inflammation simply went away when its cause, such as infection, was removed. This is not the case. The resolution of inflammation is an active process. Once invading organisms have been destroyed, affected tissues must switch from a killing process to a repair process. Proresolving molecules are produced that counteract the proinflammatory processes. For example, as inflammation progresses, macrophages change their polarization (Fig. 6.14). The first macrophages that enter the site are activated in the classical manner by TNF-α and GM-CSF to kill invading bacteria. However, these M1 macrophages gradually convert to M2 cells and develop antiinflammatory properties as they receive different signals from the tissues. Thus the same cell can act in a proinflammatory manner at the beginning of an infection but switch to antiinflammatory activities once the infection is overcome and it receives different signals.

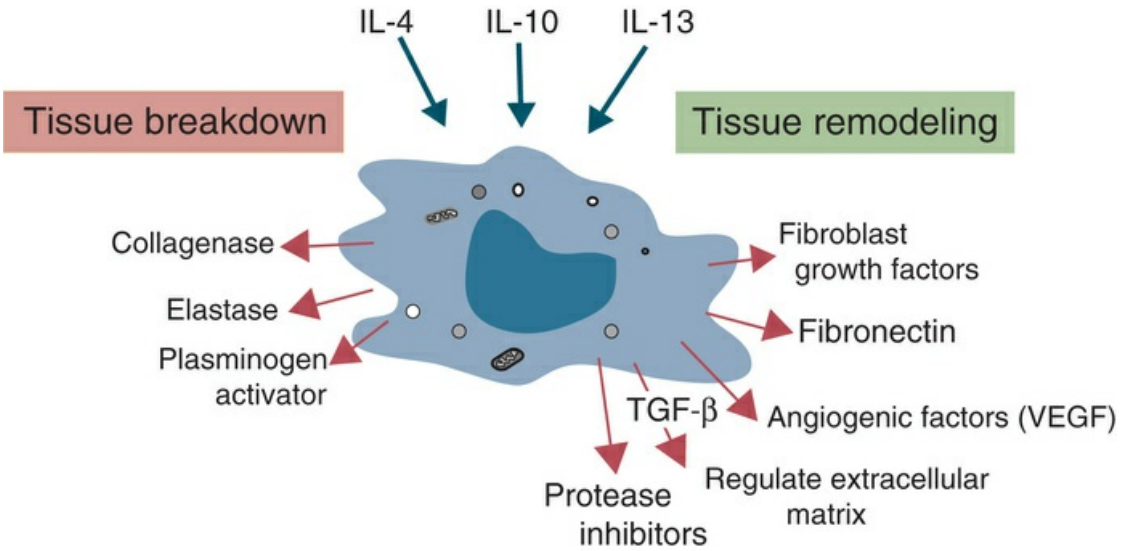


FIG. 6.14 The role of M2 macrophages in tissue breakdown and tissue repair in wound healing. In effect, damaged tissues must be removed before repair and remodeling can begin.

Inflammation is also resolved by an active coordinated process mediated by a complex mixture of lipids related to the leukotrienes. These include resolvins, protectins, maresins, and lipoxins.

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Produced by endothelial cells, resolvins and protectins promote phagocyte removal by reducing neutrophil emigration from the bloodstream and enhancing macrophage ingestion of apoptotic neutrophils. Maresins are produced by macrophages and enhance tissue repair while acting on nerves to reduce pain. Lipoxins enhance macrophage activity while reducing neutrophil emigration. Chemokines are destroyed by tissue metalloproteases. Apoptotic neutrophils exercise negative feedback by releasing lactoferrin that suppresses neutrophil recruitment. Dying neutrophils attract scavengers. Thus phagocytosis of apoptotic neutrophils by macrophages promotes production of vascular endothelial growth factor (VEGF), a cytokine that is crucial for revascularization and wound repair. Once generated, M2 cells secrete SLP1, a serine protease inhibitor. This molecule inhibits the release of elastase and oxidants by TNF-α-stimulated neutrophils and inhibits the activity of the elastase. SLP1 also protects the antiinflammatory cytokine transforming growth factor-β (TGF-β) from breakdown, and TGF-β, in turn, inhibits the release of TNF-α.

Even in normal healthy animals, many cells die daily and must be promptly removed. This is the job of the macrophages. For example, dying neutrophils release the nucleotides adenosine triphosphate and uridine triphosphate. These attract macrophages that therefore move rapidly toward the apoptotic cells. Macrophages "palpate" any neutrophils that they encounter. If the neutrophil is healthy, the cells separate. If, however, the neutrophil is dead or dying, the macrophage remains in contact and eats the neutrophil. This interaction (efferocytosis) operates through CD31 (Fig. 6.15). Thus CD31 on a neutrophil binds to CD31 on a macrophage. If the neutrophil is healthy, it sends a signal to the macrophage, causing it to disengage. On the other hand, if the neutrophil fails to signal, it will be eaten. It is interesting to note that this failure in CD31 signaling occurs long before a neutrophil begins to leak its enzyme contents and cause damage. Likewise the macrophages that consume these neutrophils do not release cytokines or vasoactive lipids. Ingestion of apoptotic neutrophils does, however, cause the macrophages to secrete more TGF-β, which in turn promotes tissue repair. Efferocytosis is thus an efficient way of removing apoptotic neutrophils without causing

tissue damage or inflammation.

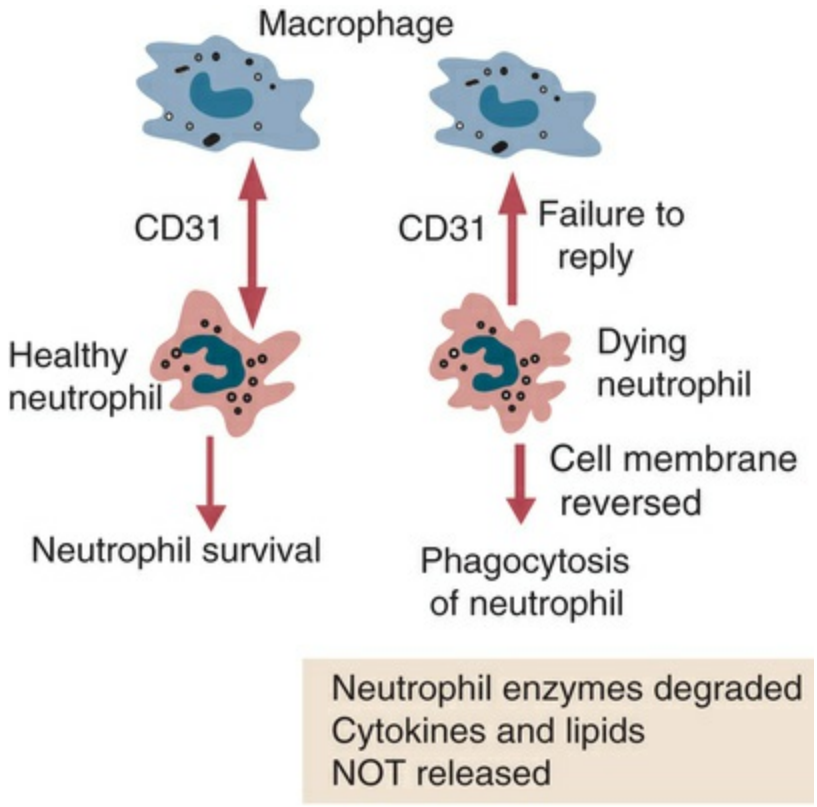


FIG. 6.15 The removal of apoptotic neutrophils. The reaction is initiated by interactions between CD31 on neutrophils and macrophages. If the neutrophil fails to reply when interrogated by a macrophage, it will be ingested and destroyed.

By secreting IL-1β, macrophages attract and activate fibroblasts. These fibroblasts enter the damaged area and produce collagen. Once sufficient collagen fibers have been deposited, their synthesis stops. This collagen is then remodeled over several weeks or months as the tissue returns to normal. The reduced oxygen tension in dead tissues stimulates macrophages to secrete cytokines such as VEGF that promote the growth of new blood vessels. Once the oxygen tension is restored to normal, new blood vessel formation ceases.

The final result of this healing process depends on the effectiveness of the inflammatory response. If the cause is rapidly and completely removed, healing will follow uneventfully. If tissue

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health is not restored, either because the invaders are not eliminated or because tissue repair is inadequate, inflammation may persist and become a damaging, chronic condition. Examples of persistent invaders include bacteria such as *M. tuberculosis,* fungi such as *Cryptococcus* species, parasites such as liver fluke, or inorganic material such as asbestos crystals. Macrophages, fibroblasts, and lymphocytes may accumulate in large numbers around the persistent material for months or years. Because they resemble epithelium in histological sections, these accumulated macrophages are called epithelioid cells. Persistent inflammation and especially prolonged TLR stimulation also causes macrophages to form various multinucleated giant cells. These develop as a result of an activated DNA damage response, repeated attempts at cell division and defects in mitosis. In all these cases, the persistence of foreign material results in the continual influx of new M2 macrophages that continue to attract fibroblasts and stimulate the deposition of collagen. The compact aggregate of immune cells that develops around this foreign material is called a granuloma (Fig. 6.16). Granulomas consist of granulation tissue—an accumulation of macrophages, lymphocytes, fibroblasts, loose connective tissue, and new blood vessels. The term *granulation tissue* is derived from the granular appearance of this tissue when cut. The "granules" are in fact new blood vessels. If the irritant is antigenic (e.g., some persistent bacteria, fungi, and parasites), the granuloma may contain many lymphocytes as well as macrophages, fibroblasts, and probably some neutrophils, eosinophils, and basophils (Fig. 6.17). The chronically activated M2 cells within these granulomas produce IL-1 that stimulates collagen deposition by fibroblasts and eventually "walls off" the lesion from the rest of the body. If the persistent foreign material is not antigenic (e.g., silica, talc, or mineral oil), few neutrophils or lymphocytes will be attracted to the lesion. Epithelioid and multinucleate giant cells, however, may continue their attempts to destroy the offending material. If the material is toxic for macrophages (as is asbestos), leaking enzymes and cytokines such as IL-6 may lead to central necrosis, chronic tissue damage, local fibrosis, and scarring.

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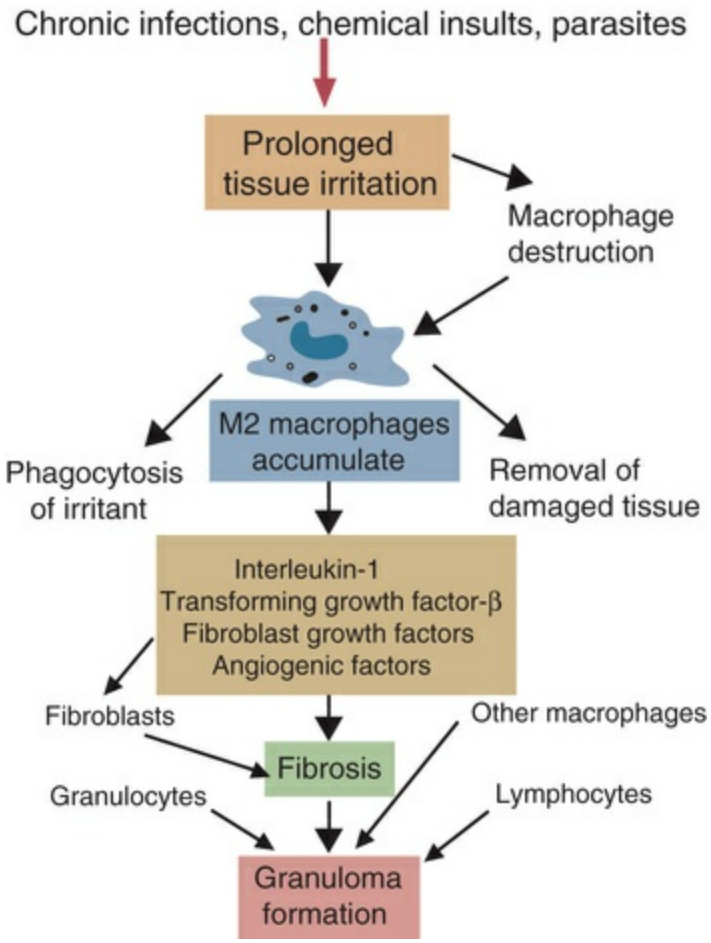


FIG. 6.16 The pathogenesis of chronic inflammation. Macrophages undergoing prolonged stimulation may switch from an M1 to an M2 phenotype. M2 cells secrete cytokine mixtures that not only promote wound healing, but also promote the "walling-off" of persistent irritants by fibroblasts and extracellular matrix. Other cell types are attracted to the persistent antigen. Their precise composition will vary with the antigens involved.

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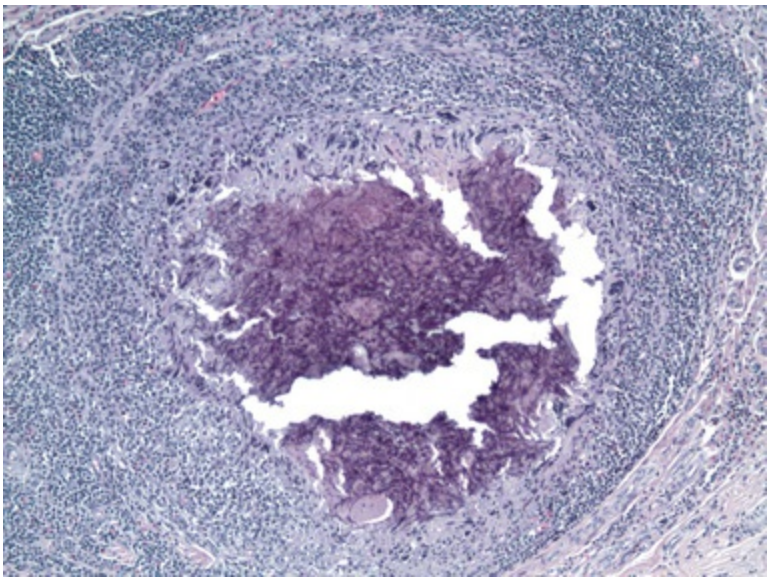


FIG. 6.17 A granulomatous inflammatory reaction around a degenerating tapeworm cyst in a bovine heart. The mass of cells around the central organism is a mixture of macrophages and fibroblasts serving to wall it off from the rest of the body. This would be classified as a type II granuloma. Original magnification ×250. (Courtesy Dr. J. Edwards.)

Chronic granulomas, whether due to immunological or foreign body reactions, may enlarge and destroy normal tissues. In liver fluke infestations, for example, death may result from the gradual replacement of normal liver cells by fibrous tissue formed as a result of the persistence of the parasites. Granulomas may be classified into type I granulomas as typified by tubercles. These contain macrophages and dendritic cells and are associated with significantly increased production of IFN-γ and IL-17 from Th1 and Th17 cells. Type II granulomas as typified by those that form around parasitic worms in tissues contain macrophages and dendritic cells, and may contain eosinophils, have increased production of IL-4, -5 and -13 and large numbers of fibroblasts.

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Sickness

The Body's Innate Responses

CHAPTER OUTLINE

Sickness Behavior, 60
Systemic Innate Responses, 62
Metabolic Changes, 62
Acute-Phase Proteins, 62
Soluble Pattern-Recognition Receptors, 62
Iron-Binding Molecules, 63
Other Acute-Phase Proteins, 64
Acute-Phase Proteins as "Biomarkers" of Disease, 65
Systemic Inflammatory Response Syndrome, 65
Bacterial Septic Shock, 65
Disseminated Intravascular Coagulation, 66
Multiple-Organ Dysfunction Syndrome (MODS), 68
Bacterial Toxic Shock, 68

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SIRS-Associated Laminitis, 68
Graft-Versus-Host Disease, 69
Protein Misfolding Diseases, 69

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Explain how inflammation triggers reactions involving the whole body.
- List the cytokines that cause a fever and outline their mechanism of action.
- Outline the properties and significance of HMGB1.
- List the benefits of a raised body temperature.
- Define the acute-phase response and its physiological benefits.
- List the major acute-phase proteins in domestic animals.
- Explain how iron metabolism is altered during an infection and the benefits and disadvantages of these processes.
- Define ferroportin, siderophore, acute-phase protein, pentraxins, amyloid, systemic inflammatory response syndrome, septic shock, disseminated intravascular coagulation, and multiple-organ dysfunction syndrome.
- Understand why excessive production of cytokines (a cytokine storm) may lead to the development of a lethal shock syndrome.
- Explain the pathogenesis of septic shock.
- Explain the mechanisms of disseminated intravascular coagulation.
- Explain how excessive, chronic release of inflammatory cytokines may result in tissue deposition of insoluble misfolded proteins called amyloid.

While inflammation appears to be a very local response, restricted to sites of tissue damage or microbial invasion, it can also have significant effects on distant parts of the body. If the local inflammation is minor, such systemic effects may not be noticed. If, however, inflammation affects multiple organ systems, or if the microbial invader succeeds in spreading throughout the body, these systemic effects become clinically significant. Collectively we call

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them sickness. It need hardly be pointed out that in veterinary medicine, it is these signs of sickness that draw the attention of an owner to an animal's illness.

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Sickness Behavior

When an animal is invaded by pathogens, a response occurs throughout the body—a response that we call sickness. The subjective feelings of sickness (e.g., malaise, lassitude, fatigue, loss of appetite, and muscle and joint pains, as well as a fever) are signs of a systemic innate immune response. These reflect a change in the body's priorities as it fights off the invaders. Microbial pathogen-associated molecular patterns (PAMPs) acting through the pattern-recognition receptors (PRRs) stimulate the production of interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α). All three of these cytokines signal to the brain using two pathways (Fig. 7.1). One pathway acts through the sensory neurons that serve damaged tissue. Sensory stimulation by IL-1β through the vagus nerve can trigger signaling to the brain. (IL-1 will not trigger a fever if the vagus nerve is cut.) Lipopolysaccharide (LPS) binding to toll-like receptor 4 (TLR4) can also trigger these vagal signals. As a result, they initiate fever, nausea, and other sickness responses in the brain.

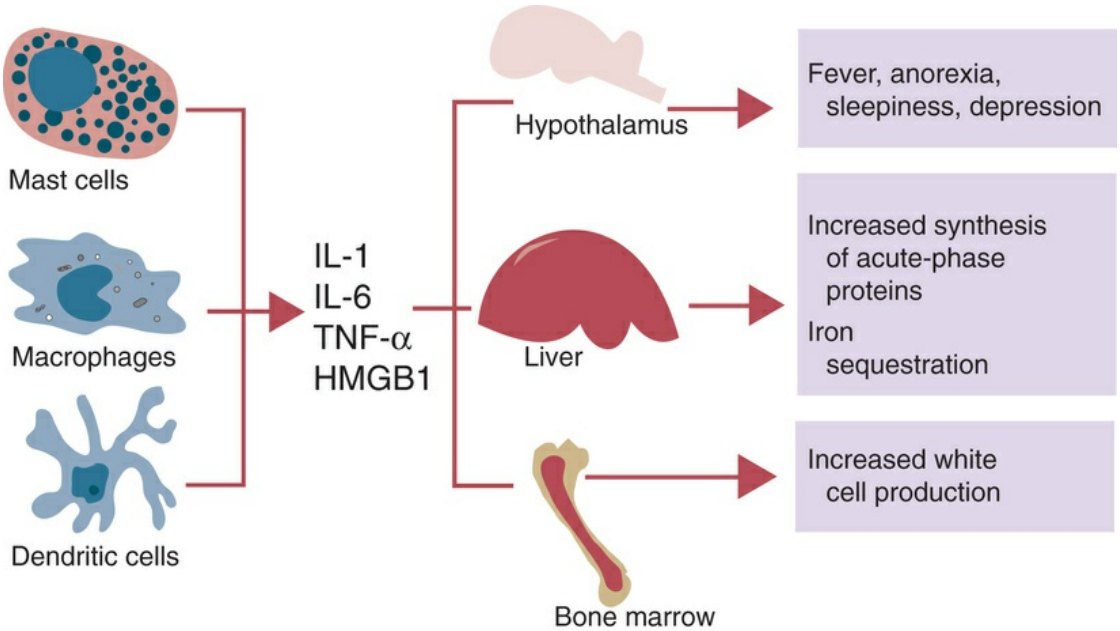


FIG. 7.1 Sickness behavior is part of the response of the body to inflammatory stimuli. Multiple systemic effects are due to the four major cytokines secreted by sentinel cells, mast cells, macrophages and dendritic cells. The major sickness-inducing cytokines are IL-1,

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A second pathway uses cytokines that either diffuse directly into the brain from the bloodstream or are produced within the brain. These cytokines can act on neurons or microglia to modify behavior and alter pain perception. Type I interferons can also enter the brain and trigger malaise, depression and sickness behavior (Box 7.1)

Box 7.1

Sex Differences in Sickness Behavior?

When beef cattle were challenged with bacterial lipopolysaccharide, cows and bulls responded in a very different manner! All the animals demonstrated sickness behavior, but heifers showed less sickness behavior than bulls. Nevertheless, the heifers developed a higher fever than the bulls. Average serum concentrations of TNF-α were greater in heifers, while concentrations of IFN-γ were higher in bulls. Bulls developed higher total leukocyte counts, but the heifers had higher neutrophil counts. In general, in many mammals, males are more susceptible to mortality and morbidity caused by bacterial infections than females. Man-flu is real!

Carroll JA et al. Sexually dimorphic innate immunological responses of prepubertal Brahman cattle following an intravenous lipopolysaccharide challenge. *Vet Immunol Immunopathol,* 166:108-115, 2015.

The most obvious systemic response to infection is the development of a fever. IL-1, IL-6, and TNF-α all trigger a rise in body temperature. These cytokines induce cyclooxygenase-2 (COX-2) in the hypothalamus. This results in prostaglandin-E_2 production. This acts on temperature-sensitive neurons to alter the body's thermostatic set-point. As a result, animals conserve heat by vasoconstriction and increase their heat production by shivering, thus raising their body temperature until it reaches the new set-point.

Fevers are protective in that they enhance both innate and adaptive immune responses (Fig. 7.2). For example, a high body temperature enhances many neutrophil functions. It promotes their

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release from the bone marrow, their transendothelial migration, and chemotaxis, thus increasing their accumulation within tissues. Fever enhances their respiratory burst and accelerates caspase-dependent neutrophil apoptosis. It also enhances the cytotoxic activities of NK cells. With respect to adaptive immunity, raised body temperatures cause dendritic cells to mature and enhance multiple macrophage functions such as phagocytosis, release of NO and cytokines, and expression of TLR2, TLR4, and MHC molecules. Fevers enhance the passage of T cells across high endothelial venules (Chapter 12), promote immunological synapse formation (Chapter 14), and inhibit T cell apoptosis.

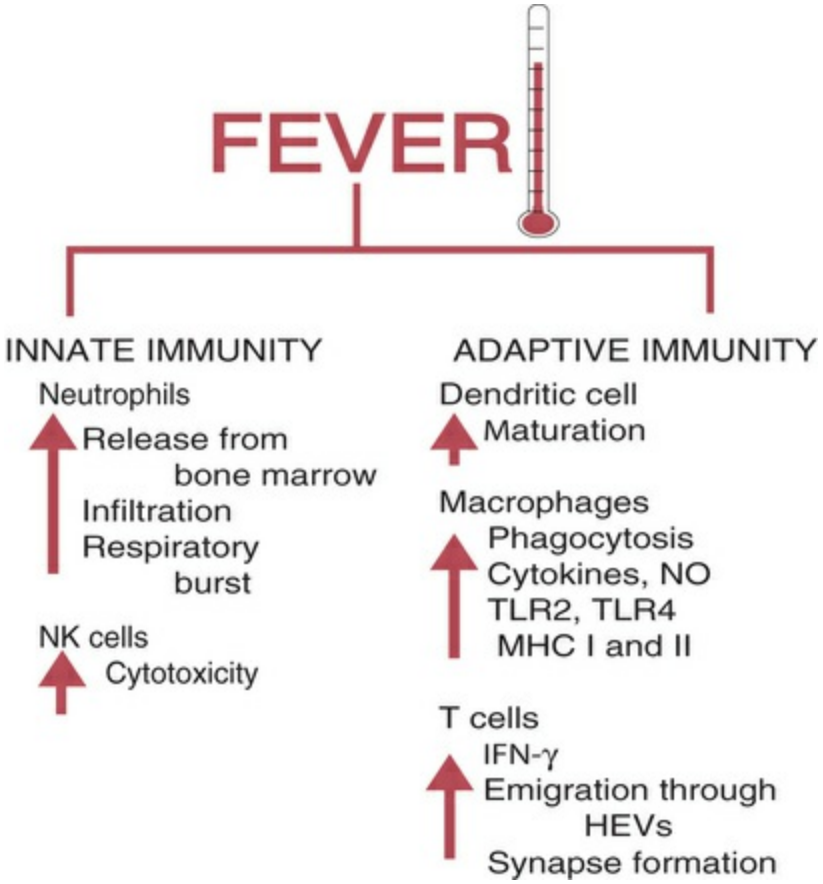


FIG. 7.2 The upregulation of both innate and adaptive immune responses as a result of a fever.

High-mobility group box protein-1 (HMGB1) (Chapter 3) is a potent sickness-inducing cytokine. Although IL-1, IL-6, and TNF-α have long been known to cause septic shock and sickness behavior, it is now clear that these three molecules induce slow release of

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HMGB1 from macrophages. HMGB1 has been implicated in food aversion and weight loss by its actions on the hypothalamic-pituitary axis. It mediates endotoxin lethality, arthritis, and macrophage activation. The inflammation induced by necrotic cells is caused in part by the escape of HMGB1 from disrupted nuclei and damaged mitochondria.

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Systemic Innate Responses

Hematopoietic stem cells (HSCs) respond to systemic infections as the body needs to replenish its supply of neutrophils and macrophages. This response is mediated by many cytokines, including granulocyte colony-stimulating factor as described in Chapter 5. Inflammation-associated cytokines such as interferons and TNF-α also have a direct effect on HSCs and stimulate them to proliferate. Since HSCs express TLR2 and TLR4, bacterial PAMPs such as lipopolysaccharides also promote their proliferation and an elevated white cell count.

Metabolic Changes

In addition to causing a fever, inflammatory cytokines, especially IL-1, promote the release of sleep-inducing molecules. Thus lethargy is commonly associated with a fever. This response may reduce other energy demands of an animal, and increase that available for defense and repair.

IL-1, IL-18, and leptin also suppresses the hunger centers in the hypothalamus and so are responsible for the loss of appetite associated with infections. The benefits of this are unclear, but it may permit the animal to be more selective about its food

IL-1, IL-6, and TNF-α also act on skeletal muscle to increase protein catabolism and release amino acids. Although this eventually results in myalgia and muscle wastage, the newly available amino acids are available for increased antibody and cytokine synthesis. If animals are subjected to long-term, low-grade inflammation, they will be exposed to chronic, low doses of TNF-α. As a result, they lose weight and become anemic and protein depleted. TNF-α inhibits the uptake of lipids by preadipocytes and causes mature adipocytes to lose stored lipids. TNF-α is thus responsible for the weight loss seen in animals with cancer or chronic parasitic and bacterial diseases. Weight loss is a common response to infection (and sometimes to vaccination) and is of special significance to livestock producers.

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Acute-Phase Proteins

Under the influence of IL-1β, TNF-α, and especially IL-6, hepatocytes synthesize new proteins. Because this increase is associated with acute infections and inflammation, these newly produced proteins are called acute-phase proteins (APPs). Type I APPs are induced by IL-1 and TNF-α, while type II APPs are induced by IL-6. These proteins are also synthesized in lymph nodes, tonsils, and the spleen, as well as by blood leukocytes. The increase in APPs begins about 90 minutes after injury or systemic inflammation and subsides within 48 hours (Fig. 7.3). It may also occur following prolonged stress such as road transportation or confinement. About 30 acute-phase proteins have been recognized, and many are important components of the innate immune system. They include soluble PRRs, complement components, clotting molecules, protease inhibitors, and iron-binding proteins. Different species produce different acute-phase proteins (Fig. 7.4).

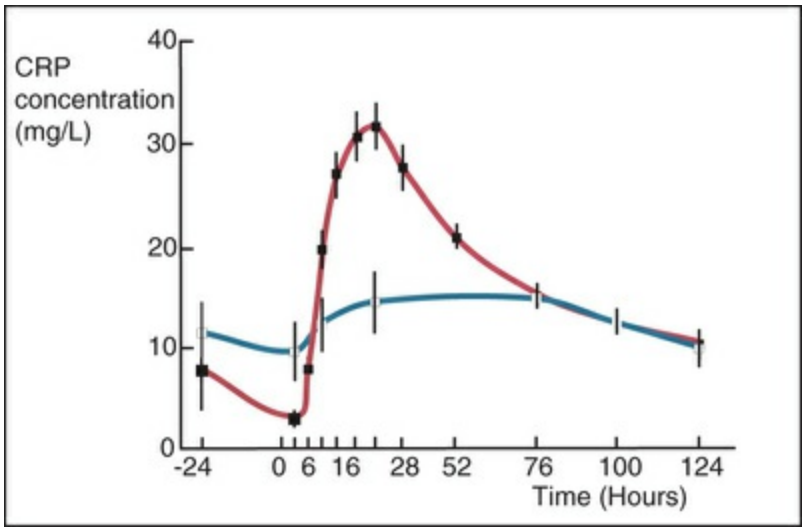


FIG. 7.3 The rise in C-reactive protein levels in six dogs following anesthesia and surgery *(red line)* and in six dogs undergoing anesthesia alone *(blue line)*. (From Burton SA, Honor DJ, Mackenzie AL, et al: C-reactive protein concentration in dogs with inflammatory leukograms, *Am J Vet Res* 55:615, 1994.)

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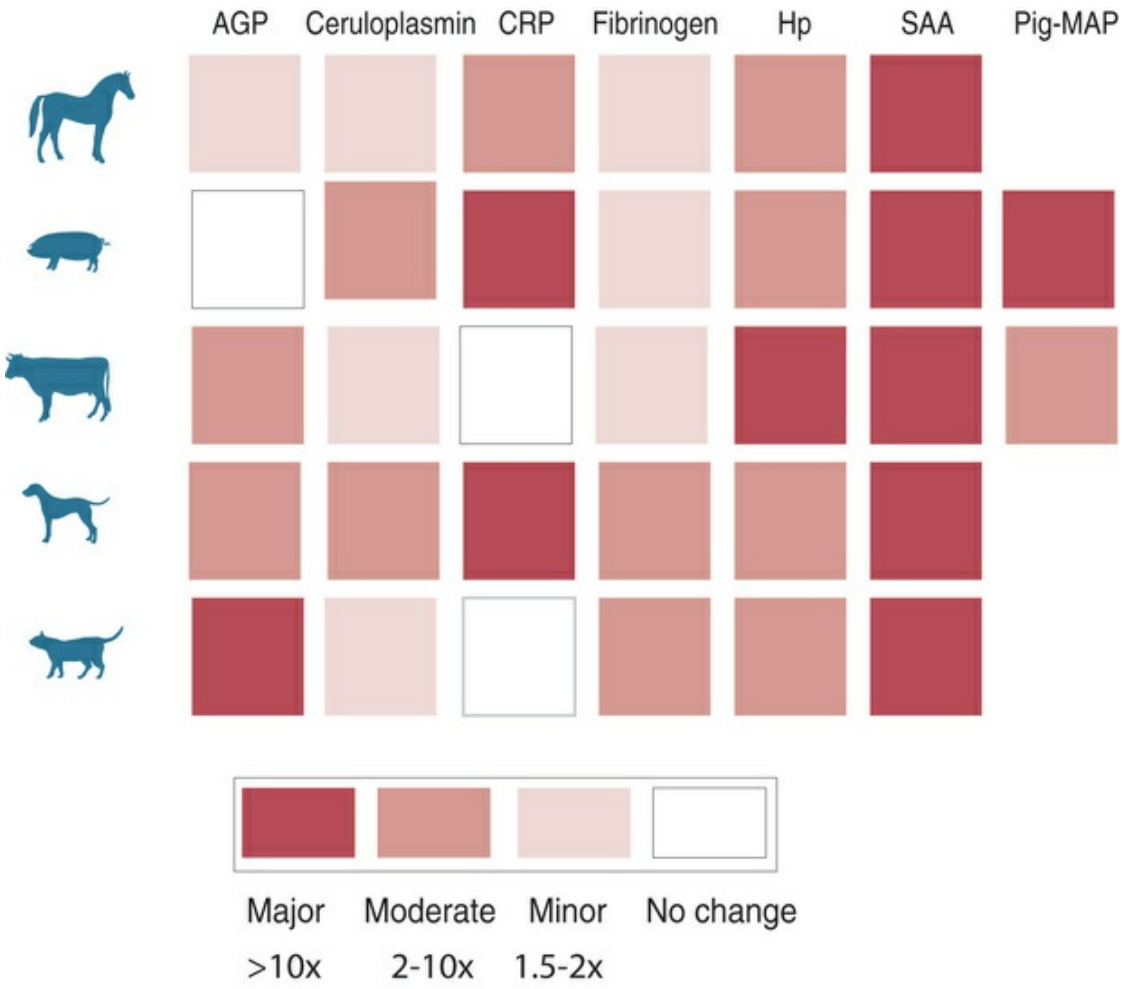


FIG. 7.4 Species differences in the major acute-phase proteins produced by the domestic mammals.

Soluble Pattern-Recognition Receptors

Pentraxins are P-type lectins that act as soluble PRRs. Two of the most important are C-reactive protein and serum amyloid P. C-reactive protein (CRP) is the major acute-phase protein produced in primates, rabbits, hamsters, and dogs, and is important in pigs. CRP has a pentameric structure (five 20-kDa units arranged in a circle) with two faces. One face binds phosphocholine, a common side chain found in all cell membranes and many bacteria and protozoa. The other face binds to the antibody receptors FcγRI and FcγRIIa on neutrophils. CRP thus acts as an opsonin and promotes the phagocytosis and removal of damaged, dying, or dead cells in addition to microorganisms. CRP can bind to bacterial polysaccharides and glycolipids and to necrotic cells, where it activates complement C1q. (Its name derives from its ability to bind

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and precipitate the C-polysaccharide of *Streptococcus pneumoniae*.) CRP also has an antiinflammatory role since it inhibits neutrophil superoxide production and degranulation and blocks platelet aggregation. CRP stimulates fibrosis and may promote healing by reducing damage and enhancing the repair of damaged tissue. In lactating cows serum CRP rises two- to five-fold. The reasons for this are unknown.

Serum amyloid P (SAP) is a pentraxin and the major acute-phase protein in rodents. Like CRP, it is a PRR, where one face of the molecule can bind nuclear constituents such as DNA, chromatin, and histones, as well as cell membrane phospholipids. The other face binds and activates C1q and thus triggers the classical complement pathway. A major function of SAP is to regulate innate immune responses. It interacts with macrophage Fc receptors, reduces binding of neutrophils to the extracellular matrix, reduces the differentiation of macrophages into fibroblasts (thus inhibiting fibrosis), and promotes phagocytosis of cell debris. Other soluble PRRs that act as acute-phase proteins include LPS-binding protein in cattle, humans and rabbits, CD14 in humans, horses and mice, and C-type lectins such as mannose-binding lectin and conglutinin in other species. LPS-binding protein is a type II APP that presents LPS to CD14 and TLR4 on phagocytic cells and enhances their proinflammatory activity up to 1000-fold. It can also bind lipoteichoic acids on the cell wall of Gram-positive bacteria and trigger inflammation through TLR2 activation.

Iron-Binding Molecules

One of the most important factors that determines the success or failure of bacterial invasion is the availability of iron (Fig. 7.5).

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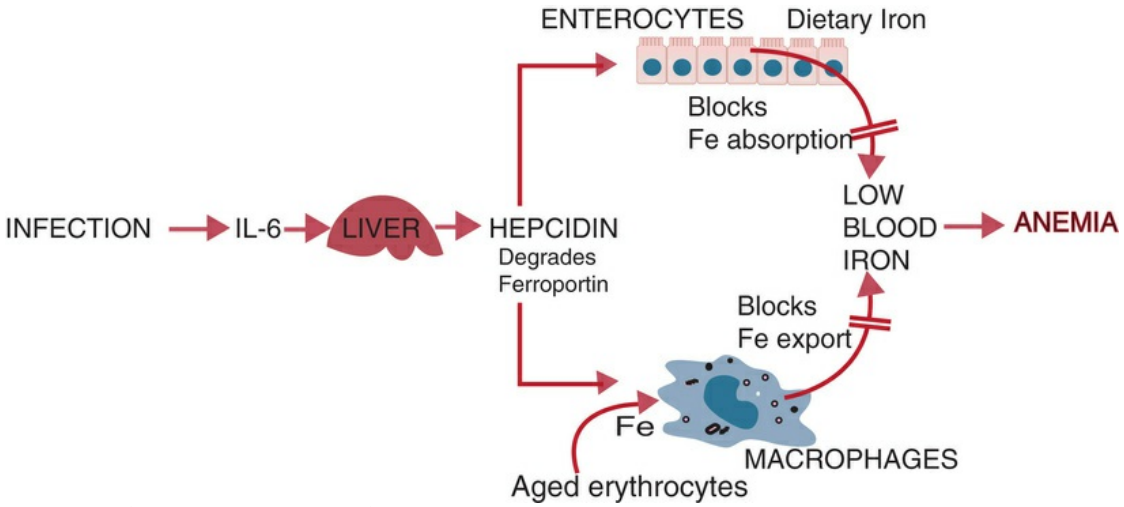


FIG. 7.5 The role of hepcidin in regulating iron availability. This protein prevents iron efflux from enterocytes and macrophages by binding to ferroportin and triggering its degradation. The net effect is to retain iron within these cells, making it unavailable for hemoglobin synthesis and leading to the development of anemia.

Most pathogenic bacteria, such as *Staphylococcus aureus, Escherichia coli, Bacillus anthracis, Pasteurella multocida,* and *Mycobacterium tuberculosis,* require iron for growth since iron forms the key catalytic site in many of their enzymes. Animals, however, also require iron for vital functions such as oxygen transport and energy production. As a result, bacteria and their hosts compete for the same metal. The result of this competition may determine the outcome of an infection.

Free iron concentrations within animal tissues are normally very low. Mammalian blood has just 10^{-26}M free iron since almost all of the available iron is bound to proteins. These iron-binding proteins include transferrin, lactoferrin, hepcidin, siderocalin, haptoglobin, and ferritin.

Iron is usually absorbed from food by duodenal enterocytes. It binds to a cell surface iron carrier called ferroportin and is transported to the bloodstream where it is bound by transferrin. Most of this iron is incorporated into hemoglobin in red cells. Less than 10% of our daily needs, however, are met by importing dietary iron. Most is derived by recycling aged or damaged red cells. Aged red cells give up their iron when ingested by splenic macrophages. The macrophages phagocytose these cells and catabolize the hemoglobin using hemoxygenase. They release the iron obtained from hemoglobin into the circulation via ferroportin. The exported

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iron binds to transferrin and is carried to erythroblasts for use in new red cell production.

Despite the low availability of iron, bacteria such as *M. tuberculosis, B. anthracis,* and *E. coli* can successfully invade an animal because they produce their own iron-binding proteins called siderophores. Mycobacteria use their siderophore (called carboxymycobactin) to strip iron from mammalian ferritin. *S. aureus* uses staphyloferrins to lyse red cells and access the iron in hemoglobin. Enterochelin is a very potent iron chelator produced and secreted by many enteric bacteria. When serum iron levels are elevated, as occurs after red cell destruction, animals become more susceptible to bacterial infections.

When bacteria invade the body, the body responds by producing two acute-phase proteins, hepcidin and haptoglobin. Hepcidin is produced by hepatocytes under the influence of IL-1 and IL-6. Hepcidin binds to ferroportin and triggers its internalization and degradation. Hepcidin also suppresses intestinal iron absorption by downregulating ferroportin expression in enterocytes. In healthy individuals, hepcidin production is regulated by systemic iron availability, or by erythropoietic signals and hypoxia. In inflammation, however, IL-6 and IL-1 stimulate the hepcidin promoter. As a result, hepcidin increases, ferroportin decreases, iron absorption by enterocytes is blocked, and macrophages can no longer export their iron. Iron availability for red blood cell production drops, and a hypoferremia develops. Chronically infected animals become anemic—the anemia of infection. This is usually a mild normocytic, normochromic, nonregenerative anemia.

Haptoglobin is another major acute-phase protein in ruminants, horses, and cats. It can rise from virtually undetectable levels in normal calves to as high as 1 mg/mL in calves with acute respiratory disease. Haptoglobin also binds iron and makes it unavailable to invading bacteria.

Mammals may also capture iron by stealing bacterial siderophores. Thus during bacterial infections, the mammalian liver, spleen, and macrophages synthesize a protein called lipocalin 2. Lipocalin 2 (also called siderocalin) binds the bacterial siderophore enterochelin with very high affinity. Lipocalin 2 is essential for limiting the growth of enterochelin-producing bacteria

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such as *E. coli* but does not affect bacteria that employ other methods to acquire iron.

Other iron-binding acute-phase proteins include transferrin (important in birds) and hemopexin. Activation of macrophages by interferon-γ (IFN-γ) leads to downregulation of macrophage iron acquisition through transferrin receptors and deprives intracellular bacteria of needed iron. A similar situation occurs in the mammary gland when, in response to bacterial invasion, milk neutrophils release stored lactoferrin. The lactoferrin binds iron and makes it unavailable to the bacteria.

Other Acute-Phase Proteins

α_1-acid glycoprotein is a minor acute-phase protein in cattle. Neutrophils exposed to activating agents such as phorbol myristate acetate rapidly release their stores of α_1-acid glycoprotein. This protein inhibits the respiratory burst and may reduce the damage caused by excessive inflammation.

Some serum protease inhibitors such as α_1-antitrypsin, α_1-antichymotrypsin, and α_2-macroglobulin are acute-phase proteins. They inhibit neutrophil proteases in sites of acute inflammation and thus reduce tissue damage. Major acute-phase protein (MAP) is a significant protease inhibitor in pigs and a moderate one in cattle. MAP shares homology with inter-α-trypsin inhibitor heavy chain 4, a moderate acute phase protein in dogs.

Serum amyloid A (SAA), a protein of 15 kDa, is the major acute-phase protein in cattle, cats, pigs, and horses and is also important in humans and dogs. Equine SAA concentrations rise several 100-fold during noninfectious arthritis, whereas canine SAA concentrations increase up to 20-fold in bacterial disease. Bacterial lipopolysaccharide induces a 1000-fold increase in SAA in mice. The functions of SAA are unclear, but it binds to TLR2 and may also be an endogenous TLR4 agonist. This will lead to NF-κB activation and the production of multiple inflammatory cytokines. It recruits lymphocytes to inflammatory sites and induces enzymes that degrade the extracellular matrix. SAA is a chemoattractant for neutrophils, monocytes, and T cells. It increases significantly in mastitic milk. Other acute-phase proteins include ceruloplasmin,

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haptoglobin, and fibrinogen in sheep, and CRP, haptoglobin, sialic acid, and ceruloplasmin in pigs.

Some protein levels fall during acute inflammation. These are called "negative" acute-phase proteins. Two of the most important are albumin and transferrin. The albumin serves as a source of amino acids that can be used on demand, such as during infections and inflammation.

Acute-Phase Proteins as "Biomarkers" of Disease

It is possible to identify animals with severe infections or inflammation by measuring acute-phase protein levels in blood. This may be helpful, for example, in antemortem meat inspections by identifying animals that are suffering from inapparent inflammation or infection and hence are unfit to eat. Numerous studies have examined the specificity and sensitivity of these assays by determining their ROC curves (Chapter 42). Different APPs increase in different inflammatory states, and different APPS may predominate at different stages of a disease. In cattle, haptoglobin increases in chronic infections such as mastitis, enteritis, respiratory disease, traumatic pericarditis, and endometritis. SAA and lipopolysaccharide-binding protein are sensitive markers of respiratory infection in calves. In calves with umbilical abscesses, fibrinogen is elevated followed by haptoglobin and SAA. A mammary-associated isoform of SAA (M-SAA3) is elevated in mastitis. The acute-phase response in sheep is similar to that in cattle.

In horses, very high SAA levels accompanied by low iron concentrations probably reflect the presence of widespread systemic infection. Fibrinogen is a less sensitive indicator in this species.

Pigs with tail lesions and carcass abscesses due to biting had elevated levels of CRP, SAA, and haptoglobin compared with control pigs. These tail lesions were associated with increased carcass condemnation. In experimental *Actinobacillus pleuropneumoniae* infections, CRP and SAA are significantly increased.

In cats with feline infectious peritonitis (FIP), blood SAA levels increase 10- to 50-fold. They are also elevated in diabetes mellitus,

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other infectious diseases, injury, and cancer. α_1-acid glycoprotein also increases in FIP, although this is usually less than 10-fold. It also increases in calicivirus, chlamydiosis, feline leukemia, and feline immunodeficiency virus infections. Haptoglobin usually increases 2- to 10-fold and is especially high in FIP.

In dogs, CRP is the major acute-phase protein, increasing 100-fold in infectious diseases such as pyometra-associated sepsis, babesiosis, leishmaniasis, parvovirus infection, and colibacillosis. APP levels increase moderately in canine inflammatory bowel disease. CRP, haptoglobin, and SAA are significantly elevated in the cerebrospinal fluid and serum of dogs with canine steroid-responsive arteritis-meningitis (Chapter 37). In pregnant dogs, haptoglobin, ceruloplasmin, and fibrinogen levels increase midway through gestation. SAA also increases in pyometra.

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Systemic Inflammatory Response Syndrome

When animals die from infectious diseases, death is not always attributable to a direct effect of the pathogen or its toxins but may be a result of a systemic inflammatory response syndrome (SIRS) (Fig. 7.6).

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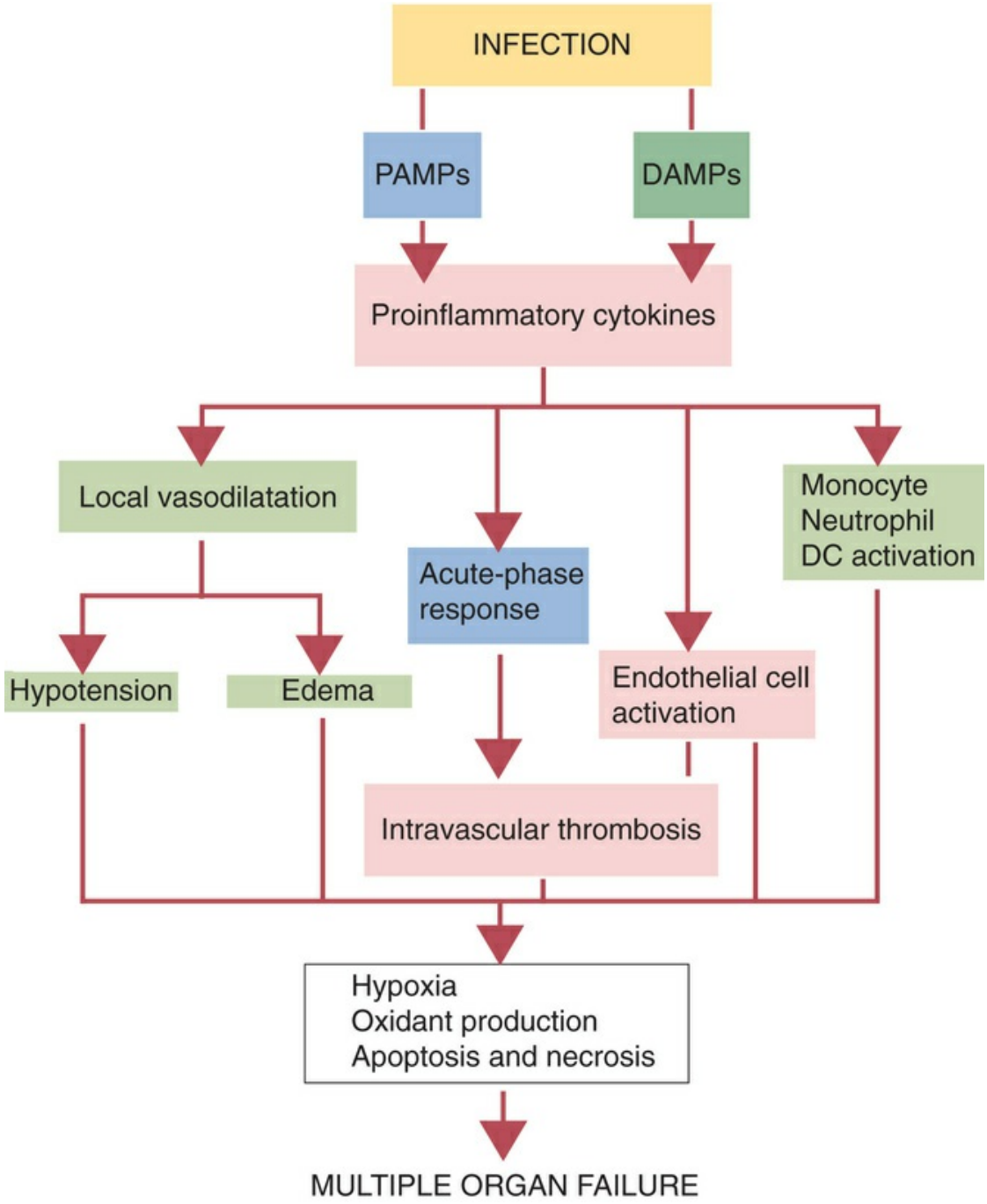


FIG. 7.6 The pathogenesis of the systemic inflammatory response syndrome. In effect, the syndrome results from overexpression of multiple cytokines. These cytokines trigger a series of events that result in widespread cell death and multiple organ failure.

After massive tissue damage, large amounts of DAMPs including HMGB1, mitochondrial components, and oxidants may escape into the bloodstream and trigger a lethal form of shock, known as SIRS. These DAMPs activate large numbers of sentinel cells and as a result generate large quantities of inflammatory mediators such as TNF-α, IFN-γ, CXCL8, and IL-6. They also generate large amounts

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of the complement fragment, C5a. The cytokines and C5a in turn trigger the activation of more T cells and the release of more cytokines, leading to even greater cell destruction. This "cytokine storm" can cause severe illness or death. The most important of these cytokine storms results from massive tissue trauma or burns. However, many viruses such as influenza and dengue, can also trigger cell destruction, leading to excessive cytokine release and death. (Box 7.2).

Box 7.2

Immune Reconstitution Inflammatory Syndrome!

When bats hibernate their body temperature drops and they may become immunosuppressed. This permits the causal agent of white nose syndrome, a filamentous fungus called *Pseudogymnoascus destructans,* to colonize and erode the skin of their wings, ears, and muzzle. About a week after infected bats emerge from hibernation, they develop an intense inflammatory response that leads to their death. It has been suggested that the sudden reversal of immune suppression that occurs when their body temperature rises results in the development of immune reconstitution inflammatory syndrome (IRIS). This syndrome was first reported in humans following the abrupt reversal of immunosuppression. (For example, in AIDS patients following the onset of effective antiretroviral therapy.) It is believed that the recovery of T cell function drives an exaggerated immune response against the underlying infection that results in significant tissue damage. Thus in white nose syndrome, cold-loving *P. destructans* is present in infected tissues. When body temperature returns to normal, T cell function is rapidly restored, leading to a massive neutrophil influx and resulting in tissue destruction.

Meteyer CU, Barber D, and Mandl JN. Pathology in euthermic bats with white nose syndrome suggests a natural manifestation of immune reconstitution inflammatory syndrome. *Virulence* 3:10-16, 2016.

Bacterial Septic Shock

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Septic shock is the name given to a SIRS caused by severe bacterial infections and complicated by reduced blood pressure and unresponsiveness to fluid therapy. It accounts for about 9% of human deaths in the United States and is a correspondingly important cause of animal deaths. Animals or humans with mild infections develop the characteristic signs of sickness such as fever, myalgia, depression, and fatigue as a result of cytokine release. Severe infections, however, may cause excessive triggering of TLRs leading to a massive and uncontrolled release of HMGB1. Other cytokines involved are TNF-α and IL-1β, with IFN-γ, IL-6, IL-3 and CXCL8 in a supporting role. These cytokines in turn stimulate nitric oxide synthase 2 (NOS2), leading to an increase in serum nitric oxide and COX-2, and resulting in massive prostaglandin and leukotriene synthesis. This excessive cytokine and mediator release causes severe acidosis, fever, lactate release in tissues, an uncontrollable drop in blood pressure, elevation of plasma catecholamines, and eventually renal, hepatic, and lung injury, and death. Tissue damage, severe systemic inflammation, and the release of damaged cell fragments all act to raise blood levels of tissue factor, a key initiator of coagulation. Vascular endothelial cell apoptosis may cause their detachment from the basement membrane. The cytokines activate vascular endothelial cells so that procoagulant activity is enhanced, leading to blood clotting. The nitric oxide causes vasodilation and a drop in blood pressure. The prostaglandins and leukotrienes cause increases in vascular permeability. This combination may result in disseminated intravascular coagulation.

Disseminated Intravascular Coagulation

Disseminated intravascular coagulation (DIC) is a severe clinical syndrome characterized by dysregulation of the clotting and fibrinolytic cascades (Fig. 7.7). The clotting cascade can be triggered by injury to vascular endothelial cells caused by ischemia, chemical mediators or even by leukocyte adhesion. It also results from the excessive production of a transmembrane glycoprotein called "tissue factor" (TF). TF is normally hidden and does not trigger blood clotting in healthy tissues. However, TF is released on exposure to cytokines, especially IL-1 and TNF-α. It is also released

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by massive tissue damage or by exposure to bacteria and bacterial endotoxins in Gram-negative sepsis. Virtually every bacterial species can trigger TF release. The TF transforms prothrombin to thrombin to form fibrin thrombi. Simultaneous downregulation of anticoagulant systems results from reduced antithrombin synthesis (it is a "negative" acute-phase protein). Activation of the clotting cascade leads to the formation of fibrin and platelet microthrombi in small blood vessels throughout the body. These clots can cause intravascular thrombosis and can lead to ischemia, impaired organ perfusion and widespread damage.

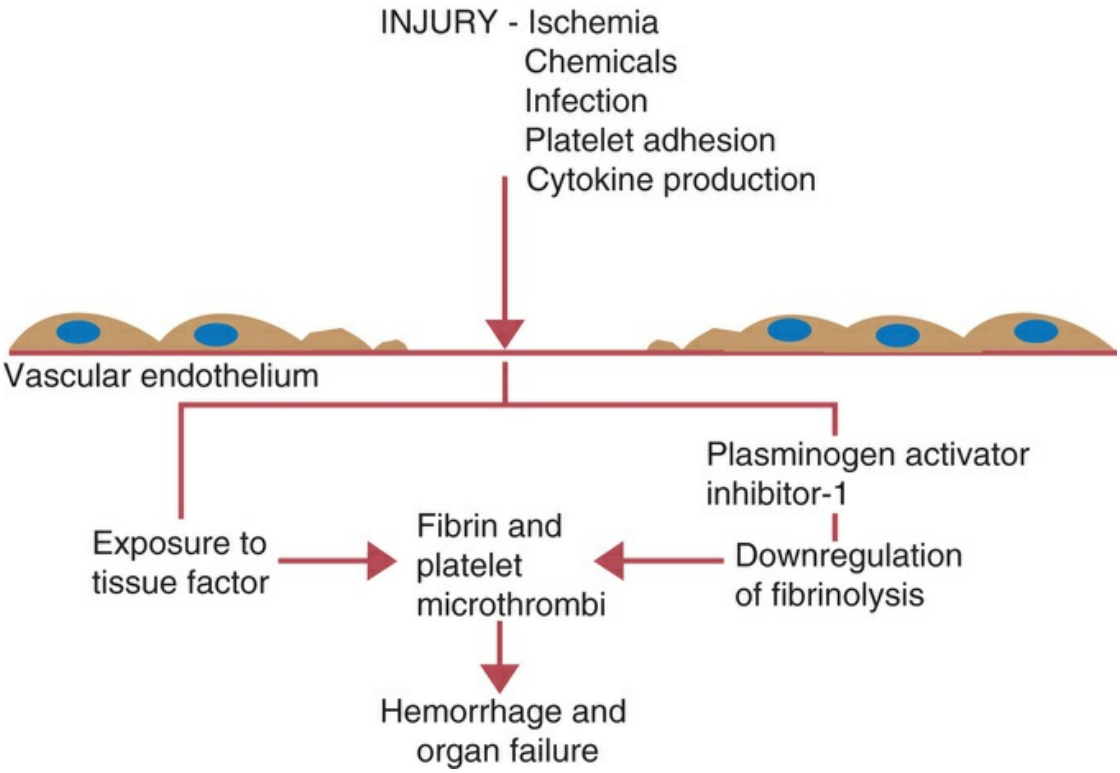


FIG. 7.7 The mechanism of disseminated intravascular coagulation. Widespread injury to vascular endothelium results in the production of microthrombi.

At same time, the excessive thrombin activates plasminogen to generate plasmin, which causes fibrinolysis. When the microthrombi break down they release anticoagulant fibrin breakdown products that therefore promote hemorrhage. In addition, the newly formed thrombi consume clotting factors and platelets. As a result, normal clotting does not occur and uncontrolled bleeding may result. Plasmin also activates the

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complement and kinin systems, and these too promote inflammation, hypotension, and increased vascular permeability. The development of DIC carries with it a poor prognosis.

Multiple-Organ Dysfunction Syndrome (MODS)

MODS is defined as the presence of altered organ function in an acutely ill animal such that homeostasis cannot be maintained without intervention. It is usually a sequel to severe sepsis or septic shock but can also develop as a result of major trauma or anything else that will induce a systemic inflammatory response syndrome (Fig. 7.8). It is characterized by hypotension, insufficient tissue perfusion, uncontrollable bleeding, and organ failure caused by hypoxia, tissue acidosis, mitochondrial dysfunction and cytopathic hypoxia, tissue necrosis, and severe local metabolic disturbances. The reported incidence of MODS in dogs is about 4% in trauma and 50% in sepsis cases. The pathogenesis of MODS is unclear, but it appears to result from immune system dysregulation and subsequent mitochondrial dysfunction. The immune system dysregulation may be due to overregulation of the inflammatory response and a compensatory antiinflammatory response, as well as the excessive release of neutrophil reactive oxygen species (ROS), leading to lethal tissue damage. MODS can affect the liver, respiratory system, brain, adrenal, the heart, and the kidneys. It permits bacterial translocation in the gastrointestinal tract and disseminated intravascular coagulation. Its development is associated with a poor outcome.

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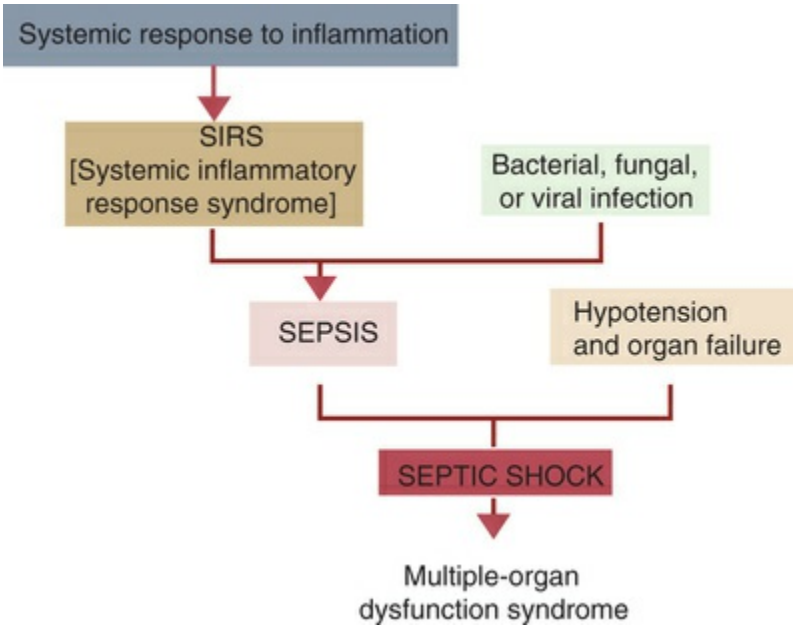


FIG. 7.8 The relationships of SIRS, sepsis, septic shock, and multiple-organ dysfunction syndrome.

The sensitivity of mammals to septic shock varies greatly. Species with pulmonary intravascular macrophages (cat, horse, sheep, and pig) tend to be more susceptible than dogs and rodents, which lack these cells and are relatively insusceptible to lung injury.

Bacterial Toxic Shock

Some strains of *S. aureus* produce enterotoxins that stimulate T cell functions (Fig. 7.9). These toxins may stimulate up to 20% of an animal's T cells, causing them to secrete enormous quantities of IL-2 and IFN-γ. These in turn stimulate overproduction of TNF-α and IL-1β. This leads to the development of a fever, hypotension, collapse, skin lesions, and damage to the liver, kidney, and intestines with multiple-organ dysfunctions. It is called toxic shock syndrome. A similar syndrome has also been observed in some streptococcal infections. In these cases, streptococcal M-protein binds to fibrinogen. The M-protein–fibrinogen complexes bind to endothelial cell integrins and trigger a respiratory burst. This causes an increase in vascular permeability and hypercoagulability, leading to toxic shock characterized by hypotension and DIC.

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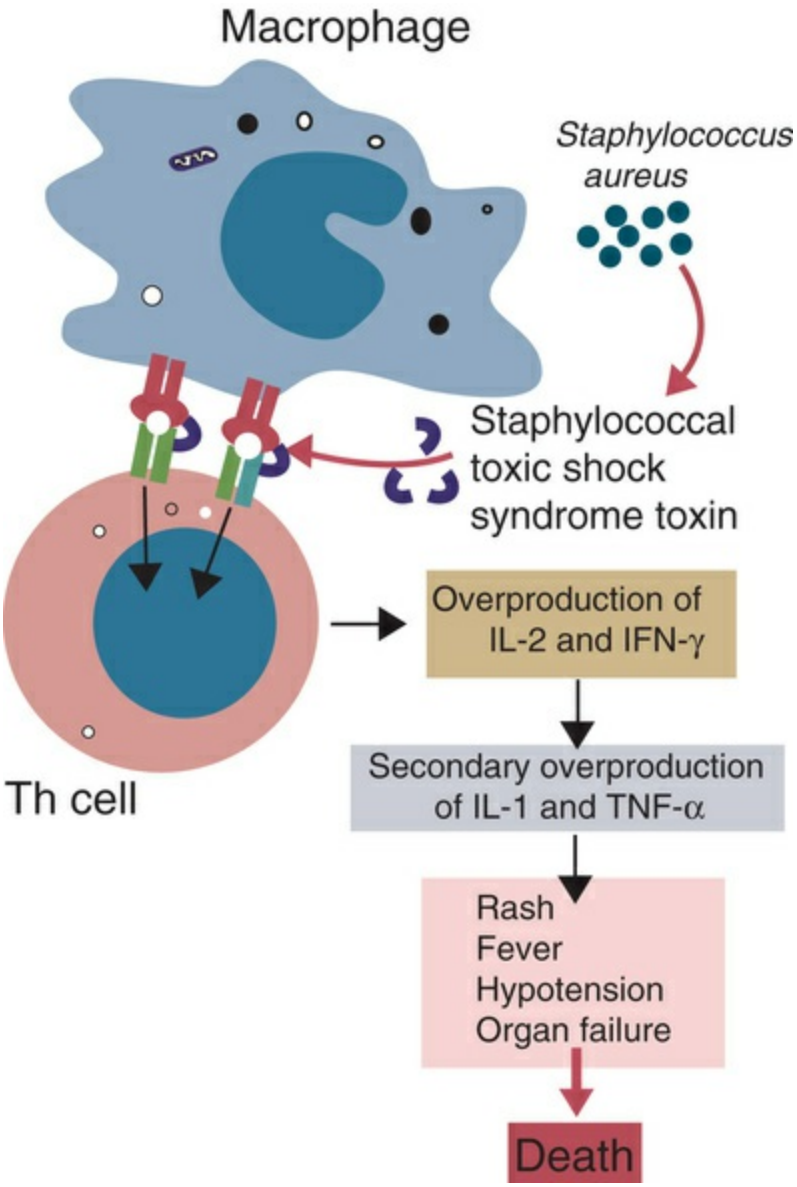


FIG. 7.9 The pathogenesis of staphylococcal toxic shock syndrome. The toxic shock syndrome toxin is a potent superantigen that acts as a powerful stimulant of IL-1 and TNF-α production.

SIRS-Associated Laminitis

Acute laminitis is a common debilitating disease in horses and ponies. While traditionally considered a single entity, laminitis is now recognized to result from several different mechanisms: these include endocrinopathic disease caused by prolonged hyperinsulinemia, support-limb laminitis caused by prolonged weight-bearing on one hoof, and inflammation-related laminitis. Experimentally, inflammation sufficient to trigger laminitis can be

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triggered by carbohydrate (starch and oligofructose) overload; black walnut poisoning, or by sepsis. All of these forms of laminitis may have a final common pathway.

In most mammals with sepsis, as described above, internal organs such as liver or lung are damaged as a result of a cytokine storm, and this results in organ failure. In the horse, in contrast, it is suggested that the epidermal laminae within the hoof are the vulnerable organs. (The laminar tissue is formed by the interdigitations between the dermis and epidermis formed by the basement membrane.) Infections that trigger laminitis may therefore include pleuropneumonia, endometritis, and gastrointestinal injury. In the latter case, this may be due to enterocolitis and severe colic, especially when associated with dysbiosis of the colonic microbiota as a result of carbohydrate overload. Massive death of Gram-negative bacteria releases large amounts of lipopolysaccharide, resulting in destruction of the epithelial barrier, Gram-negative sepsis, and endotoxemia. While endotoxin alone cannot induce laminitis, other molecules escaping from the damaged gut probably play a role. The complex mixture of PAMPs and DAMPs escaping from the colon, presumably acting through PRRs, trigger acute laminar inflammation. Endothelial cells, epidermal cells, and macrophages release COX-2 and ROS, as well as IL-1β and IL-6, and many different chemokines. They also trigger leukocyte infiltration and activation. The resulting acute inflammation within the lamina upregulates matrix and secreted metalloproteases within the hoof. These degrade the proteoglycans in the basement membrane of the lamellae as well as collagen in the intercellular matrix. This leads to basement membrane disruption and the separation of the epidermal and dermal laminar tissue. The separation may also be due to downregulation of epithelial cell adherence proteins such as integrins and to a loss of hemidesmosomes, oxidative injury, and excessive apoptosis.

Treatment includes prolonged cooling of the foot to reduce blood flow and exudation, pain management, and mechanical support for the damaged hoof. If lamellar damage is not controlled, the disease may become chronic, leading to rotation of the third phalanx, coffin bone remodeling, dropped sole, and development of a lamellar wedge.

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Graft-Versus-Host Disease

Another syndrome characterized by excessive production of cytokines, especially TNF-α, is graft-versus-host disease. In this disease, described in more detail in Chapter 34, donor lymphocytes attack the tissues of the graft recipient. TNF-α from these cells causes mucosal destruction, leading to ulceration, diarrhea, and liver destruction.

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Protein Misfolding Diseases

Amyloidosis is the name given to the deposition of insoluble proteins in body organs. These deposits appear as amorphous, eosinophilic, hyaline proteins in cells and tissues (Fig. 7.10). Amyloid proteins are produced as a result of errors in the folding of newly formed protein chains. These misfolded chains eventually aggregate to form insoluble fibrils. Amyloid proteins consist of protein fibrils, formed by peptide chains cross-linked to form β-pleated sheets (Fig. 7.11). This molecular conformation makes amyloid proteins extremely insoluble and almost totally resistant to proteases. Consequently, once deposited in cells or tissues, amyloid deposits are almost impossible to remove. Amyloid infiltration eventually leads to gradual cell loss, tissue destruction, and death. Amyloidosis may be systemic when it involves multiple organs, or it may be localized, involving only a single organ.

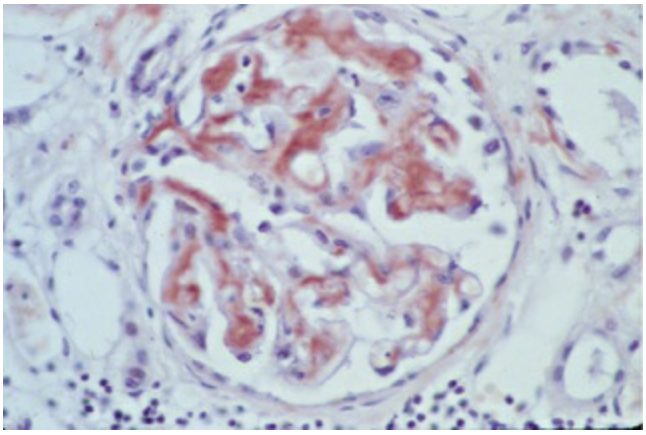


FIG. 7.10 Secondary amyloid deposited in a glomerulus. The red dye (Congo red) specifically binds to amyloid fibrils. Original magnification ×400.

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FIG. 7.11 A molecular model of an amyloid fibril derived from analysis of fibrils grown in culture. The model represents one way in which the β-pleated peptide sheets can be assembled within the fibrils. From Dobson C: *Trends Biochem Sci* 24, 331, 1999. With permission.

At least 30 different proteins have been shown to misfold and form amyloid (Fig. 7.12). The most important of these proteins is the acute-phase protein, SAA. As a result, amyloidosis develops in response to long-term, persistent inflammation. Fragments of SAA can accumulate, misfold, aggregate, and then be deposited extracellularly in organs. This material, one of the most common forms in domestic animals, is called reactive amyloid or amyloid A. Reactive amyloidosis is associated with chronic inflammation in diseases such as mastitis, osteomyelitis, abscesses, traumatic pericarditis, metritis, gangrenous pneumonia, equine recurrent uveitis, and tuberculosis. Reactive amyloidosis is a major cause of death in horses repeatedly immunized for commercial antiserum production. Familial amyloidosis of Shar-pei dogs consists of reactive amyloid deposited following chronic immune-mediated arthritis.

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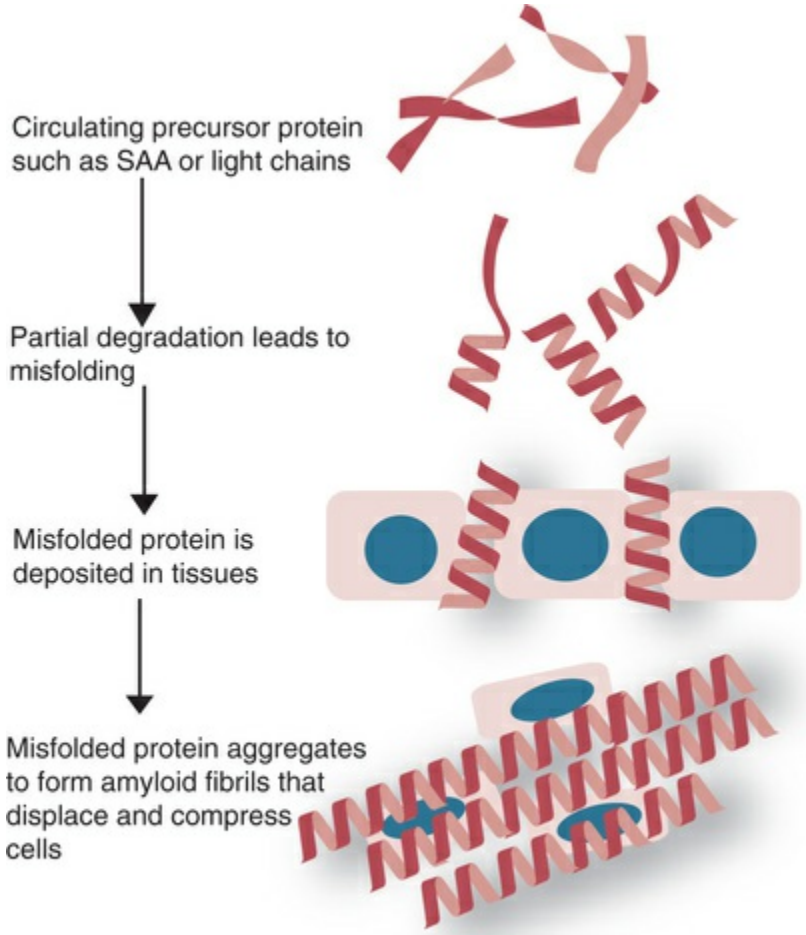


FIG. 7.12 The pathogenesis of reactive amyloid fibril deposition. Misfolded proteins aggregate to form insoluble fibrils.

Multiple myelomas are plasma cell tumors that secrete antibodies, especially antibody light chains (Chapter 15). Huge quantities of antibody light chains and their fragments are produced, and their misfolding results in the deposition of immunogenic amyloid in organs. Immunogenic amyloid is the most common form of amyloid in humans, but is very rare in domestic animals.

Several other forms of localized amyloidosis are recognized in domestic animals; for example, old dogs may suffer from vascular amyloidosis, in which amyloid is deposited in the media of leptomeningeal and cortical arteries. An inherited form of amyloid has been described in Abyssinian cats. Tumor-like amyloid nodules and subcutaneous amyloid have been reported in horses, but in general, amyloid deposits are found in the liver, spleen, and kidneys, particularly within glomeruli. In humans, amyloid fibrils

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are deposited in the neurons of patients with Alzheimer's disease.

Misfolded proteins may be transmissible (Box 7.3). They form the prion proteins that cause the spongiform encephalopathies such as bovine spongiform encephalopathy (BSE) and scrapie. Prions are protease-resistant forms of cellular proteins. In the case of BSE, the prion is a misfolded and aggregated form of a cellular protein PrP^c that is important for normal macrophage functions. These proteins normally play a role in resistance to intracellular bacteria such as *Brucella.* It is also of interest to note that even reactive amyloidosis is somewhat "transmissible" since inoculation of AA proteins into an animal will hasten the development of amyloidosis. In such cases, amyloid proteins likely act by providing a substrate on which other misfolded proteins can be deposited. There is evidence that foie gras prepared from duck or goose liver can transmit AA amyloidosis when fed to mice. Similarly, silk fibers formed from a protein composed of β-sheets, may promote amyloidosis when inserted into mice!

Box 7.3

Transmissible Amyloidosis?

AA amyloidosis is unusually common in captive cheetahs and poses a significant threat to the species' survival. Upon investigation, however, it was found that cheetahs suffering from amyloidosis excreted AA amyloid fibrils in their feces. These fecal amyloid fibers could be purified and injected into mice, where they were much more effective than tissue amyloid in inducing amyloidosis. Thus cheetahs appear to transmit AA amyloid in their feces. This material may cause disease in mice that happen to ingest cheetah feces. It is also possible that amyloidosis may develop in cheetahs that happen to eat these mice! It may also be that other cheetahs may acquire the amyloidosis by fecal contamination of cuts or abrasions. Given that cheetahs are genetically homogeneous and tend to suffer from many chronic inflammatory diseases, they may be more susceptible to amyloidosis than other species. The transferred amyloid might simply enhance an ongoing disease. Thus AA amyloidosis in cheetahs may be a prion disease.

Data from Zhang B, Une Y, Fu X, Yan J et al: Fecal transmission of AA amyloidosis in the cheetah contributes to high incidence of disease, *Proc Natl Acad Sci U S A* 105:7263-7268, 2008.

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How Immune Cells Communicate

Cytokines and Their Receptors

CHAPTER OUTLINE

Cytokine Nomenclature, 72
Cytokine Functions, 73
Cytokine Structure, 74
Cytokine Receptors, 74
Receptor Families, 76
Cytokine Regulation, 76
Signal Transduction, 77
Protein Phosphorylation, 77
Transduction Pathways, 78
NF-κB Pathway, 78
NF-AT Pathway, 79
JAK-STAT Pathway, 80
Gene Transcription, 81

LEARNING OBJECTIVES

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After reading this chapter, you should be able to:

- Understand that the immune responses result from multiple complex interactions among many cell populations.
- Explain the differences between cytokines and hormones.
- Describe how these signaling molecules bind to specific receptors on target cells.
- Explain the basic mechanisms involved in signal transduction and the generation of transcription factors.
- List some important transcription factors.
- Explain the importance of protein phosphorylation in cell signaling.
- Explain the importance of tyrosine kinases in cell signaling.
- Explain the central role of NF-κB in immunity.
- Explain how cytokine activity is controlled.
- Explain in general terms how cytokines can cause changes in gene transcription, how new proteins are produced and secreted, and how affected cells alter their behavior.
- Define interleukin, interferon, NF-κB, JAK-STAT, tumor necrosis factors, tyrosine kinase, protein phosphorylation, and gene transcription.

The immune systems are complex networks that involve many different cell populations, each sending and receiving multiple messages from many sources. These intercellular signals are transmitted in two general ways. In volume transmission, a mediator molecule is released by the signaling cell and simply diffuses through the extracellular fluid to the receiving cell, where it binds to cell surface receptors. The second method, called network transmission, occurs when two cells come into direct contact using complementary receptors. Signals are then transmitted directly through these two receptors.

Irrespective of how the signals are transmitted, target cells can be directed to behave in a specific manner by signaling through appropriate receptors. They may be told to divide or stop dividing; they may be stimulated to synthesize and secrete other signaling molecules or express new receptors; they may be told to commit suicide. Each cell may be exposed to hundreds of signals at any one

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time. The target cell must integrate these signals and respond appropriately. In this chapter, we review the signaling molecules produced by immune cells, the receptors that receive these signals, and the way in which these incoming signals are interpreted by a cell.

The cells of the immune system produce hundreds of proteins that control the immune responses. These proteins are called cytokines (Box 8.1). Cytokines differ from conventional hormones in several important respects. Unlike hormones, which tend to affect a single target cell type, cytokines can affect many cell types. Second, immune system cells rarely produce a single cytokine at a time. For instance, macrophages secrete at least four interleukins (IL-1, IL-6, IL-12, and IL-18) as well as tumor necrosis factor-α (TNF-α). Third, cytokines are "redundant" in their biological activities in that many cytokines have similar effects. For example, IL-1, TNF-α, TNF-β, IL-6, and high mobility group box protein-1 (HMGB1), all act on the brain to cause a fever. Finally, cytokine-mediated signals are transient, and the messages delivered may vary over time as the cytokine environment changes.

Box 8.1

Properties of Cytokines

- Short-lived proteins
- Highly diverse structures and receptors
- Can act locally and/or systemically
- Pleiotropic: affect many different cells
- Redundant: exhibit biologically overlapping functions
- Carefully regulated
- Toxic in high doses

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Cytokine Nomenclature

The nomenclature and classification of the cytokines is not based on any systematic relationship among these proteins. Many were originally named after their cell of origin or the bioassay used to identify them. The interleukins, for example, are cytokines that signal between lymphocytes and other leukocytes. They are numbered sequentially in the order of their discovery. Because their definition is so broad, the interleukins are a heterogeneous mixture of proteins with little in common except their name. As of 2016, 39 numbered interleukins have been described. As might be expected, we know a lot about some of these molecules and very little about others. Likewise, some are clearly critical to a successful immune response, whereas others appear to be less essential.

The interferons are a family of cytokines produced in response to virus infection or immune stimulation. Their name is derived from the fact that they interfere with viral RNA and protein synthesis and so have antiviral activity (Chapter 27). There are three major types of interferon. Type I interferons are a diverse mixture, the most important of which are interferon-α (IFN-α) and IFN-β. There is a single type II interferon, called IFN-γ. Three type III interferons (IFN−λ) have been identified. Type I interferons are primarily antiviral with a secondary immunoregulatory role. For type II and type III interferons such as IFN-γ and IFN−λ, the reverse is the case. Many type I interferons also play an important role in the maintenance of pregnancy (Chapter 34).

The tumor necrosis factors (TNFs) are cytokines produced by macrophages and T cells. As their name suggests, they can kill tumor cells, although this is not their primary function. Thus TNF-α is a key mediator of acute inflammation. The TNFs belong to a family of related cytokines, the TNF superfamily, which is involved in the coordination of host defenses, cell survival, immune regulation and inflammation. It consists of at least 50 cytokines and their receptors. Important members of the TNF superfamily include CD178 (also called CD95L or Fas ligand) (Chapter 18), and CD154 (CD40 ligand) (Chapter 15).

Many cytokines act as growth factors (or colony-stimulating

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factors) and so control cell production by regulating stem cell activities. They thereby ensure that the body is supplied with sufficient cells to defend itself.

Chemokines are a family of at least 50 small proteins that play a role in leukocyte chemotaxis, circulation, migration, and activation, especially in inflammation. A typical example of a chemokine is CXCL8 (also known as IL-8). Chemokines are described in detail in Chapter 3.

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Cytokine Functions

Cytokines are produced in response to many stimuli. Examples of these stimuli include antigens acting through the T cell or B cell antigen receptors; antigen-antibody complexes acting through antibody receptors (FcR); pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides acting through pattern-recognition receptors (PRRs); and other cytokines acting through specific cytokine receptors (Fig. 8.1).

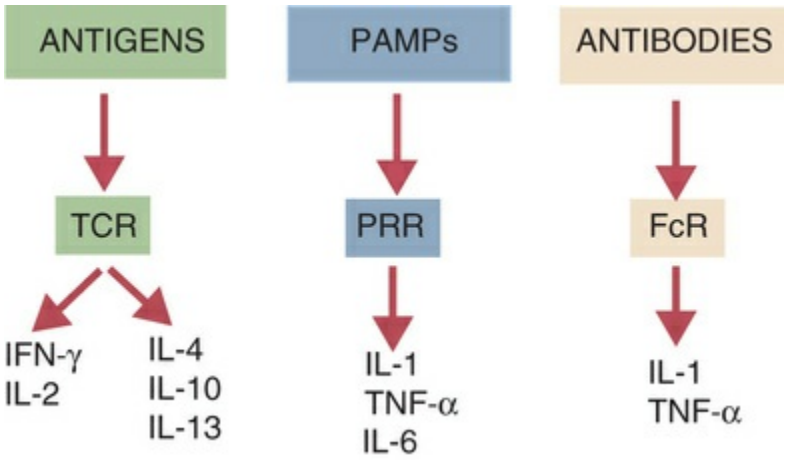


FIG. 8.1 Three of the most important pathways that trigger cytokine release are the binding of antigens to their receptors on T and B cells, the binding of PAMPs to pattern-recognition receptors on sentinel cells; and the binding of antibodies to Fc receptors on phagocytic cells.

Cytokines act on many targets. They may, for example, bind to receptors on the cell that produced them and thus have an autocrine effect. Alternatively, they may bind only to receptors on nearby cells; this is called a paracrine effect. Some cytokines may spread throughout the body, affecting target cells in distant locations, and thus have an endocrine effect (Fig. 8.2).

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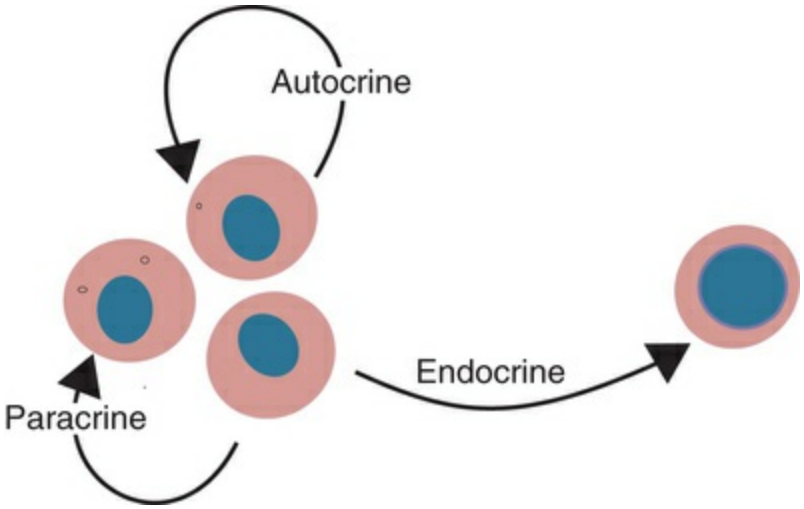


FIG. 8.2 The distinction among autocrine, paracrine, and endocrine effects. Cytokines differ from hormones in that most of their effects are autocrine or paracrine, whereas hormones usually act on distant cells in an endocrine fashion.

When cytokines bind to receptors on target cells, they affect cell behavior. They may induce the target cell to divide or differentiate, or they may stimulate the production of new proteins. Alternatively, they may inhibit these effects—preventing division, differentiation, or new protein synthesis. Most cytokines act on many target cell types, perhaps inducing different responses in each one, a feature that is called pleiotropy. Conversely, many cytokines may act on a single target, a feature known as redundancy. For example, IL-3, IL-4, IL-5, and IL-6 all affect B cell function. Some cytokines work best when paired with other cytokines in a process called synergy. For example, the combination of IL-4 and IL-5 stimulates B cells to make immunoglobulin E (IgE) and hence triggers an allergic response. Synergy can also occur in sequence when, for example, one cytokine induces the target cell to express the receptor for another cytokine. Finally, some cytokines have opposing effects and may antagonize the effects of others. The best example of this is the mutual antagonism of IL-4 and IFN-γ.

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Cytokine Structure

Cytokines are very diverse proteins. They can be classified based on their structures or on their functions (Table 8.1). Structurally, many fall into four major groups. The group I cytokines (or hematopoietins), consist of four α helices bundled together. This group can be subdivided into three families: the interferon family, the IL-2 family, and the IL-10 family. All facilitate communication between lymphocytes and epithelial cells. Group II cytokines consist of long-chain β-sheet structures. They include the TNF family and the IL-1 family. The IL-1 family includes seven agonist cytokines, three receptor antagonists, and one antiinflammatory cytokine. IL-1 family members require proteolytic processing by enzymes such as caspases to achieve full biological activity. Group III cytokines include the chemokines and related molecules, small proteins with both α helices and β sheets. Group IV cytokines are heterodimers that use mixtures of domains and structural motifs and include the IL-12 family and the IL-17 family. Many cytokines, such as IL-14, IL-16, and IL-32, are structurally unique and do not belong to any of these major families.

TABLE 8.1

Molecular Classification of Selected Cytokines

Structural Families	Structure	Examples
Group 1	Four α helix bundle	IL-2, -3, -4, -5, -6, -7, -9, -11, -13, -15, -21; GM-CSF, erythropoietin, G-CSF, prolactin, leptin, TSLP.
	IFN subfamily	IFN-α/β, IFN-γ,IFN−λ
	IL-10 subfamily	IL-10, -19, -20, -22, -24, -26, -28, -29
Group 2	β Sheets	TGF-β
	IL-1 family	IL-1α, -1β, -18, -33, -36, -37, -38,
	TNF family	TNFs, BAFF
Group 3	α Helices and β sheets	Chemokines
Group 4	Mixed motifs	
	IL-12 family	IL-12, -23, -27, -30, -35, -39
	IL-17 family	IL-17A-F. IL-17F is also called IL-25
Ungrouped	Unique structures	IL-14, -16, -32

Patterns may also be seen in their biological activities. Thus group I cytokines tend to be involved in immune regulation or stem cell regulation. Group II cytokines are mainly involved in the

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growth and regulation of cells, cell death, and inflammation. Group III cytokines are involved in inflammation. The activities of the group IV cytokines depend on their subcomponents. For example, IL-12 is formed by a combination of a group I structure with a stem cell receptor, but it acts like a group I cytokine.

A more practical cytokine classification is based on their roles in adaptive immunity. Put simply, type I cytokines mediate cell-mediated or type 1 immune responses, while type II cytokines mainly mediate antibody or type 2 responses. The major type I cytokines are IL-1 and IFN-γ. The major type II cytokines are IL-4 and IL-13.

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Cytokine Receptors

Cytokines act through cell surface receptors. These receptors consist of at least two functional units, one for ligand binding and one for signal transduction (Fig. 8.3). These units may or may not be on the same protein. Cytokine receptors can also be classified into classes based on their structure.

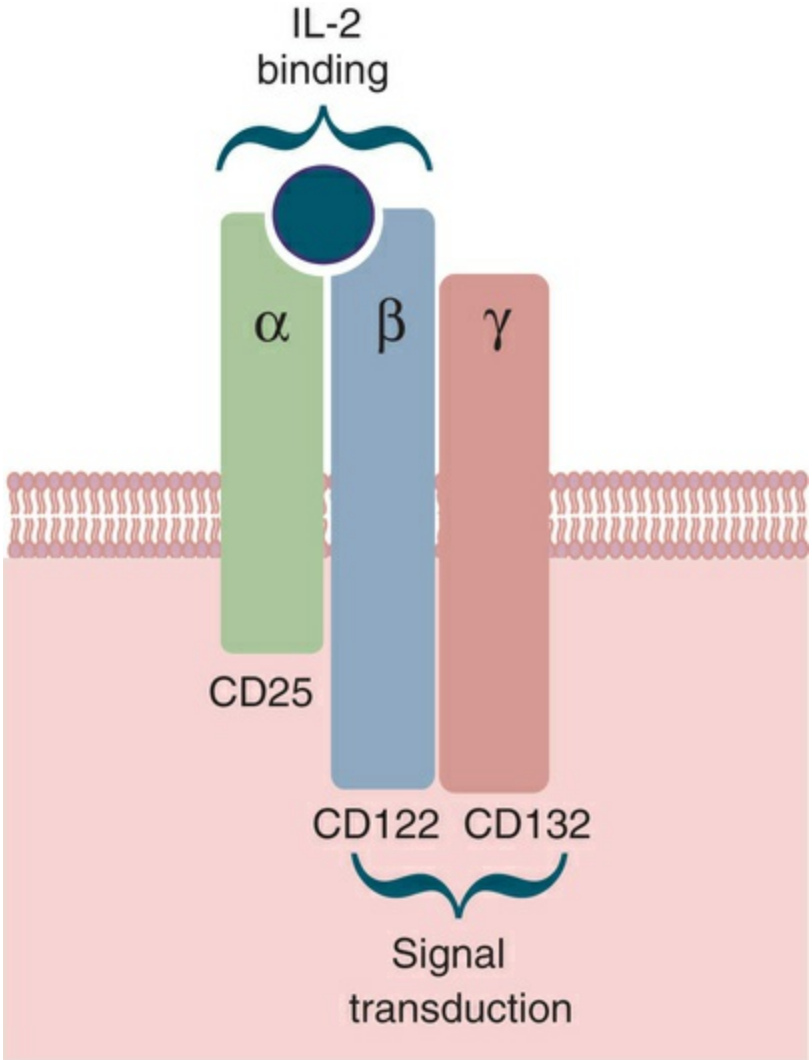


FIG. 8.3 The structure of a cytokine receptor, in this case, the IL-2 receptor complex. The complete trimer serves as a high-affinity receptor, whereas the β-γ dimer serves as a low-affinity receptor. Note that different receptor components each serve different functions.

One class of receptor includes the channel-linked receptors,

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which act as transmitter-gated ion channels. Thus the receptor itself is a channel, and binding of its ligand opens that channel, allowing ions to pass through it. Channel-linked receptors are found in inflammatory and immune cells, but their roles are unclear. They do not serve as cytokine receptors.

A second class of receptor consists of proteins that also act as tyrosine kinases (Fig. 8.4). These are typically growth factor and cytokine receptors. In these, binding of the ligand to two adjacent receptors forms an active dimer. The ligand-binding site, the membrane-spanning region, and the tyrosine kinase are separate domains of a single protein. Thus when the ligand binds to the extracellular domains, the two receptor chains come together so that the two tyrosine kinases can activate each other. These kinases phosphorylate tyrosine residues on other proteins or even the receptor itself (auto-phosphorylation). Since many of these other proteins are also tyrosine kinases, phosphorylation also activates them. In this way a cascade of phosphorylations develops within the cell (Fig. 8.5). Phosphorylation causes changes in cellular activities. Many cytokines operate through this type of receptor, especially through tyrosine kinases of the src family.

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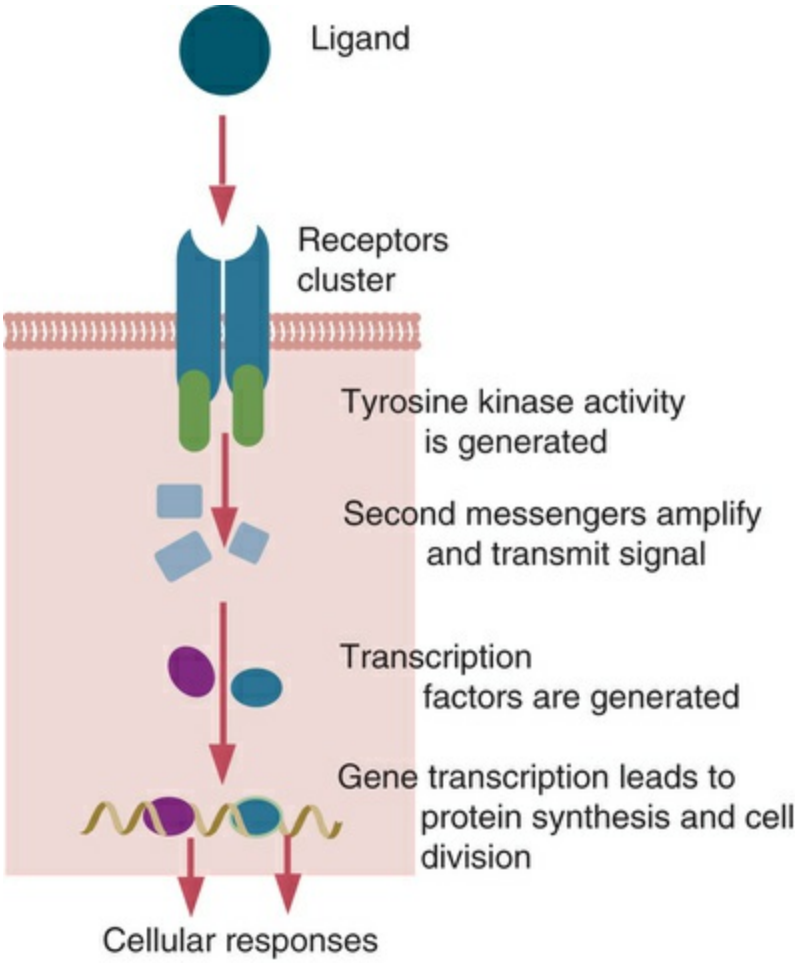


FIG. 8.4 A generic view of signal transduction involving the activation of tyrosine kinases. Although receptor signaling varies in its details and intricacy, the overall process of signal transduction has some consistent features as shown here.

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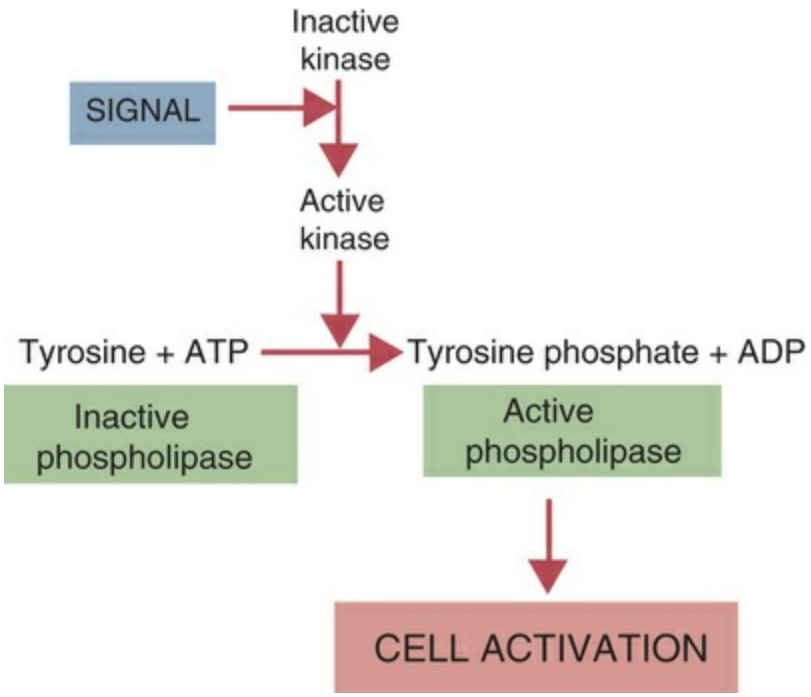


FIG. 8.5 The key to signal transduction and cellular activation is the phosphorylation of the amino acid tyrosine by the actions of a tyrosine kinase. For example, phosphorylation of tyrosine by a protein kinase can result in phospholipase activation, which leads eventually to cell activation. Phosphorylation can have many other effects on protein functions and fate.

A related class of receptor consists of proteins that are not themselves tyrosine kinases but can activate linked tyrosine kinases. This type of receptor is also widely employed in the cells of the immune system. Examples of tyrosine kinase-linked receptors include the T cell antigen receptor (TCR) and the B cell antigen receptor (BCR). Some of these tyrosine kinases may transfer their phosphate groups to transcription factors within the nucleus and activate them. Others act indirectly through the production of second messenger molecules.

A large class of receptors are coupled to membrane-bound guanosine triphosphate (GTP)-binding proteins, called G-proteins. G-proteins act as chemical switches and control many cellular processes. When the receptor is inactive, they bind guanosine diphosphate (GDP). When bound to their ligand, they add another phosphate group to form GTP (Fig. 8.6). The activated G-protein then activates other substrates, resulting in a biological response. The GTP is rapidly hydrolyzed to GDP so that the G-protein is then turned off. The targets of G-proteins include ion channels, enzymes

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such as adenylate cyclase, phospholipase C, and some protein kinases.

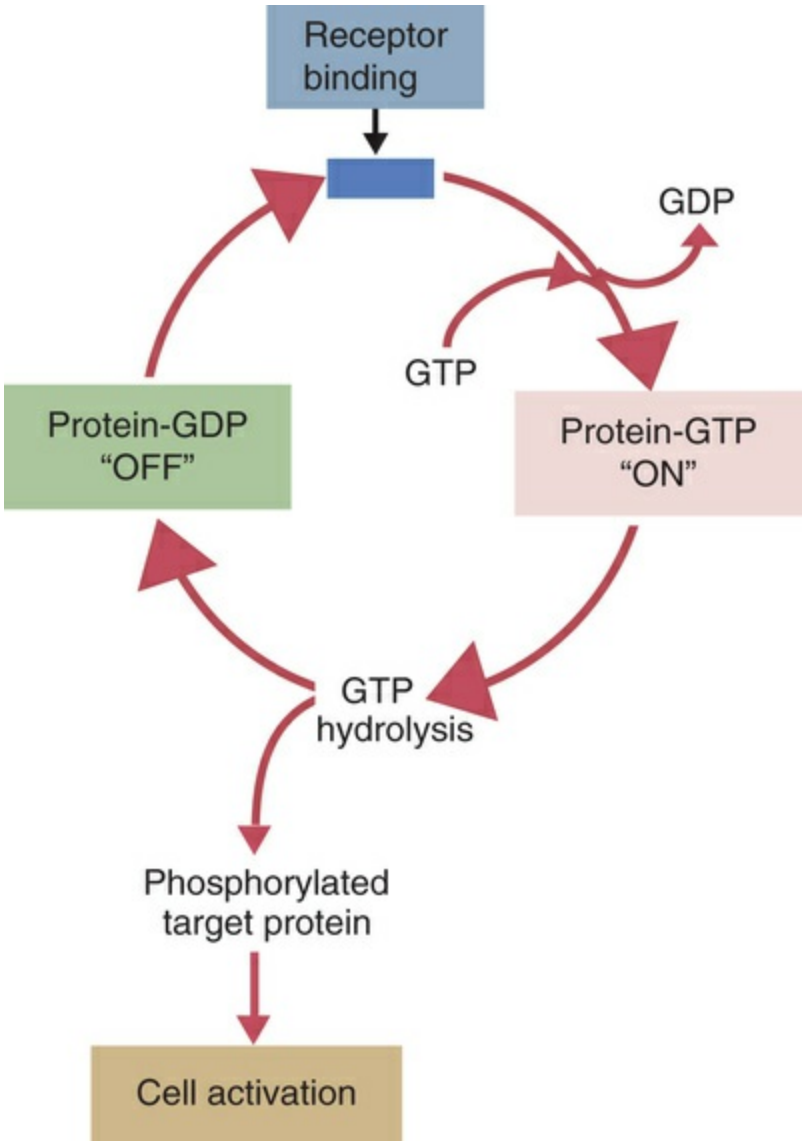


FIG. 8.6 GTP-binding proteins (G-proteins) can act as signaling switches to turn cell functions on and off. They are often activated in the initial stages of signal transduction.

When activated by a G-protein, phospholipase C splits the membrane-bound lipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), into two messenger molecules, inositol trisphosphate and diacylglycerol (Fig. 8.7). Inositol trisphosphate binds to intracellular receptors releasing Ca^{2+} from internal stores and increases the concentration of intracellular Ca^{2+}. These calcium ions can activate many proteins. The diacylglycerol remains in the plasma membrane

and along with calcium activates an enzyme called protein kinase C. Immunological receptors that employ G-proteins include the receptors for C5a, chemokine receptors, leukotriene receptors, and the platelet-activating factor receptor. In neutrophils, G-proteins control responses to chemoattractants.

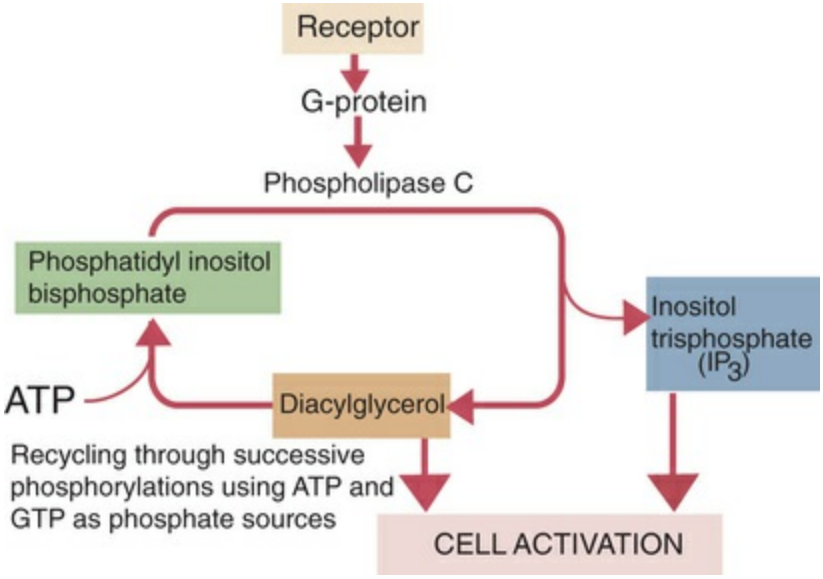


FIG. 8.7 Activation of cell membrane phospholipase C generates both inositol trisphosphate and diacylglycerol. These two molecules are messengers that initiate cell activation. The signaling molecules can then be recycled by phosphorylation.

A fourth class of receptor acts in a totally different manner. It activates a sphingomyelinase that then hydrolyzes the cell membrane phospholipid, sphingomyelin, to form ceramide. The ceramide then activates a serine-threonine protein kinase that phosphorylates cellular proteins. This mechanism of signal transduction is used by the receptors for IL-1 and IFN-α.

Receptor Families

In general, most cytokines use receptors that act through tyrosine kinases. However, within this class, one can identify related families of receptors. For example, the IL-1/toll-like receptor (TLR) family participates in host responses to injury and infection. The family can be split into the molecules that are IL-1R–like (IL-1R1, IL-18R) and the molecules that are toll-like (TLR). Ligation of these

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receptors triggers activation of the transcription factor NF-κB (see Fig. 8.4).

Another large receptor family, the group I cytokine receptor family, includes among its members the receptors for IL-2 (β chain), IL-4, IL-5, IL-6, IL-12, granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Fig. 8.8). It also includes the common γ chain of IL-2, IL-4, IL-7, IL-9, and IL-15 receptors. These receptor chains dimerize in the presence of the ligand and form complexes with a group of kinases called Janus kinases (JAKs). JAKs in turn phosphorylate a cytosolic protein called STAT (signal transducers and activators of transcription). The STAT then dimerizes to form an active transcription factor. Depending on the specific JAKs and STATs used, these may enhance or suppress cytokine signaling.

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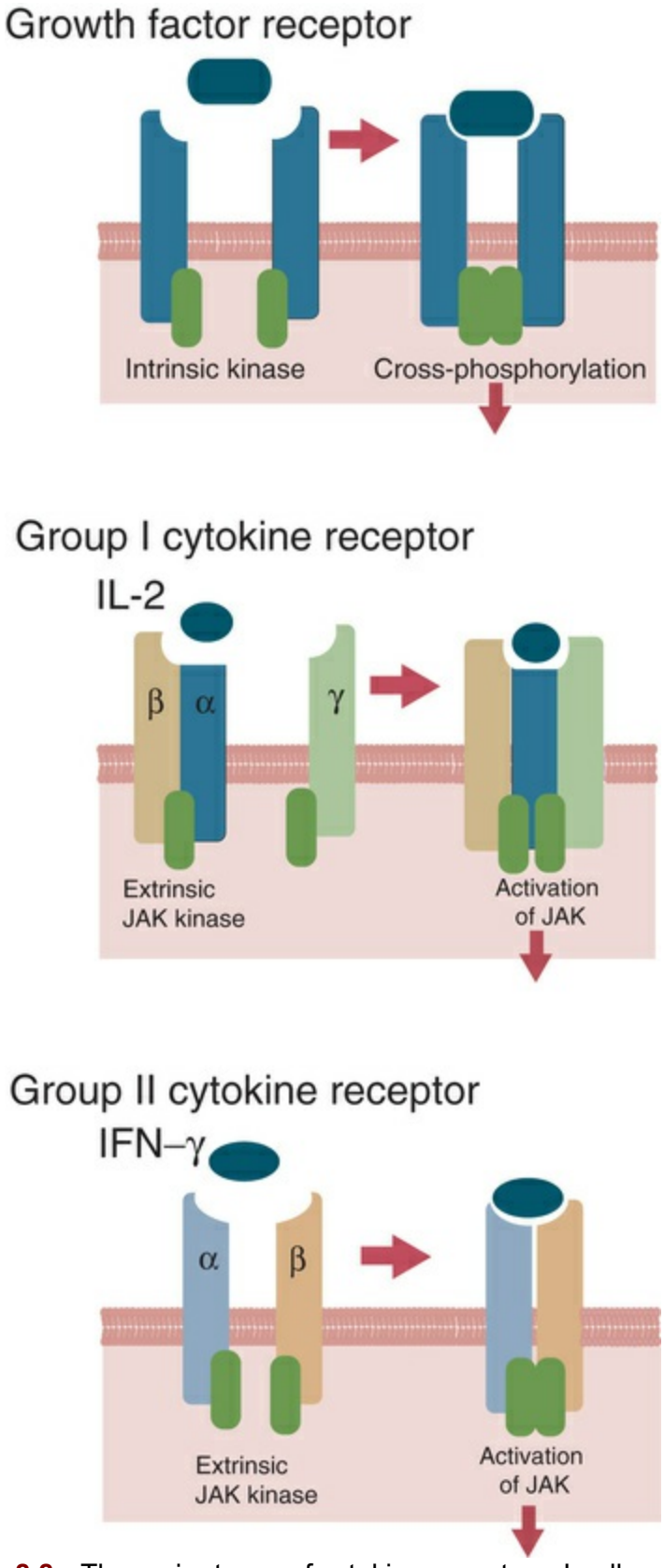


FIG. 8.8 The major types of cytokine receptors. In all cases, protein kinases are activated by ligand binding. For example, growth factor receptors associate in the presence of their ligand to form a dimer. This brings the two tyrosine kinases on the cytoplasmic domains close together. The enzymes then activate

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each other by cross-phosphorylation. Group I cytokine receptors, so called because they bind the type I cytokines, associate in the presence of the ligand to form oligomers and form complexes with JAK kinases. When brought together, the JAK kinases are activated and in turn activate STAT proteins. The activated STAT proteins then dissociate and activate transcription factors. Group II cytokines such as the interferons and IL-10 bind receptors that have a similar mode of action to type I receptors.

The members of the group II cytokine receptor family have a very different structure. They bind interferons (α, β, γ, and λ) and members of the IL-10 cytokine family (IL-19, IL-20, IL-22, IL-24, and IL-26). These receptors also form heterodimers in the presence of the ligand and signal through the JAK-STAT pathway.

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Cytokine Regulation

Cytokine signaling is regulated in three ways: by changes in receptor expression, by the presence of specific binding proteins, and by cytokines that exert opposing effects. For example, IL-2 receptor expression determines the response of T cells to IL-2. Resting T cells express few receptors for IL-2, but when activated, their expression of these receptors increases enormously. In contrast, IL-1 is regulated by a receptor antagonist called IL-1RA. IL-1RA is a form of IL-1 that binds to the IL-1 receptor but does not stimulate signal transduction. It therefore blocks active IL-1 (Fig. 8.9). Other cytokines may bind to soluble receptors in body fluids. Examples include the soluble receptors for IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, TNF-α, and macrophage colony-stimulating factor (M-CSF). In most cases, these soluble receptors compete for cytokine binding with the cell surface receptors and hence inhibit cytokine activity. Cytokines such as IL-1, IL-12, and TGF-β may bind to connective tissue glycosaminoglycans such as heparin or CD44 that then serve as a reservoir of readily available molecules.

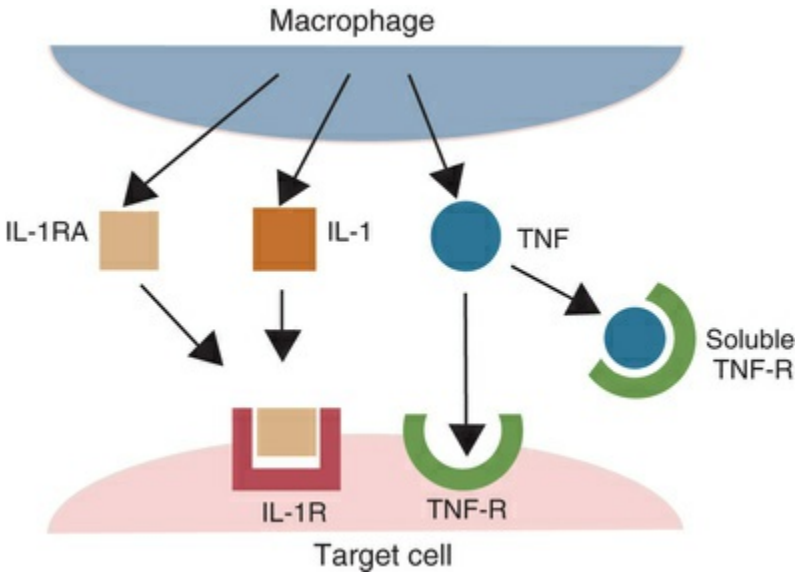


FIG. 8.9 The control of cytokine activities as exemplified by IL-1 and TNF-α. IL-1 activity is regulated by the presence of IL-1RA, an inert form of IL-1 that binds and blocks the IL-1 receptor, preventing signal transduction. Soluble TNF-αR, in contrast, competes for TNF-α with the cell membrane receptor but also serves to inhibit cytokine

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Some cytokine receptors are decoys. They bind cytokines but do not transmit signals. The IL-1 type II receptor is such a receptor. Other decoy receptors have been identified for the IL1/IL-18 family as well as for the TNF, IL-10, and IL-13 receptor families.

The most important mechanism of cytokine regulation is through the opposing effects of different cytokines. For example, the type II cytokine IL-4 stimulates IgE production, whereas the type I cytokine, IFN-γ suppresses IgE production (Chapter 29). Likewise IL-10 and IL-37 inhibit the activities of many other cytokines (see Fig. 20.12). Suppressors of cytokine signaling (SOCS) proteins play an important role in the control of the inflammatory response. These act on both the NF-κB and the JAK-STAT pathways and so regulate inflammation. It is also important to bear in mind that at any given time, a single cell may receive signals from multiple cytokine receptors. It must somehow integrate these multiple signals to produce a coherent response.

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Signal Transduction

When a cytokine binds its receptor, the receptor transmits a signal to the cell to modify its behavior. This conversion of an extracellular signal into a series of intracellular events is called signal transduction. The key components of signal transduction include binding of an agonist such as a cytokine to its receptor, possibly clustering of receptor chains, activation of kinases by the receptor, secondary activation of second messengers, generation of new transcription factors, and gene activation leading to altered protein synthesis and cell behavior (see Fig. 8.4). Because cell signaling must be fast and precise, this is best accomplished by enzyme cascades. Since enzymes can produce or modify a large number of molecules in a short time, a pathway that involves the use of several enzymes in sequence can amplify responses very rapidly.

Protein Phosphorylation

Central to most cell signaling is the reversible modification of proteins by addition of a phosphate group to selected amino acids. Signal transduction systems use a high-energy phosphate-rich compound such as adenosine triphosphate (ATP) to modify proteins and hence send a signal to a cell. Cell growth, cell division, and other critical processes are all regulated by protein phosphorylation. The enzymes that do this, called protein kinases, enzymatically phosphorylate the amino acids serine, threonine, or tyrosine.

$$\text{Protein} + \text{ATP} \xrightarrow{\text{Protein kinase}} \text{protein-P} + \text{ADP}$$

In some proteins, only one amino acid is phosphorylated; in others, multiple amino acids are phosphorylated. Phosphorylated and nonphosphorylated proteins have different functional properties. For example, the phosphorylation of serine or threonine activates some enzymes, whereas dephosphorylation has the opposite effect. In most cells, about 90% of the phosphate groups

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are linked to serine and about 10% to threonine. Only about 1/2000 of the phosphate is linked to tyrosine. Thus tyrosine phosphorylation is a rare event. Nevertheless it is a key mechanism in almost all the signal transduction pathways described in this book.

Transduction Pathways

Although there are many signal transduction pathways, three play key roles in the immune system. These involve the generation of the transcription factors: NF-κB, NF-AT, and STAT.

NF-κB Pathway

The NF-κB pathway is the most significant signal transduction pathway in the immune system. It is the pathway that is activated when antigens bind to the T cell and B cell antigen receptors (TCR and BCR); when PAMPs bind to the pattern-recognition receptors (PRRs), such as the TLRs and NODs; and when TNF-α binds to its receptor. Thus NF-κB plays a critical role in both innate and adaptive immunity. The term NF-κB refers to a family of five transcription factors. These factors can form many heterodimers that activate different genes. More than 150 unique stimuli can activate NF-κB, and more than 150 genes are expressed after NF-κB activation. In a resting cell, NF-κB is found in the cytosol in an inactive form bound to a protein called IκB. IκB inhibits NF-κB activity by masking its nuclear binding site. Thus in resting cells, NF-κB cannot move to the nucleus or activate genes.

The major NF-κB activation pathway is triggered by inflammatory cytokines IL-1 and TNF-α, by TLRs, and by antigen receptors, and it is essential for innate immunity. The signals induced by these stimuli converge on a central regulator of NF-κB, the IKK (IκB kinase) complex. This complex consists of multiple subunits with kinase activity. When activated, IKK phosphorylates IκB. As a result, the IκB dissociates from the NF-κB and is destroyed. This releases the NF-κB so that it can enter the nucleus and activate selected genes including those encoding IL-1β, IL-6, IL-18, IL-33, TNF-α, GM-CSF, and IL-4. NF-κB also activates the genes coding for chemokines, proangiogenic factors, adhesion molecules,

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antiapoptotic proteins, inducible enzymes such as iNOS and cyclooxygenase-2 (COX-2), as well as IκB. This newly synthesized IκB will eventually bind and suppress NF-κB activation. Molecules or organisms that block the destruction of IκB have antiinflammatory and immunosuppressive effects. For example, corticosteroids stimulate the production of excess IκB, whereas some bacteria can block its degradation. Either way, the activation of cells and the development of inflammation and immune responses may be blocked.

An example of the importance of the NF-κB pathway is seen when macrophages respond to a PAMP that binds to their TLRs (Fig. 8.10). This immediately causes the receptor to dimerize. As a result, it binds several adaptor molecules, of which one, myeloid differentiation primary response gene 88 (MyD88), is the most important (Chapter 2). When MyD88 binds to the TLR, it also binds two kinases called IRAK-1 and IRAK-4. IRAK-4 activates IRAK-1, and these in turn recruit TRAF6. TRAF6 and other proteins then activate the IKK complex. Activation of IKK phosphorylates IκB, causing its destruction and the release of active NF-κB. The NF-κB in turn enters the nucleus and activates the genes that encode precursors of TNF-α, IL-1β, and caspase-1. Caspase-1 activates these newly produced cytokines that then trigger inflammation.

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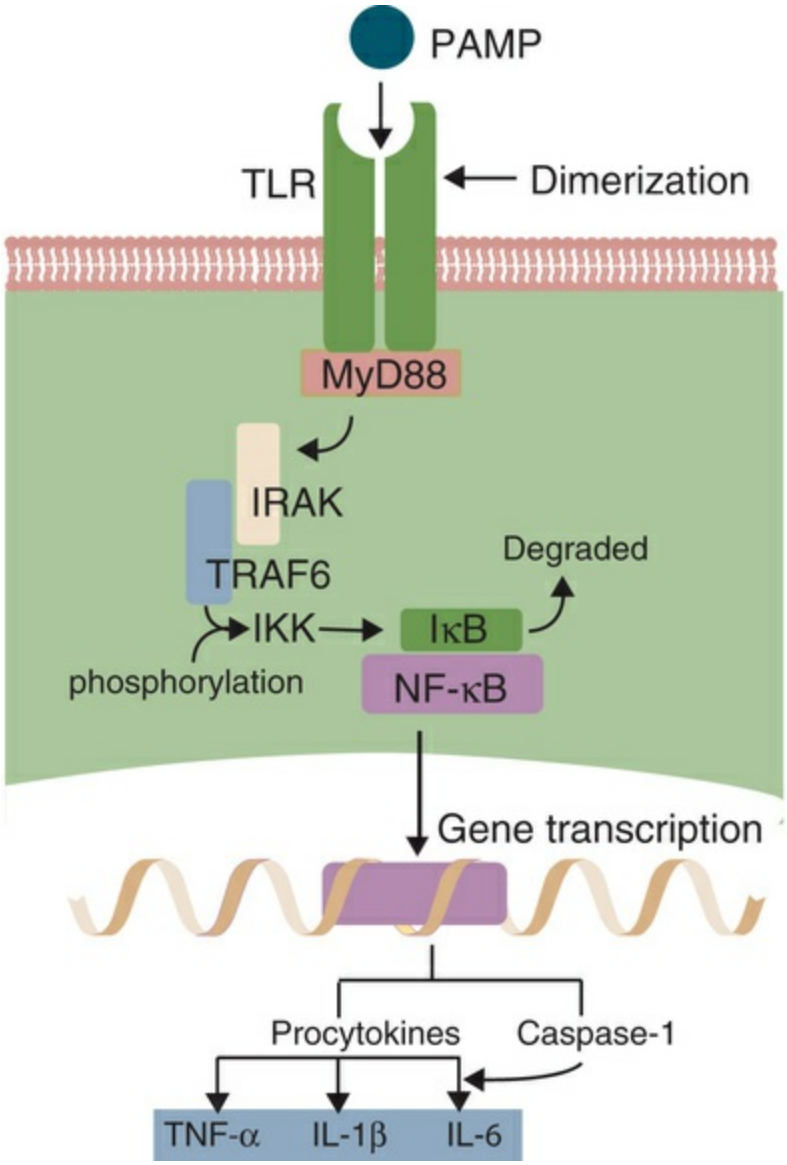


FIG. 8.10 One major pathway of signal transduction is mediated by the transcription factor NF-κB. This is widely employed in immunological signaling such as the response to sentinel cells to activation of their TLRs by PAMPs.

Binding of antigen to TCRs also activates NF-κB. Thus binding to the TCR activates a protein kinase C to form a protein complex that degrades IKK. Another pathway involves stabilization of NF-κB–inducing kinase (NIK). This activates IKKα, that then promotes the destruction of IκB by proteasomes. All NF-κB members affect TCR-induced T cell proliferation. Antigen dose and oscillations in calcium concentrations are also important because they modulate the movement of transcription factors between the nucleus and cytoplasm.

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NF-AT Pathway

When an antigen binds to its receptor on a T cell, the signal is first transmitted from the antigen-binding receptor (TCR) to a signal transducing complex called CD3, where it causes the CD3 chains to cluster together in lipid rafts (Fig. 8.11). Each CD3 protein has amino acid sequences in its cytoplasmic domains called immunoreceptor tyrosine-based, activation motifs (ITAMs). When the CD3 chains cluster, their ITAMs collectively activate several tyrosine kinases. These tyrosine kinases (TKs) are members of the src kinase family. They include lck and fyn in T cells and NK cells, and lyn and fyn in B cells and mast cells. In T cells, the first TK activated, called lck, phosphorylates the ITAMs. As a result, these sites then bind a second TK, called zeta-associated protein-70 (ZAP-70). The bound ZAP-70 is phosphorylated and after binding many other proteins forms a multimolecular proximal signaling complex (PSC). Signals generated by the PSC then activate at least three families of transcription factors. One pathway generates the second messengers, diacylglycerol and inositol trisphosphate. The inositol trisphosphate releases calcium ions from intracellular organelles and opens transmembrane channels, allowing Ca^{2+} to enter the cell and raising intracellular calcium. This in turn activates a phosphatase called calcineurin. Calcineurin removes a phosphate from NF-AT. Dephosphorylated NF-AT enters the nucleus and with the help of another transcription factor called activator protein-1 (AP-1), binds to the promoters of at least 100 genes. The potent immunosuppressive drugs tacrolimus and cyclosporine bind to calcineurin and so block T cell-mediated responses (see Figs. 41.4 and 41.5). If the T cell receives suppressive signals, such as those provided by IL-10 or TGF-β, NF-AT will associate with a transcription factor called Foxp3. Foxp3 activates a very different set of genes and converts the cell into a regulatory T cell (Treg) that suppresses immune responses (Chapter 20).

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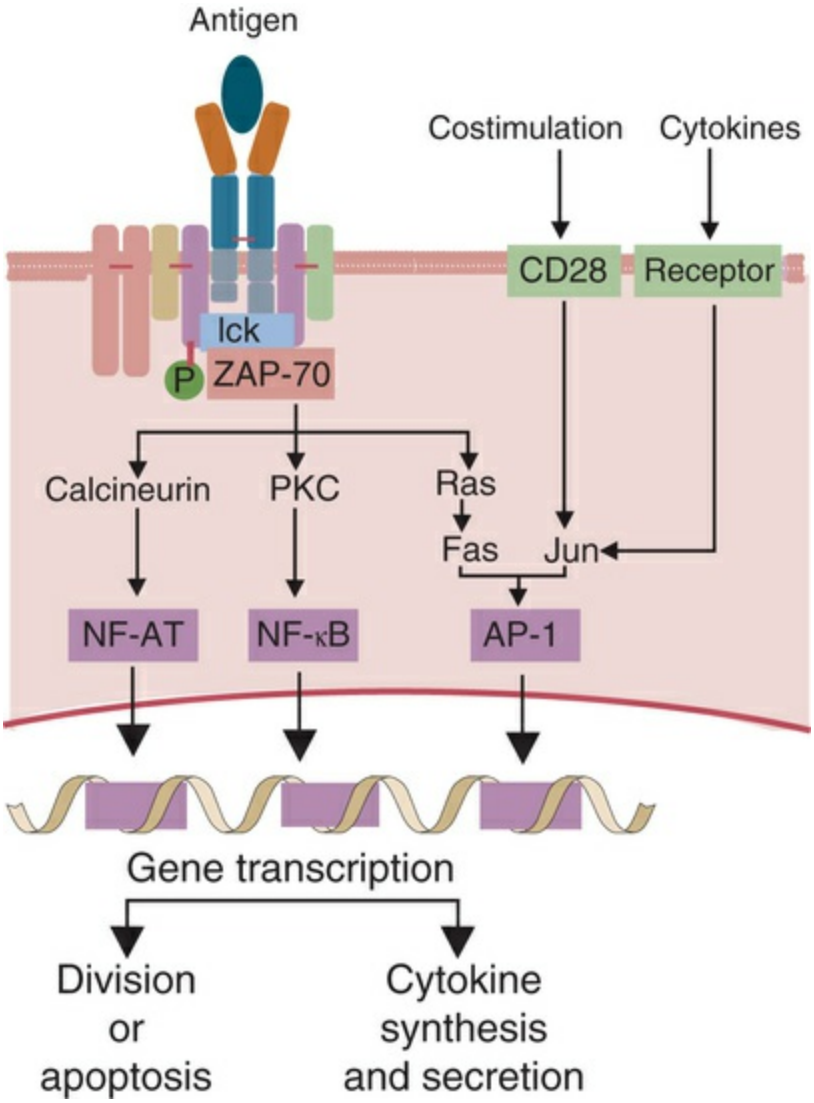


FIG. 8.11 Signal transduction mediated through T cell antigen receptors generates three transcription factors: NF-AT, NF-κB, and AP-1. When TCRs cluster, they activate several protein kinases. The most important of these is called ZAP-70. This in turn triggers three signaling pathways, and with appropriate costimulation, generates multiple transduction factors. The jun-fos heterodimer (AP-1) is required to stimulate the genes for cytokines and their receptors. The final results of the stimulus include cell division or apoptosis as well as cytokine production.

In B cell antigen receptors, the adaptor molecules Ig-α and Ig-β also have ITAMs. When aggregated by antigen and costimulated by CD19, they activate the src kinases, lyn and fyn. These in turn activate phospholipase C and eventually generate both NF-κB and NF-AT (Fig. 8.12).

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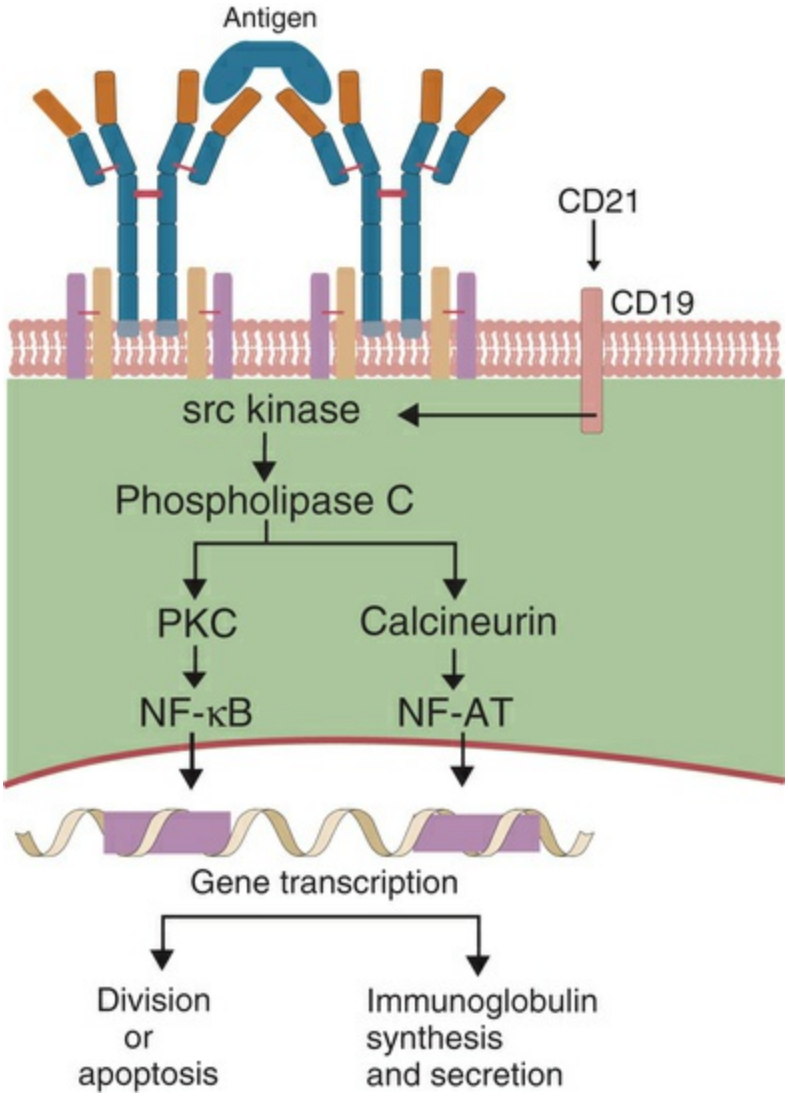


FIG. 8.12 Signal transduction by two cross-linked BCRs activates B cells, triggering cell division, differentiation, and immunoglobulin synthesis. Both NF-κB and NF-AT are involved in B cell signal transduction.

AP-1 belongs to a family of transcription factors consisting of fos and jun heterodimers. Production of AP-1 is also triggered by the proximal signaling complex (PSC). The PSC activates a protein kinase C, which in turn leads to activation of ras-mitogen protein kinase (MAPK) and increased production of c-fos. The c-fos moves to the nucleus, where it combines with preexisting jun proteins to form AP-1. AP-1 binds to NF-AT proteins integrating the calcium signaling with the ras-MPCK pathway. Collectively they activate the genes coding for IL-2, IFN-γ, GM-CSF, TNF-α, IL-3, IL-4, IL-13, IL-5, FasL, and CD25.

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JAK-STAT Pathway

Almost 40 cytokines use the JAK-STAT pathway, including IL-4, IL-7, IL-11, IL-12, IL-13, and IL-31, leptin, GM-CSF, and IFN-γ. These ligands use group I cytokine receptors that consist of two identical transmembrane proteins. When a cytokine binds to its receptor the two proteins come together. This dimerization leads in turn to phosphorylation of two tightly associated JAK proteins. These activated JAK molecules then phosphorylate STAT proteins. The phosphorylated STAT proteins dissociate from JAK and move to the nucleus, where they act as transcription factors and induce the expression of target genes. There are four JAK and seven STAT family members currently recognized. A specific JAK-STAT combination is paired with each cytokine receptor. For example, hematopoietic growth factor receptors usually use JAK2. Receptors with the common γ chain preferentially use JAK1 and JAK3. The IFN-γ receptor uses JAK1 and JAK2. The IL-4R uses JAK1 and JAK3. Presumably the genes turned on by this signaling depend on these specific combinations of JAK and STAT as well as the cell type involved (Box 8.2).

Box 8.2

Inhibition of JAK-STAT Signaling

Inhibition of JAK-STAT signaling can have immunosuppressive and antiinflammatory effects. This may be useful in suppressing unwanted immune responses or inflammation. Drugs directed against specific JAK proteins can have profound antiinflammatory responses. For example, oclacitinib (Apoquel) is a synthetic selective inhibitor of JAK1 and JAK3. As a result, it blocks signal transduction and hence inhibits the itching caused by IL-31 (Chapter 30). Specifically, it is used for the treatment of severe itch (pruritus) in dogs with allergic dermatitis including flea and food allergies, atopic dermatitis, and contact dermatitis (Chapters 30 and 33).

Although the pathways described above are of greatest importance in cells of the immune system, many other transcription

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factors can trigger cell differentiation. This is especially important in the T cell system, where the cells are plastic and can readily transform from one subset to another. These transcription factors include T-bet, which controls transcription of IFN-γ in Th1 cells; GATA3, which controls cytokine transcription in Th2 cells; ROR γT, which acts in Th17 cells; and FoxP3, which acts in Treg cells.

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Gene Transcription

The activity of each gene in a cell is carefully regulated. Central to gene control, however, are the transcription factors. Activation of genes depends on the presence of an appropriate mixture of transcription factors. As described earlier, these transcription factors are only generated when a cell receives an appropriate signal. The transcription factors then collectively activate the appropriate RNA polymerase, and gene transcription begins.

Transcription factors have two binding sites. One site binds DNA; the other binds regulatory proteins. When a transcription factor is generated, it enters the nucleus and binds to specific DNA control elements located between 50 and 200 bases upstream from the start site of the gene (Fig. 8.13). Transcription factors may also bind to enhancer elements located thousands of bases upstream. These bound transcription factors then use their other binding site to bind either directly to a basal transcription complex or to coactivator molecules. The basal transcription complex, together with attached coactivator molecules, then binds to the RNA polymerase and activates it. It is believed that the conformation of the polymerase changes once activated. The polymerase then begins the process of transcribing the selected genes leading eventually to the production of the required proteins (Box 8.3).

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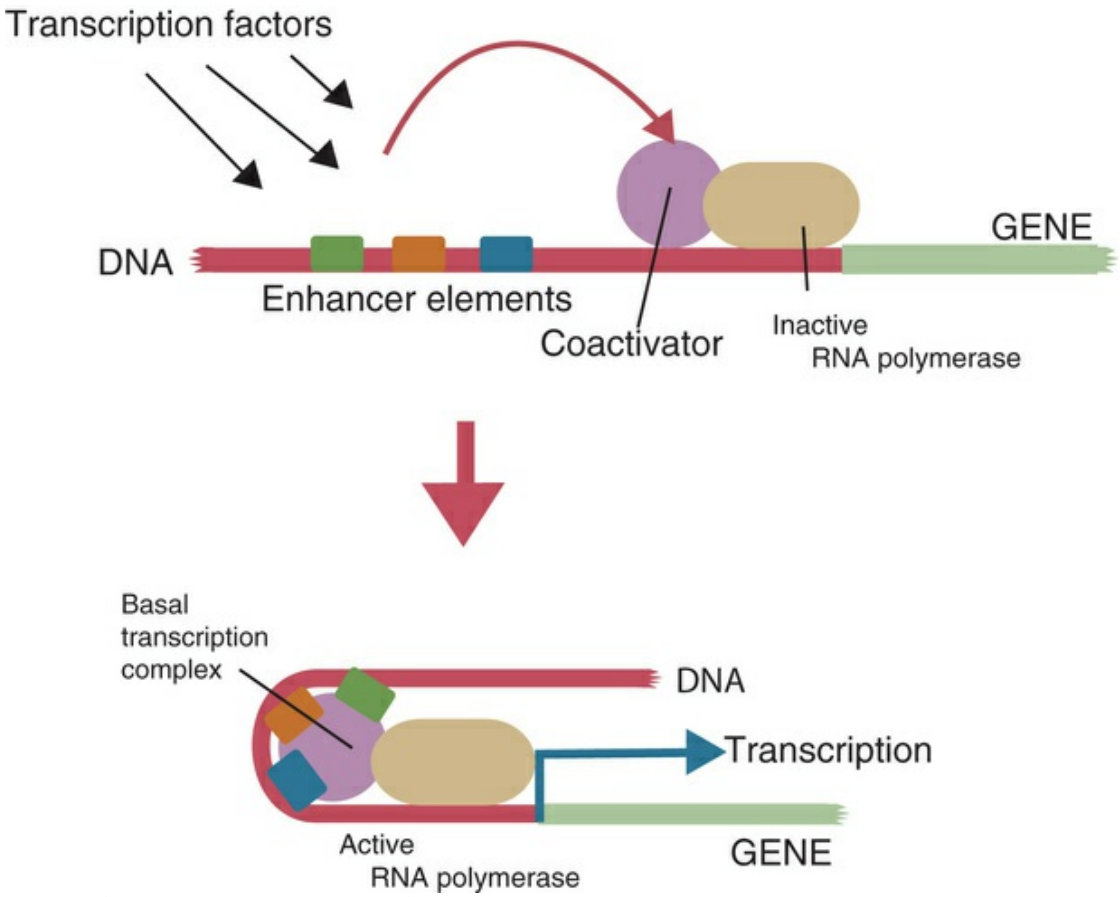


FIG. 8.13 Transcription factors bind to enhancer elements on DNA located upstream of the genes they activate. Gene transcription is turned on by a carefully regulated RNA polymerase. However, the polymerase can only be turned on when transcription factors form a basal transcription complex and activate the basic transcriptional machinery.

Box 8.3

No Interleukin-26 in Horses

Interleukin-26 (IL-26) is a member of the IL-10 family. Its gene is located in a cluster with the IFN-γ gene on one side and the IL-22 gene on the other. As a result of a one-base pair frame-shift mutation, the IL-26 gene is inactivated in horses and related equids such as zebras and donkeys. Since IL-26 is a stimulator of T cells and may promote inflammation and autoimmunity, this inactivation may explain, in part, why some aspects of acute inflammation differ between horses and other mammals.

Data from Shakhsi-Niaei M, Drogemuller M, Jagannathan V, Gerber V, Leeb T: IL-26 gene inactivation in Equidae, *Anim Genet* 44:770-772, 2013.

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Antigens

Triggers of Adaptive Immunity

CHAPTER OUTLINE

Microbial Antigens, 82
- Bacterial Antigens, 82
- Viral Antigens, 83
- Other Microbial Antigens, 83

Nonmicrobial Antigens, 84
- Cell Surface Antigens, 84
- Autoantigens, 84

What Makes a Good Antigen?, 84
- Foreignness, 85

Epitopes, 86
- Haptens, 86
- Some Examples of Haptens, 87

Cross-Reactions, 87

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

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- Explain how the adaptive immune system is optimized to recognize microbial macromolecules, especially, but not exclusively, proteins.
- Understand why the best antigens are therefore large, complex, stable, foreign proteins.
- Explain why small molecules of less than 5 kDa are usually poor antigens.
- List important bacterial and viral antigens.
- Describe how small molecules may be made antigenic by linking them to large proteins to act as haptens.
- Recognize that the cells of the adaptive immune system use receptors that can recognize specific sites on the surface of foreign molecules.
- Define antigenic determinant, epitope, hapten, antigenicity, autoantigen, and cross-reaction.
- List the key factors that influence antigenicity.

Up to now we have considered only the body's innate reactions to microbial invasion. Innate responses are triggered by recognition of a limited number of conserved microbial pathogen-associated molecular patterns (PAMPs) such as microbial nucleic acids or lipopolysaccharides. The triggering of inflammation and the mobilization of phagocytic cells such as neutrophils and macrophages by these molecules contributes to the rapid destruction of microbial invaders. Although effective in the short term, innate immunity cannot be guaranteed to provide complete resistance to infection. Nor does the body learn from the experience. Thus a more potent immune response should ideally recognize all the foreign molecules on an invading microbe. In addition, such a response should be able to learn from this experience and, given time, evolve more efficient procedures to combat subsequent invasions. This new and improved response is the function of the adaptive immune system.

During an adaptive immune response, molecules from invading organisms are captured, processed, and presented to the cells of the immune system. These cells have surface receptors that can bind appropriately presented molecules. These bound molecules or antigens then trigger a powerful immune response that ensures an animal's survival. In addition, the immune system “remembers”

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these antigens, makes some improvements, and, by adapting, responds even more effectively when it encounters these organisms again.

Since the function of the adaptive immune system is to defend the body against invading microorganisms, it is essential that these organisms be recognized as soon as they invade the body. The body must be able to recognize that these are foreign (and dangerous) if they are to stimulate an immune response. The innate immune system recognizes only a limited number of PAMPs—those that are characteristic of major groups of pathogens. The adaptive immune system, in contrast, uses receptors that can bind and respond to almost all the foreign macromolecules present in an invading microorganism. These foreign macromolecules are called antigens.

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Microbial Antigens

Bacterial Antigens

Bacteria are single-celled prokaryotic organisms consisting of a cytoplasm containing the essential elements of cell structure surrounded by a lipid-rich cytoplasmic membrane (Fig. 9.1). Outside the cytoplasmic membrane is a thick, carbohydrate-rich cell wall. The major components of the bacterial surface thus include the cell wall and its associated protein structures, the capsule, the pili, and the flagella. The cell wall of Gram-positive organisms is largely composed of peptidoglycan (chains of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid cross-linked by short peptide side chains) (see Fig. 2.2). Gram-positive cell walls also contain lipoteichoic acids that are involved in the transport of ions across the cell wall. The cell wall in Gram-negative organisms, in contrast, consists of a thin layer of peptidoglycan covered by an outer membrane consisting of a lipopolysaccharide. Most of the antigenicity of Gram-negative bacteria is associated with the lipopolysaccharide. This consists of an oligosaccharide attached to a lipid (lipid A) and to a series of repeating trisaccharides. The structure of these trisaccharides determines the antigenicity of the organism. Many bacteria are classified according to this antigenic structure. For example, the genus *Salmonella* contains a major species, *Salmonella enterica,* that is classified into more than 2500 serovars based on antigenicity. These polysaccharide antigens are called O antigens. The outer cell wall lipopolysaccharides of Gram-negative bacteria bind to toll-like receptors (TLRs) and other pattern-recognition receptors and induce the production of inflammatory cytokines when an animal is infected. These cytokines cause a fever and sickness, so bacterial lipopolysaccharides are also called endotoxins.

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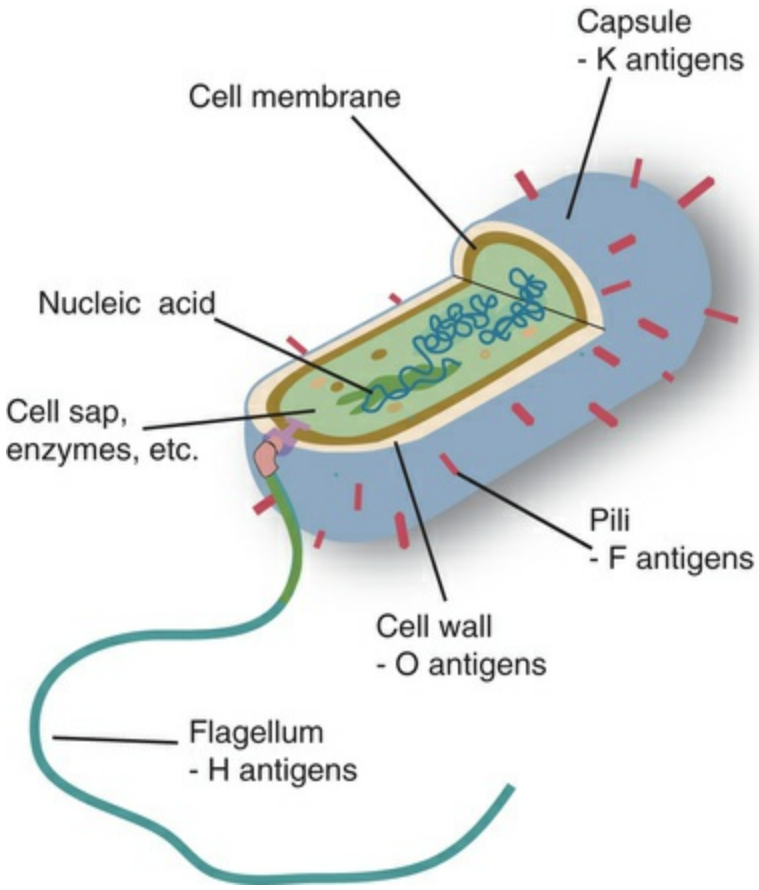


FIG. 9.1 The structure of a typical bacterium and the location of its most important antigens.

Bacterial capsules consist mainly of polysaccharides that are usually good antigens. The capsules protect bacteria against phagocytosis and intracellular destruction, whereas anticapsular antibodies can overcome the effects of the capsule and protect an infected animal. Capsular antigens are collectively called K antigens.

Pili and fimbriae are short projections that cover the surfaces of some Gram-negative bacteria; they are classified as F or K antigens. Pili bind bacteria together and play a role in bacterial conjugation and movement. Fimbriae bind bacteria to cell surfaces. Antibodies to fimbrial proteins may be protective since they can prevent bacteria from sticking to body surfaces. Bacterial flagella are long filaments used for bacterial movement. They consist of a single protein called flagellin. Flagellar antigens are collectively called H antigens.

Other significant bacterial antigens include the porins, the heat-shock proteins, and the exotoxins. The porins are proteins that form

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pores on the surface of Gram-negative organisms. Heat-shock proteins are generated in large amounts in stressed bacteria. The exotoxins are toxic proteins secreted by bacteria or released into the surrounding environment when they die. Exotoxins are highly immunogenic proteins and stimulate the production of antibodies called antitoxins. Many exotoxins, when treated with a mild protein-denaturing agent such as formaldehyde, lose their toxicity but retain their antigenicity. Toxins modified in this way are called toxoids. Toxoids may be used as vaccines to prevent disease caused by toxigenic bacteria such as *Clostridium tetani*. Bacterial nucleic acids rich in unmethylated CpG dinucleotides serve both as effective antigens for the adaptive immune system and as potent stimulators of innate immunity acting through TLRs. The presence of the enormous and diverse intestinal microbiota provides a source of many different microbial antigens. These too can stimulate adaptive immune responses.

Viral Antigens

Viruses are very small structures that grow only inside living cells. They are thus "obligate," intracellular parasites. Viruses usually have a relatively simple structure consisting of a nucleic acid core covered by a protein layer (Fig. 9.2). This protein layer is termed the capsid, and consists of multiple subunits called capsomeres. Capsid proteins are good antigens, well capable of stimulating antibody responses. Some viruses may also be surrounded by an envelope containing lipoproteins and glycoproteins. A complete viral particle is called a virion. When a virus infects an animal, its proteins are processed, recognized, and trigger adaptive immune responses. Viruses, however, are not always found free in the circulation but live within cells, where they are protected from the unwelcome attentions of antibodies. Indeed, viral nucleic acids may be integrated into a cell's genome. In this situation, the integrated viral genes code for new proteins, some of which are expressed on the surface of infected cells. These proteins, although they are synthesized inside an animal's own cells, can still bind to antigen receptors and provoke adaptive immunity. These newly synthesized foreign proteins are called endogenous antigens to

distinguish them from the foreign antigens that enter from the outside and are called exogenous antigens.

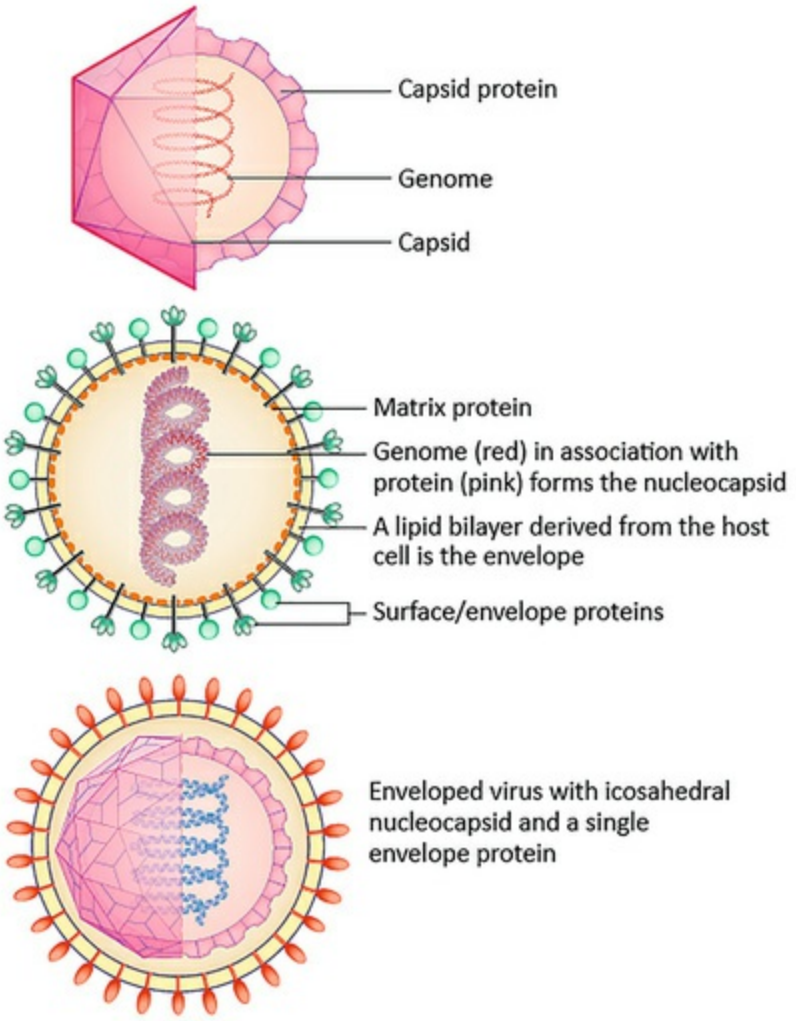


FIG. 9.2 The structure of some viruses. The antigenic proteins include the capsids and envelope proteins. (Courtesy Dr. S. Payne.)

Other Microbial Antigens

In addition to bacteria and viruses, animals may be invaded by fungi, protozoan parasites, arthropods, and even parasitic worms (helminths). Each of these organisms consists of many different structures composed of proteins, carbohydrates, lipids, and nucleic acids. Many of these molecules can serve as antigens and trigger adaptive immunity. However, their antigenicity does vary, and the

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adaptive responses triggered by these organisms are not always successful in protecting an animal or eliminating the invader.

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Nonmicrobial Antigens

Invading microorganisms are not the only source of foreign material entering the body. Food contains many foreign molecules that under some circumstances may trigger immune responses and cause allergic reactions. Likewise, inhaled dusts can contain antigenic particles such as pollen grains, and these may enter the body through the respiratory system. Foreign molecules may be injected directly into the body through a snake or mosquito bite, or by a veterinarian. Furthermore, foreign proteins may be injected into animals for experimental or therapeutic purposes. Organ grafts are an effective way of administering a large amount of foreign material to an animal.

Cell Surface Antigens

The surface of mammalian cells consists of a fluid lipid bilayer with a complex mixture of protein molecules embedded in it. Most of these proteins can act as antigens if they are injected into another species or even into a different individual of the same species. For example, glycoproteins known as blood-group antigens are found on the surface of red blood cells. Early attempts to transfuse blood between unrelated individuals usually meet with disaster because the transfused cells were rapidly destroyed. Investigation revealed that the problem was due to the presence of naturally occurring antibodies against these foreign red cell glycoproteins.

Nucleated cells, such as leukocytes, possess hundreds of different protein molecules on their surface. These proteins are good antigens and readily provoke an immune response when injected experimentally into a different species. These surface molecules are classified by the CD system (see Box 2.4). Other cell surface proteins may provoke an immune response (such as graft rejection) if transferred into a genetically different individual of the same species. The cell surface proteins that trigger graft rejection are called histocompatibility antigens. Histocompatibility antigens are of such importance in immunology that they warrant a complete chapter of their own (Chapter 11).

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Autoantigens

In some situations (and not always abnormal ones), an animal may mount immune responses against normal body components. These are called autoimmune responses. Antigens that induce autoimmunity are called autoantigens. They can include hormones, such as thyroglobulin; structural components, such as basement membranes; complex lipids, such as myelin; intracellular components, such as the mitochondrial proteins, nucleic acids, or nucleoproteins; and cell surface proteins, such as hormone receptors. The production of autoantibodies and the consequences of this are discussed in detail in Chapter 36.

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What Makes a Good Antigen?

Molecules vary in their ability to act as antigens (their antigenicity) (Fig. 9.3). In general, foreign proteins make the best antigens, especially if they are big (greater than 1000 Da is best). Many of the major antigens of microorganisms such as the clostridial toxins, bacterial flagella, virus capsids, and protozoan cell membranes are large proteins. Other important antigenic proteins include components of snake venoms, serum proteins, cell surface proteins, milk and food proteins, hormones, and even antibody molecules themselves.

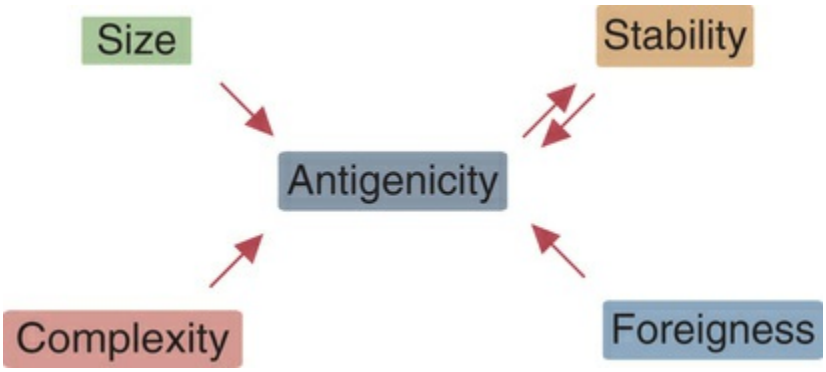


FIG. 9.3 The factors that significantly influence the antigenicity of a molecule. Of these, either excessive or insufficient stability will reduce antigenicity. The best antigens are large, complex, and foreign. However, their ability to stimulate an immune response is also determined by their route of administration, by the amount of antigen administered, and by the genetic makeup of the immunized animal.

Simple polysaccharides, such as starch or glycogen, are not good antigens simply because they are often degraded before the immune system has time to respond to them. More complex carbohydrates may be effective antigens, especially if bound to proteins. These include the major cell wall antigens of Gram-negative bacteria and the blood-group glycoproteins of red blood cells. Many of the so-called natural antibodies found in the serum of unimmunized animals are directed against polysaccharides and probably arise as a result of exposure to glycoproteins or carbohydrates from the intestinal microbiota or from food. To this

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extent they can also be considered part of the innate immune system.

Lipids tend to be poor antigens because of their wide distribution, relative simplicity, structural instability, and rapid metabolism. Nevertheless, when linked to proteins or polysaccharides, lipoproteins, glycolipids and lipopolysaccharides can trigger immune responses. Some lymphocytes possess specific receptors, called CD1 molecules, that can bind lipid, lipoprotein, and glycolipid antigens and present them to the antigen-sensitive cells (Chapter 19).

Mammalian nucleic acids are very poor antigens because of their relative simplicity and flexibility, and because they are very rapidly degraded. Microbial nucleic acids, on the other hand, have a structure very different from that found in eukaryotes with many unmethylated CpG sequences. As a result, they can stimulate potent immune responses. It is perhaps for this reason that autoantibodies to nucleic acids are produced in some important autoimmune diseases (Chapter 38).

Proteins are the most effective antigens because they have properties that best trigger an immune response. (More correctly, the adaptive immune system has evolved to trap, process, and then recognize foreign proteins.) Thus large molecules are better antigens than small molecules, and proteins can be very large indeed (Fig. 9.4). For example, hemocyanin, a very large protein from invertebrate blood (670 kDa) is a potent antigen. Serum albumin from other mammals (69 kDa) is a fairly good antigen but may also provoke tolerance. The small peptide hormone angiotensin (1031 Da) is a poor antigen.

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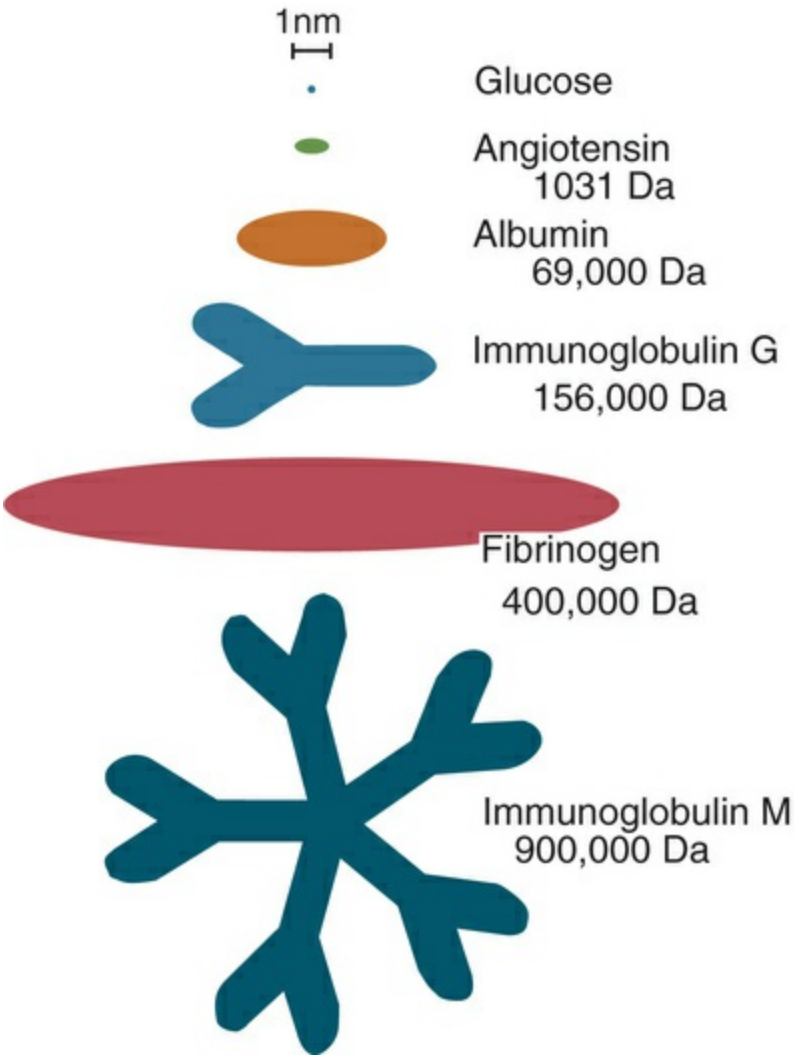


FIG. 9.4 The relative sizes of several significant antigens. Size does matter! Big molecules are generally much more antigenic than small molecules. Molecules as small as angiotensin are poor antigens.

Similarly, the more complex an antigen is, the better. For example, starch and other simple repeating polymers are poor antigens, but complex bacterial lipopolysaccharides are good. Complex proteins containing many different amino acids, especially aromatic ones, are better antigens than large, repeating polymers, such as lipids, carbohydrates, and nucleic acids.

Structural stability is an important feature of good antigens, especially those that trigger antibody responses. To bind to specific foreign molecules, the cell surface receptors of the adaptive immune system must recognize their shape. Consequently, highly flexible molecules that have no fixed shape are poor antigens. For example, gelatin, a protein well known for its structural instability

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(which is why it can wobble), is a poor antigen unless it is stabilized by the incorporation of tyrosine or tryptophan molecules, which cross-link the peptide chains. Similarly, flagellin, the major protein of bacterial flagella, is a flexible, weak antigen. Its rigidity, and thus its antigenicity, is greatly enhanced by polymerization. Remember too that the route of antigen administration, its dose, and the genetics of the recipient animal also influence antigenicity.

Not all foreign molecules can stimulate an immune response. Stainless steel bone pins and plastic heart valves are commonly implanted in animals without triggering an immune response. The lack of antigenicity of metals or large organic polymers, such as plastics, is due not only to their molecular uniformity but also to their inertness. These polymers cannot be degraded and processed by cells to a form suitable for triggering an immune response. Conversely, since immune responses are antigen driven, foreign molecules that are unstable and destroyed very rapidly may not persist for a sufficient time to stimulate an immune response.

Foreignness

The cells that respond to antigens (antigen-sensitive cells) are lymphocytes that have been selected so that their receptors do not normally bind to molecules originating within an animal (self-antigens). Cells that bind self-antigens are eliminated, but that still leaves a huge variety of lymphocytes with receptors that can bind foreign molecules. These are sufficient to protect animals against almost all potential pathogens. They will bind and respond, however, to a huge variety of foreign molecules that differ even in minor respects from those normally found within the body.

The immunogenicity of a molecule also depends on its degree of foreignness. The greater the difference in molecular structure between a foreign antigen and an animal's own antigens, the greater will be the intensity of the immune response. For example, a kidney graft from an identical twin will be readily accepted because its proteins are identical to those on the recipient's own kidney. A kidney graft from an unrelated animal of the same species will be rejected in about 10 days unless prevented by drugs. A kidney graft between different species such as from a pig to a dog will be

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rejected within a few hours despite the use of immunosuppressive drugs.

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Epitopes

Foreign particles, such as invading bacteria, transplanted nucleated cells, and transfused red blood cells, consist of an enormously complex mixture of proteins, glycoproteins, polysaccharides, lipopolysaccharides, lipids, and nucleoproteins. The adaptive immune response against such foreign invaders or cells is therefore a mixture of many simultaneous immune responses directed against each of the foreign molecules in the mixture.

A large complex molecule such as a protein can be recognized by many different lymphocytes and thus stimulate multiple immune responses. Large molecules have regions on their surface that bind to lymphocyte antigen receptors and against which immune responses are therefore directed. These regions, usually on the surface of the molecule, are called epitopes, or antigenic determinants (Fig. 9.5). In a large, complex protein molecule, many different epitopes may be recognized by lymphocytes, but some are much more immunogenic than others. Thus animals may respond to a few favored epitopes, and the remainder of the molecule may be ignored. Such favored epitopes are said to be immunodominant. In general, the number of epitopes on a molecule is directly related to its size, and there is usually about one epitope for each 5 kDa of a protein. When we describe a molecule as "foreign," therefore, we are implying that it contains epitopes that are not found on self-antigens. The cells of the immune system bind and respond to these foreign epitopes. A good example of a well-defined epitope is the peptide, proline-glutamic acid-proline-lysine, which binds to antibodies against the bacterium *Streptococcus equi.* Presumably the shape of this peptide is identical to the major antigenic determinant on *S. equi.*

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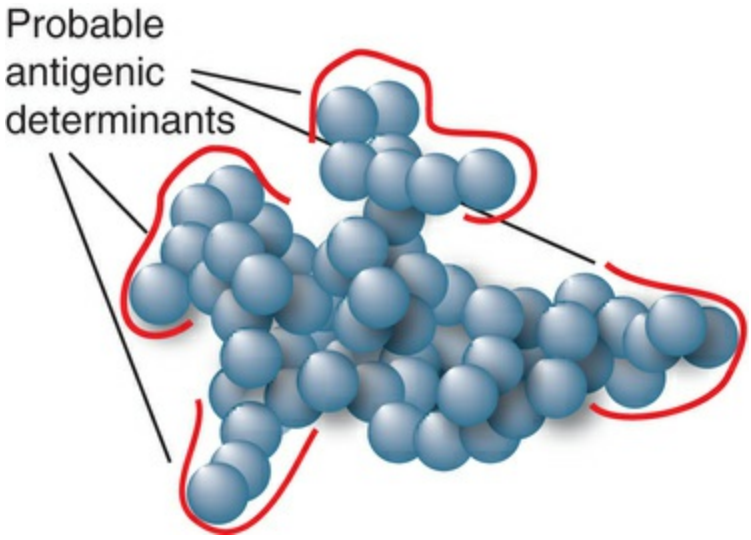


FIG. 9.5 A hypothetical antigen. Note that the cells of the immune system preferentially recognize the prominent structures on the surface of the molecule as a result of their characteristic shapes.

Haptens

Small molecules, such as many drugs or hormones of less than 1000 Da, are far too small to be appropriately processed and presented to the immune system. As a result, they are not immunogenic. If, however, these small molecules are chemically linked to a large protein molecule, they will form new epitopes on the surface of the larger molecule (Fig. 9.6). If this complex molecule is injected into an animal, immune responses will be triggered against all its epitopes. Some of the antibodies made in response to the complex will be directed against new epitopes formed by the small molecule. Small molecules that can function as epitopes only when bound to other larger molecules are called haptens (in Greek, *haptein* means "to grasp or fasten"). The antigenic molecule to which the haptens are attached is called the carrier. Many drug allergies occur because the drug molecules, although small, can bind covalently to normal body proteins and so act as haptens.

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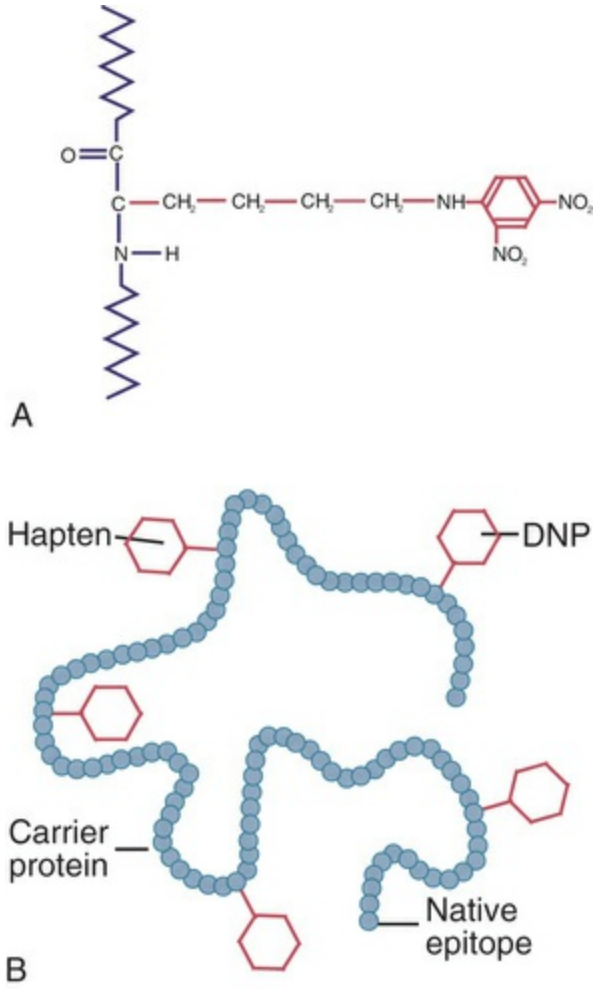


FIG. 9.6 **A,** A typical hapten; in this case, dinitrophenol attached to a lysine side chain. **B,** When several haptens are attached to a peptide chain, they serve as new epitopes and will stimulate immune responses.

By using haptens of known chemical structure, it is possible to study the interaction between antibodies and epitopes in great detail. For example, antibodies raised against one hapten can be tested for their ability to bind to other, structurally related molecules. Simple tests have shown that any alteration in the shape, size, or charge of a hapten alters its ability to bind to antibodies. Even very minor modifications to the shape of a hapten may influence its ability to bind an antigen receptor or an antibody. Since there exists an enormous number of potential haptens, and since each hapten can provoke its own specific antibodies, it follows that animals must be able to generate an extremely large variety of antigen receptors and specific antibody molecules. It is this enormous diversity that enables animals to successfully fight the multitude of pathogenic microbes encountered throughout life.

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Some Examples of Haptens

Although the concept of haptens and carrier molecules provides the basis for much of our knowledge concerning the specificity of the antibody response, haptens may also be of clinical importance. For example, the antibiotic penicillin is a small nonimmunogenic molecule. Once degraded within the body, however, it forms a very reactive "penicilloyl" group, which can bind to serum proteins such as albumin to form penicilloyl-albumin complexes (Fig. 9.7). The penicilloyl hapten can be recognized as a foreign epitope in some individuals and so provokes an immune response, resulting in penicillin allergy.

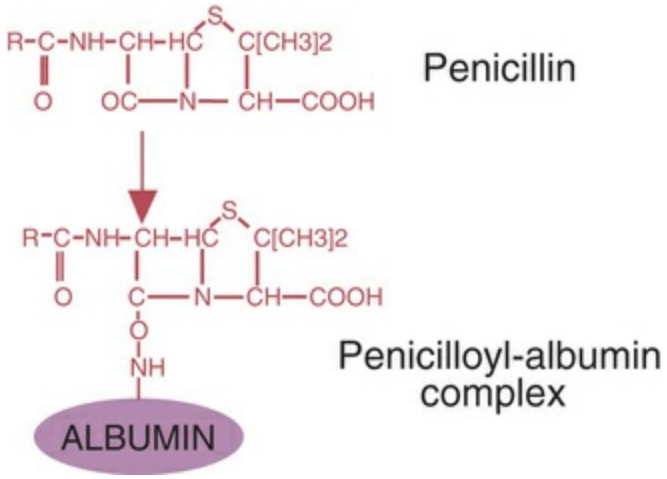


FIG. 9.7 Penicillin as a hapten. Penicillin can break down in vivo by several different pathways. The most important derivative is a penicillanic acid that combines with amino groups in a protein such as serum albumin to form a penicilloyl-protein complex. This complex may provoke an immune response and result in a penicillin allergy.

A second example of a naturally occurring reactive chemical that binds spontaneously to normal proteins and so acts as a hapten is the toxic component of the poison ivy plant *(Rhus radicans)*. The resin of this plant, called urushiol, will react with any protein with which it comes into contact, including the skin proteins of a person who rubs against the plant. The modified skin proteins are then regarded as foreign and attacked by lymphocytes in a manner similar to the rejection of a skin graft. The result is the uncomfortable skin rash called allergic contact dermatitis (Chapter

33).

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Cross-Reactions

Identical or similar epitopes may sometimes be found on apparently unrelated molecules. As a result, antibodies directed against one antigen may react unexpectedly with an unrelated antigen. In another situation, the epitopes on a protein may differ in only minor respects from those on the same protein obtained from an animal of a related species. Consequently, antibodies directed against a protein in one species may also react in a detectable manner with the homologous or similar protein in another species. Both phenomena are called cross-reactions.

An example of a cross-reaction of the first type is seen when blood typing. Many bacteria possess cell wall glycoproteins with carbohydrate side chains that are identical to those found on mammalian red blood cell glycoproteins. For example, some of the intestinal microbiota possess glycoproteins with A or B side chains on their cell wall (Chapter 31). These glycoproteins are absorbed through the intestinal wall and trigger an antibody response. Blood-group glycoprotein side chain A is foreign to a pig of blood group O (Fig. 9.8). Pigs of blood group O therefore develop antibodies that react with red cells from pigs of blood group A. These antibodies arise not as a response to previous immunization with group A red cells but following exposure to glycoproteins from the intestinal microbiota. Another example of cross-reactivity occurs between *Brucella abortus* and some strains of *Yersinia enterocolitica. Y. enterocolitica,* a relatively unimportant organism, may provoke cattle to make antibodies that cross-react with *B. abortus.* Since *Brucella*-infected animals are detected by testing for the presence of serum antibodies, a *Yersinia*-infected animal may be wrongly thought to carry *B. abortus* and so be killed. In another example, cross-reactivity occurs between the coronavirus of feline infectious peritonitis (FIP) and the virus of pig transmissible gastroenteritis (TGE). It is difficult to grow the FIP virus in the laboratory. TGE virus, on the other hand, is readily propagated. By detecting antibodies to TGE in cats, it is possible to diagnose FIP without having to culture the FIP virus.

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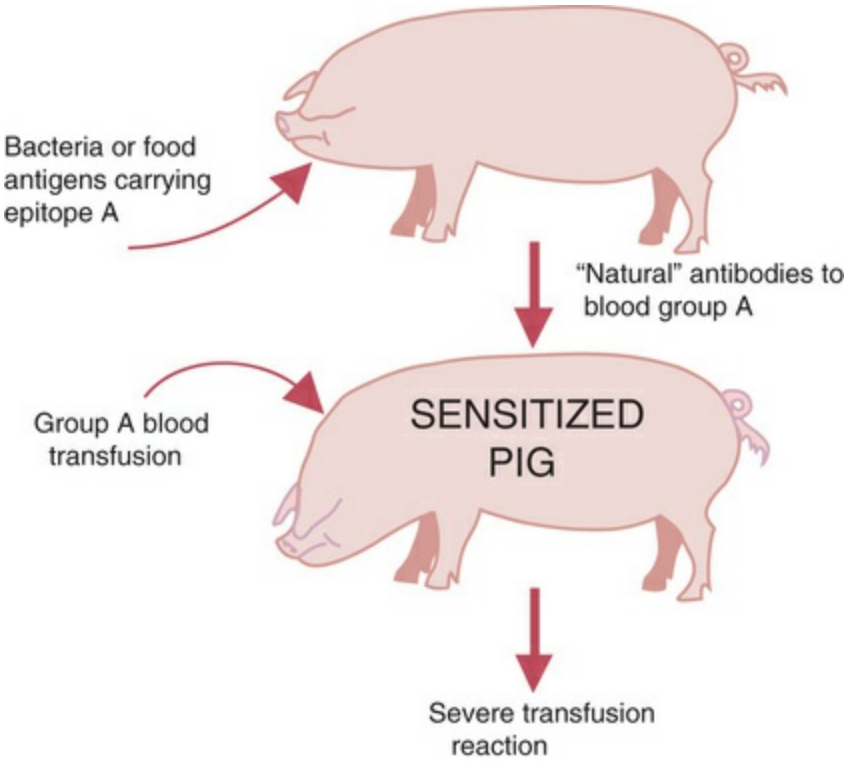


FIG. 9.8 Food or bacterial antigens encountered in the diet carry epitopes that cross-react with blood group glycoprotein A. As a result, pigs of blood group O make antibodies to the A epitope despite never having received group A red cells. Should these animals be inadvertently transfused with group A blood, they will suffer an immediate and severe transfusion reaction.

The second type of cross-reactivity, which occurs between related proteins, may be demonstrated in many different biological systems. One example is a method used to determine relationships between mammalian species. Thus antisera to bovine serum albumin cross-react strongly with sheep and goat serum albumin but weakly with serum albumin from other mammals (Table 9.1). Presumably, this reflects the degree of structural similarity between the epitopes on serum proteins and is thus a useful tool in determining evolutionary relationships.

TABLE 9.1

Degree of Cross-Reaction Between a Specific Antibody (Antibovine Light Chain Antibodies) and Related Proteins (Light Chains) From Other Mammals

Cow	*Bos Taurus*	100
Bison	*Bos bison*	100
Sheep	*Ovis aires*	100

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Yak	*Pocphagus grunniens*	68
Goat	*Capra hircus*	68
Elk	*Cervus Canadensis*	64
Buffalo	*Bubalus*	54
Reindeer	*Rangifer tarandus*	37
Human	*Homo sapiens*	17
Horse	*Equus caballus*	10
Rat	*Rattus*	10
Mouse	*Mus musculus*	10
Pig	*Sus scrofa*	8
Camel	*Camelus dromedaries*	7

Data from Henning D, Nielsen K: Cross-reactivity of monoclonal antibodies to bovine immunoglobulins with immunoglobulins of other species, *Vet Immunol Immunopathol* 34:235-243, 1992.

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10

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Dendritic Cells and Antigen Processing

CHAPTER OUTLINE

Dendritic Cells, 90
- Origin, 90
- Structure, 90
- Subpopulations, 90
- *Classical DCs, 91*
- *Plasmacytoid DCs, 92*
- *Langerhans Cells, 92*
- *Follicular DCs, 92*
- Dendritic Cell Maturation, 92
- *Immature DCs, 92*
- *Mature DCs, 93*
- Tolerance Induction, 93
- cDC1 and cDC2 Cells, 94
- Interleukin-12, 94

Dendritic Cells in Domestic Animals, 95

Other Antigen-Processing Cells, 96
- Macrophages, 96

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B Cells, 96
Other Cells, 96
Antigen Processing, 96
MHC Class II Pathway, 96
MHC Class I Pathway, 97
Cross-Priming, 99
Histiocytosis and Histiocytomas, 99

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Explain the importance of antigen processing in the development of adaptive immunity.
- Compare the processing of exogenous and endogenous antigens.
- Describe the characteristic properties of dendritic cells (DCs), their subpopulations, and their origins.
- Recognize that DCs, macrophages, and B cells are the major cell types that capture and process foreign antigens.
- Describe why DCs are the most efficient of these antigen-processing cells.
- Explain how immature DCs are especially equipped to capture and process antigens.
- Explain how mature DCs are highly effective in presenting these processed antigens to T cells.
- Describe how macrophages also act as antigen-presenting cells, and why they are much less efficient than DCs.
- Explain how B cells can also act as antigen-presenting cells and are especially effective during secondary immune responses.
- Define dendritic cells, antigen processing, antigen presentation, Langerhans cells, exogenous antigen, endogenous antigen, and proteasome.
- Compare dendritic cells and macrophages.
- Describe the major histocompatibility complex (MHC) class I pathway of

endogenous antigen presentation

- Describe the MHC class II pathway of exogenous antigen presentation.

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The innate immune defenses have evolved to destroy microbes as soon as they enter the body. Most invaders, especially if they are of low virulence, are rapidly eliminated. However, in addition to being uncomfortable and damaging, inflammation is not a foolproof process. If the body is to be defended effectively, an animal must have defenses that detect and eliminate all microbial invaders without the damage and discomfort associated with inflammation. This is the task of the adaptive immune system.

In order to trigger this adaptive immunity, a sample of foreign material must first be captured, processed, and presented in the correct fashion to cells that can recognize it. This is the responsibility of antigen-processing cells.

Antigen-processing cells are attracted by microbial products and tissue damage and are activated by the same stimuli that trigger inflammation. Indeed, dendritic cells and macrophages serve both as sentinel cells and antigen-processing cells. As a result, antigen processing can be initiated at the same time as the invader is being eliminated by the innate defenses. Once an invader has been eliminated, an animal can develop adaptive immunity against a second attack by the same organism.

Antigen processing involves breaking large protein molecules into small peptides within a cell. These peptides are then attached to specialized antigen-presenting receptors called major histocompatibility complex (MHC) molecules. Once bound to MHC molecules, the peptides are carried to the cell surface. Adaptive immunity is triggered when these MHC-bound peptides are recognized by lymphocytes bearing specific receptors for the antigen. These lymphocytes (called T cells) bind and respond only to foreign peptides that have been correctly processed and presented. This ensures that adaptive immune responses do not proceed indiscriminately.

The organisms that trigger adaptive immune responses are of two general types. One type is typified by the bacteria that invade

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the body from outside and then grow in the tissues and extracellular fluid. These are called exogenous antigens, and they are processed by specialized antigen-processing cells. A second type of invading organism is typified by viruses that invade a cell and force it to make viral proteins. These new proteins are called endogenous antigens. Endogenous antigens are processed by the cells in which they are produced. There are therefore two classes of MHC molecules, class I and class II. MHC class I molecules are made by all nucleated cells and present endogenous antigens. MHC class II molecules, in contrast, are restricted to specialized antigen-processing cells, and they present exogenous antigens. The body mainly employs three specialized cell families, dendritic cells, macrophages, and B cells, to process exogenous antigens. The most important of these are dendritic cells (DCs) (Fig. 10.1).

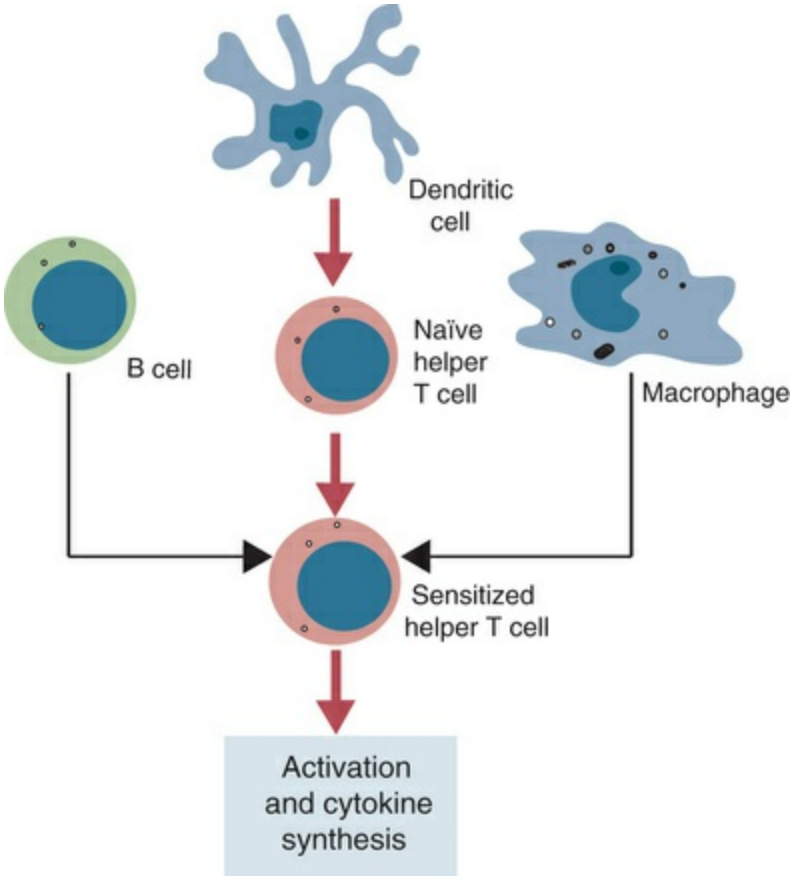


FIG. 10.1 The three major populations of antigen-presenting cells: B cells, DCs, and macrophages. Of these, only DCs can activate naïve T cells and trigger a primary immune response.

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Dendritic Cells

Dendritic cells are specialized antigen-presenting cells that can initiate adaptive immune responses. They are located at sites where they are most likely to encounter foreign antigens, that is, in lymphoid organs and under epithelial surfaces. DCs perform three major functions. First, they serve as sentinel cells and activate innate defenses when they first encounter invaders. Second, they process exogenous antigens and thus initiate adaptive immune responses. Third, they regulate adaptive immunity by determining whether an antigen will trigger an antibody-mediated or a cell-mediated response, or even prevent an immune response (a condition called tolerance). DCs are at least 100 times more effective antigen-presenting cells than macrophages or B cells. DCs can take up many different antigens, including dead microorganisms, soluble foreign antigens in tissue fluids, and antigens released by dying cells, and present them to T cells. DCs are the only antigen-processing cells that can activate T cells that have never previously encountered an antigen (naïve cells) and therefore are essential for initiating primary immune responses.

Origin

All DCs are derived from hematopoietic stem cells in the bone marrow. Immature DCs migrate through the body and form networks in virtually every tissue. Monocytes may also develop into classical DCs when exposed to appropriate cytokines. DCs are found in all organs except the brain, parts of the eye, and the testes. They are especially prominent in lymph nodes, skin, and mucosal surfaces—sites where invading microbes are most likely to be encountered.

Structure

The shape of a DC depends on its state of activation. Typically, however, they are characterized by having a small cell body, about 13-15 μm in diameter with many long cytoplasmic processes known

as dendrites (Fig. 10.2). The dendrites increase the cell surface area and thus increase the efficiency of antigen trapping and maximize contact between DCs and other cell types.

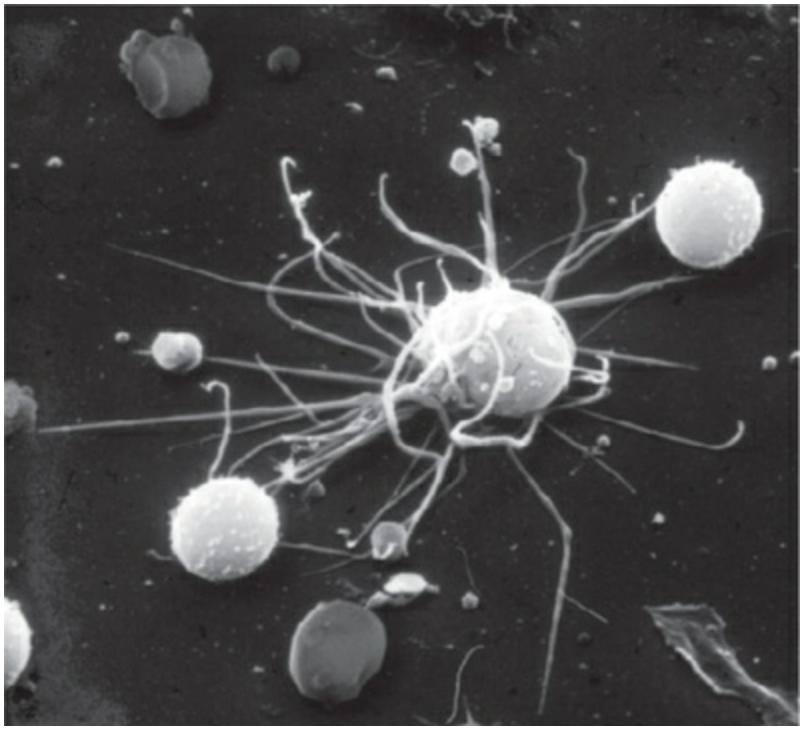


FIG. 10.2 A scanning electron micrograph of a dendritic cell from a guinea pig lymph node. Note the relatively small cell body and the numerous long dendrites. Original magnification ×4000.

Subpopulations

Like other immune cell populations, DCs belong to a heterogeneous family and consist of multiple subpopulations. The two most prominent of these are classical/conventional DC (cDC) and plasmacytoid DC (pDC) (Fig. 10.3). These cells differ in morphology, in their surface antigens, and in their functions, although they share adhesion molecules, costimulatory molecules, and activation markers. Other important dendritic cell subpopulations are found in the skin (Langerhans cells) and in lymphoid organs (follicular DCs). Each subpopulation expresses different cell surface receptors, signaling receptors such as TLRs and FcRs, and each produces a diverse and complex mixture of cytokines and chemokines. As with other cells described in this text,

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many of the details of DC function have been worked out in humans and mice. It must not be assumed that the structure and functions of DCs in domestic mammals are identical.

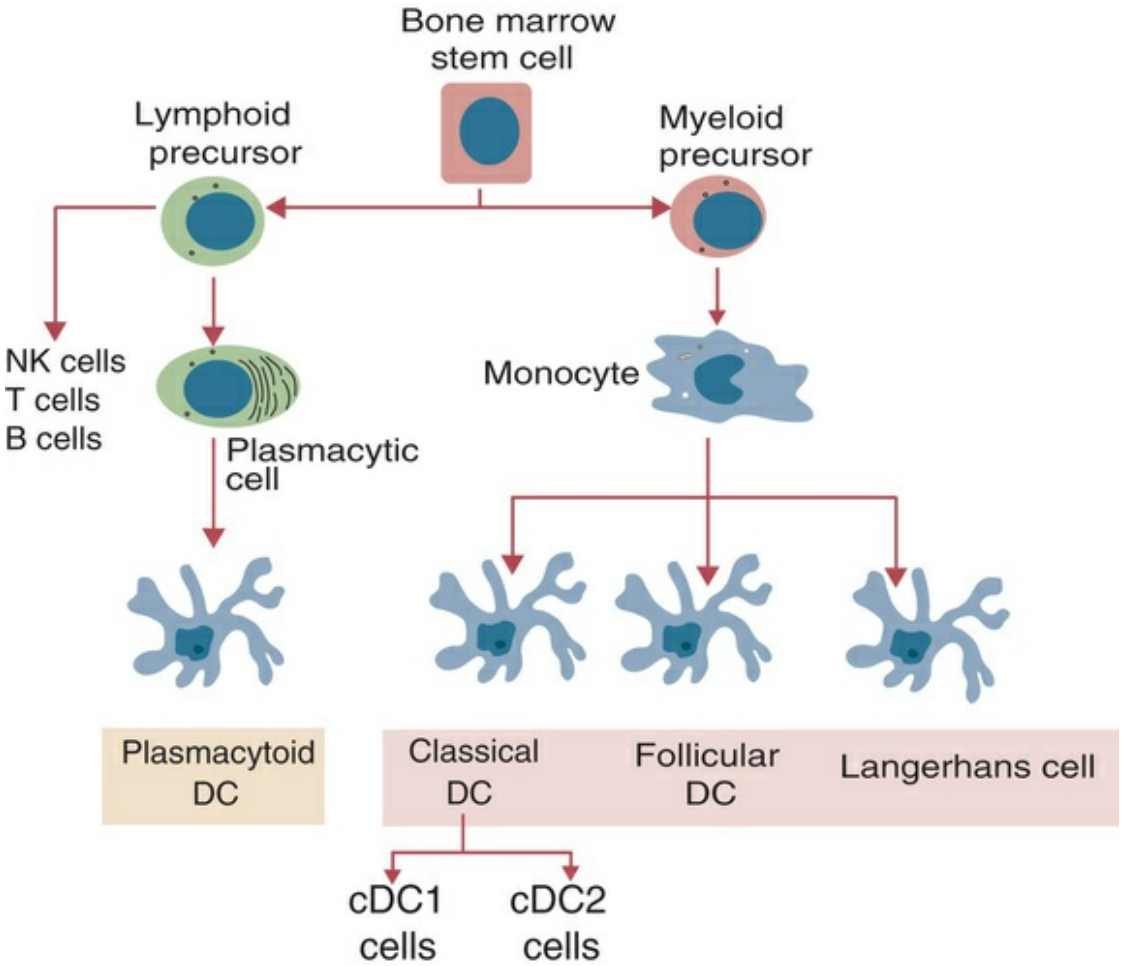


FIG. 10.3 The origins and diversity of DCs. One subpopulation, the plasmacytoid DCs, originates from lymphoid precursors. The second major subpopulation arises from myeloid precursors and is probably derived from monocytes. These monocyte-derived cells differentiate into classical DCs follicular DCs and Langerhans cells.

As pointed out earlier, the adaptive immune system mounts two types of response: the antibody-mediated and cell-mediated responses. The type of response mounted is determined by the type of helper T (Th) cells activated when an antigen is encountered. Thus there are multiple types of helper cells (see Fig. 14.12). One major type, helper 1 cells (Th1 cells), stimulates cell-mediated immune responses designed to protect animals against intracellular organisms. The other major type, helper 2 cells (Th2 cells),

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stimulates antibody-mediated immune responses designed to protect animals against extracellular invaders. Which of these helper cell types is activated depends on the DCs.

Classical DCs

The relationships between macrophages and dendritic cells is unclear and may differ between species. Blood monocytes are the immediate precursors of some tissue macrophages and some cDCs. Which cell type is produced depends on the mixture of cytokines and cells encountered by the monocyte as it differentiates. Each type can convert to the other until late in the differentiation process. cDCs can therefore be considered part of the mononuclear phagocytic system being derived from a common stem cell, respond to the same growth factors, express the same surface markers, and in effect are in no specific way uniquely different from other macrophages. Their main function is antigen presentation and induction of T cell responses.

Plasmacytoid DCs

Plasmacytoid DCs (pDCs) are long-lived cells found in blood, bone marrow, and lymphoid organs. They lack myeloid antigens but retain some lymphoid characteristics. pDCs serve as an early warning system for viral infections since they are rapidly activated by viral nucleic acids. They respond to viruses by synthesizing massive amounts of the type I interferons (IFN-α and IFN-β). (Ten to 100-fold more than other cell types.) They are also major producers of type III interferons (IFN-λ)(Chapter 27). Their numbers increase during infection. pDCs have a unique ability to link innate and adaptive immunity. After producing large amounts of type I interferon, they are still able to differentiate into mature DCs that can stimulate naïve T cells. Because pDCs secrete large amounts of IFN-α, they also activate natural killer (NK) cells (Chapter 19).

Langerhans Cells

Langerhans cells are specialized, long-lived antigen-presenting cells found in the skin. They contain characteristic rod- or racquet-

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shaped cytoplasmic granules called Birbeck granules, whose function is unclear. Their long dendrites form an extensive network that is ideally situated to capture foreign antigens (Fig. 10.4). These antigens include not only invading microbes but also topically applied antigens, such as the resins of poison ivy, or intradermally injected antigens, such as those in mosquito saliva. Langerhans cells influence the development of skin immune responses, such as delayed hypersensitivity and allergic contact dermatitis (Chapter 33). They express multiple pattern-recognition receptors (PRRs), including the C-type lectins langerin and DC-SIGN (see below) that can bind bacteria, fungi, and some viruses. Once antigens are captured, the Langerhans cells migrate to draining lymph nodes, where they present the antigen to T cells. Some immunologists regard Langerhans cells simply as specialized macrophages.

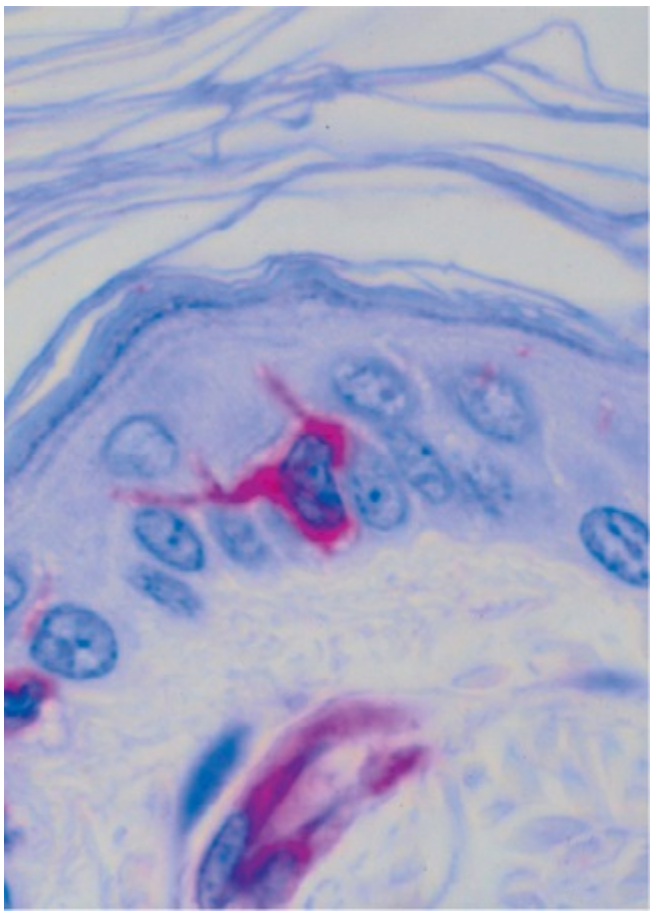


FIG. 10.4 This dark red cell in the epidermis of a dog is a Langerhans cell stained for the protein vimentin. Note that its dendrites extend between the epidermal cells so that it can effectively trap antigens. (Courtesy Dr. K.M. Credille.)

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Follicular DCs

Specialized DCs called follicular DCs (fDCs) are found in the germinal centers of the spleen, lymph nodes, and Peyer's patches (Chapter 12). They are also found around localized infection sites and chronic inflammatory reactions. Follicular DCs are essential for antibody production and the development of B cell memory. In an animal that has not previously been exposed to the antigen, antigen presentation is a passive process. The fDCs simply provide a surface on which antigen can be presented to B cells. In contrast, in animals that have previously been exposed to an antigen and possess antibodies, the antigen and antibody combine to form antibody-antigen complexes (also called immune-complexes). Follicular DCs take up these immune-complexes on their surface and then shed them in membrane vesicles called exosomes. B cells can take up these exosomes and after processing the antigen, present it to antigen-sensitive T cells. Follicular DCs can retain antigens on their surface for more than 3 months. They also integrate signals from toll-like receptors (TLRs) and other sources to support effective germinal center responses (Chapter 12).

Dendritic Cell Maturation

Although many DC subpopulations have been characterized, their most important properties are based on their state of maturity (Fig. 10.5). Thus immature DCs are specialized efficient antigen-trapping cells. As they mature, DCs undergo cellular reorganization and become specialized efficient antigen-presenting cells.

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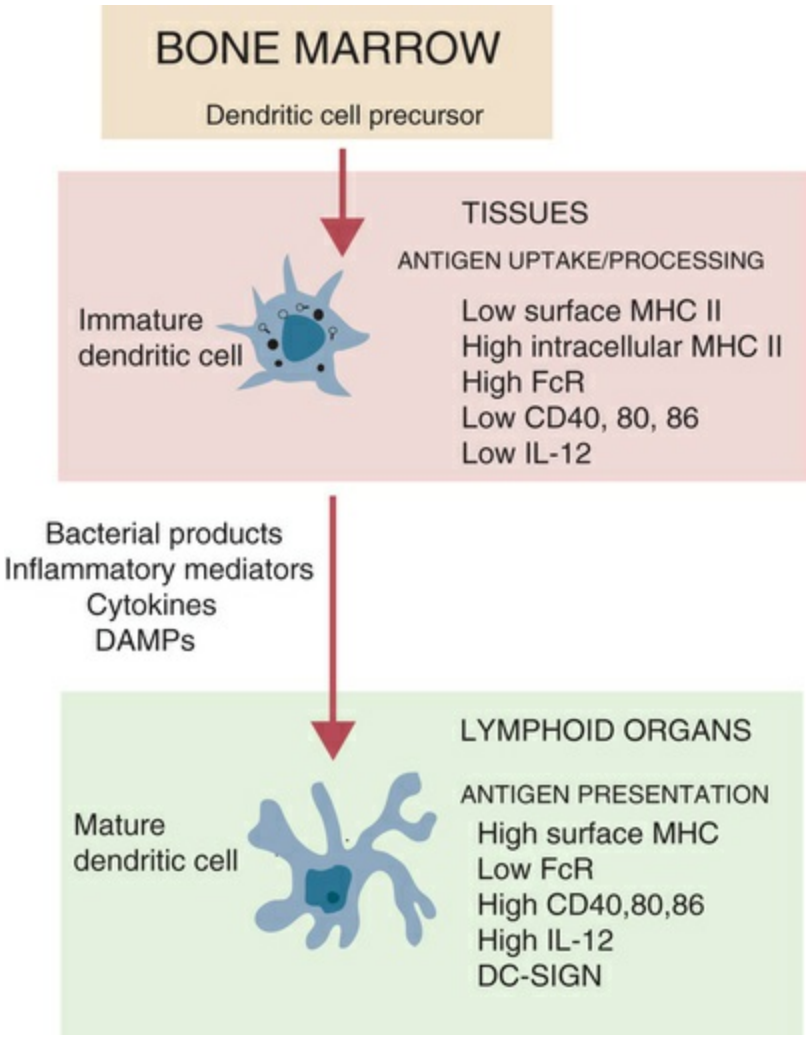


FIG. 10.5 As DCs mature, they change their function. Immature DCs are specialized antigen-trapping cells. Mature DCs, on the other hand, are specialized antigen-processing cells.

Immature DCs

Newly generated DCs migrate from the bone marrow to lymph nodes or other tissues. Here they act as sentinels whose role is to capture invading microbes. With their short life span, they can be regarded as disposable antigen-trapping cells. If they do not encounter antigens, they die in a few days. If, however, they encounter antigens and are stimulated by tissue damage or inflammation, they become activated and mature rapidly. Immature DCs have receptors that help them carry out their functions. These include the receptors for interleukin-1 (IL-1R) and tumor necrosis factor (TNFR), chemokine receptors, C-type lectins, Fc receptors (FcγR and FcεR), mannose receptors (CD206), heat-

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shock protein receptors, as well as TLRs.

Although the most important functions of DCs are to trap, process, and present antigen to the cells of the immune system, they must also be able to kill any pathogens they encounter. Thus DCs can kill invaders by mounting a respiratory burst. Activation of their TLRs by pathogen-associated molecular patterns (PAMPs) enhances their production of superoxide.

DCs mature in response to interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) as well as to PAMPs and damage-associated molecular patterns (DAMPs). Injured and inflamed tissues release large amounts of soluble heparan sulfate that binds to TLR4 and activates DCs. Breakdown of nucleic acids generates uric acid, another potent dendritic cell activator. One of the most important activators of immature DCs is high mobility group box protein-1 (HMGB1) (Chapter 7). Immature DCs are attracted to areas of inflammation by chemokines, defensins, and HMGB1.

Immature DCs capture antigens and cell fragments by phagocytosis, by pinocytosis (the uptake of fluid droplets—cell drinking), and by binding to cell surface receptors. They also capture apoptotic cell bodies. If they ingest bacteria, they can usually kill them. They can distinguish between normal tissue debris and foreign organisms by selectively sampling their environment. This differentiation depends on the ability of the foreign material to bind to TLRs. Activation of TLRs by PAMPs ensures that ingested material is processed in such a way that it triggers adaptive immunity. Material that does not activate TLRs is not processed and will not trigger an adaptive response.

The pH in the phagosomes of conventional phagocytic cells such as neutrophils and macrophages is highly acidic and hence optimized for proteolytic destruction of foreign material. The pH within dendritic cell and B cell phagosomes is, in contrast, relatively alkaline since these phagosomes do not fuse with lysosomes. Cysteine and aspartyl proteases are inhibited at these high pH levels, and as a result, ingested antigens are not completely degraded but rather are preserved for presentation by MHC class I molecules.

Mature DCs

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Once they have captured and processed antigens, immature DCs carry these antigens to sites where they can be recognized by T cells. The activated DCs are attracted to lymphoid organs by chemokines. Infection or tissue damage also promotes the migration of antigen-bearing DCs to lymph nodes or the spleen. Once they enter a lymphoid organ, the cells mature rapidly.

Mature DCs secrete chemokines that attract T cells, which accumulate in clusters around the dendritic cell (Fig. 10.6). The DCs embrace the T cells in a net of dendrites as they interact. During this time, the T cells examine the mature DCs for the presence of antigen fragments. If their antigen receptors can bind the presented fragments, the T cells will be triggered to respond.

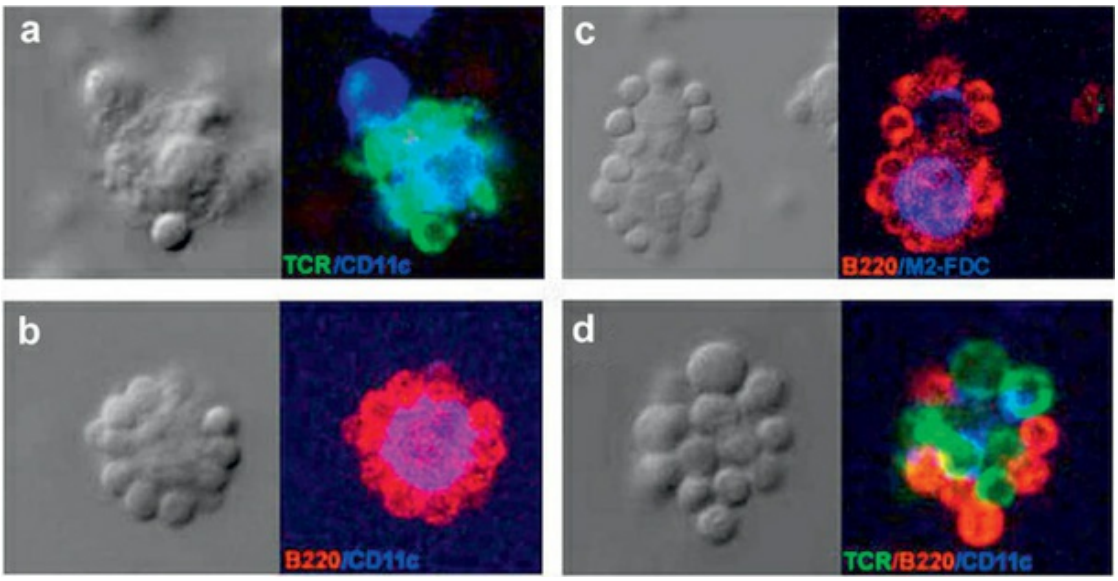


FIG. 10.6 When DCs and T cells interact, they form visible clusters as the cells converse among themselves. Thus in these figures, DCs are stained with a blue fluorescent dye (anti-CD11c), T cells are stained with a green dye (anti-CD3), and B cells are stained with a red dye (anti-B220). **A,** T cells are interacting with a dendritic cell. **B,** B cells are binding to a dendritic cell. **C,** B cells are binding to a follicular dendritic cell. **D,** There is a mixed B and T cell cluster. Note that some B cells appear to be attached to T cells. (From Hommel M, Kyewski B: Dynamic changes during the immune response in T cell-antigen-presenting cell clusters isolated from lymph nodes, *J Exp Med* 197:269-280, 2003.)

As DCs mature, their MHC molecules move from intracellular endosomes and lysosomes to the cell surface. Cell surface expression of their costimulatory molecules also increases. As a result, MHC molecules and MHC-peptide complexes are found at

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levels 100 times higher on mature DCs than on other cell types such as B cells or macrophages. Their expression of costimulatory molecules such as CD86 (Chapter 14) may also rise 100-fold.

Mature DCs are the only cells that can trigger a primary T cell response. One reason for this is that mature DCs can assemble complete T cell activation complexes (antigen-loaded MHC plus costimulatory molecules) within the cell before they are carried to the cell surface. Mature DCs also express DC-SIGN (CD209), a C-type lectin that binds a ligand called intercellular adhesion molecule-3 (ICAM-3 or CD50) on naïve T cells. DC-SIGN permits transient binding between DCs and T cells. It allows a single dendritic cell to rapidly screen thousands of T cells to find the few that are expressing a compatible antigen receptor. Because of their potency, only a few DCs are needed to trigger a strong T cell response. Thus one dendritic cell may activate as many as 3000 T cells.

Tolerance Induction

Under steady-state conditions, in the absence of inflammation or infection, some immature DCs will spontaneously mature and migrate to lymphoid tissues carrying normal tissue antigens on their MHC molecules. If a T cell recognizes this "normal" antigen, the T cell will undergo apoptosis and die. Alternatively, these DCs may trigger the production of IL-10, a suppressive cytokine that generates regulatory T cells. As a result, the processing of normal tissue antigens or even harmless environmental antigens by DCs can lead to T cell deletion and immunological tolerance (Chapter 20). In humans a subset of DCs secrete the immunosuppressive molecule indoleamine 2,3-dioxygenase (IDO). This subset has a regulatory function and may be able to promote tolerance. The decision to induce either a tolerogenic response or a defensive immune response likely depends upon the presence of microbial PAMPs or tissue DAMPs. A good example of this is the tolerance expressed in the intestine to food antigens and commensal bacteria. DCs that sample these antigens are potent inducers of retinoic acid and TGF-β (Chapter 21). This retinoic acid promotes the differentiation of regulatory T cells and thus prevents the

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development of inappropriate immune responses to commensals and food.

cDC1 and cDC2 Cells

When cDCs stimulate helper T cells, they generate three signals. The first signal is delivered when T cell antigen receptors bind antigen fragments attached to MHC molecules. The second signal provides the cells with additional critical costimulation through cell surface molecules such as CD40 and CD80/86. The third signal determines the direction in which naïve helper T cells will develop and is provided by secreted cytokines. For example, some microbial antigens trigger cDCs to secrete IL-12 (Fig. 10.7). These are called cDC1 cells since their IL-12 activates Th1 cells and triggers type 1 responses. Other microbial antigens induce cDCs to secrete IL-1 and IL-6. These cytokines stimulate Th2 differentiation and are produced by cDC2 cells. They stimulate type 2 responses. Other antigens may induce cDC2s to secrete IL-23 and so provoke the development of Th17 cells.

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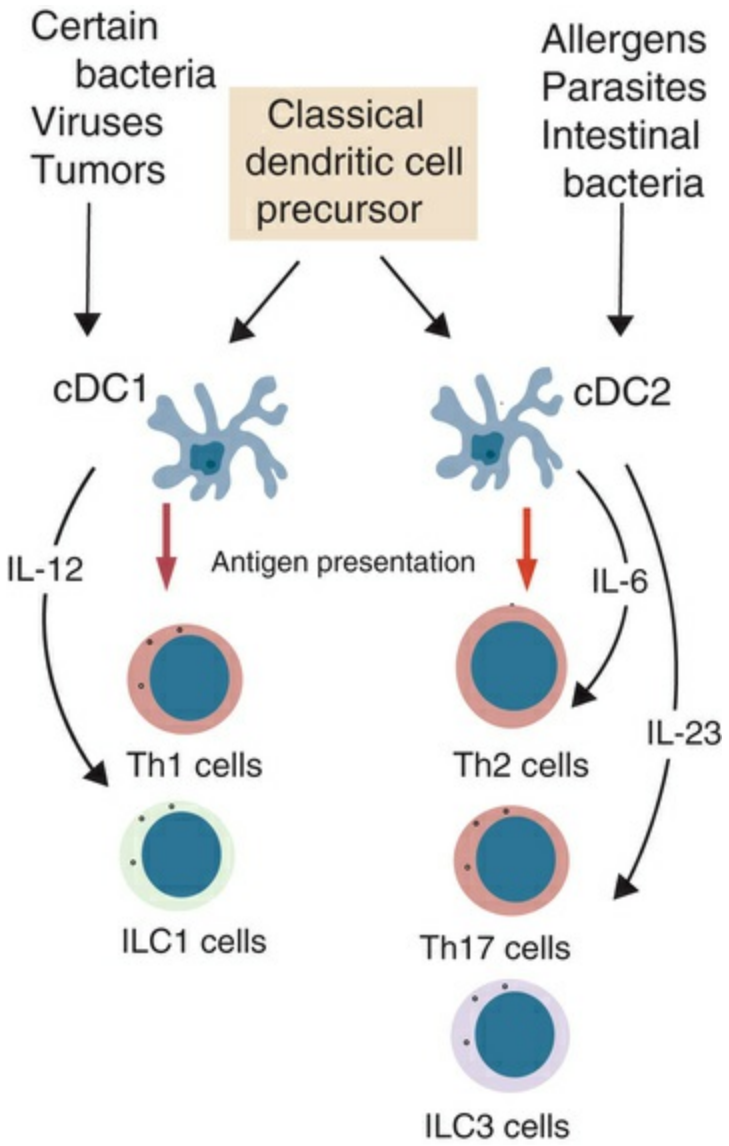


FIG. 10.7 Two populations of cDCs activate helper T cell subpopulations. cDC1 cells promote type I responses by producing IL-12 and activating Th1 cells. Whereas cDC2 cells promote type II responses by producing IL-6 and IL-23 and so activating Th2 cells and Th17 cells. The helper cell population that is activated depends on the cytokine mixture produced by these dendritic cell subpopulations. The specific dendritic cell subpopulations activated depend upon the nature of the inducing antigens. Dendritic cells also activate innate lymphoid cells (ILCs). cDC1s activate ILC1s and cDC2s activate ILC3s (Chapter 19).

Different PAMPs and DAMPs acting through different TLRs influence the development of these dendritic cell subpopulations and thus the types of responses induced. The stimuli that promote cDC1 production include double-stranded RNA acting through TLR3, lipopolysaccharide acting through TLR4, flagellin acting through TLR5, and nucleic acids acting through TLR7 and TLR9.

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On the other hand, inflammatory mediators, such as IL-10, transforming growth factor-α (TGF-α), prostaglandin E_2 (PGE_2), histamine, extracts of parasitic worms, or the toxin of *Vibrio cholerae*, promote cDC2 production. cDC2 responses may also be triggered by bacterial lipopolysaccharides and proteoglycans acting through TLR2, TLR6, or TLR1. Ligands of TLR2 promote the production of IL-23 and thus promote Th17 cell responses. As pointed out previously, a similar functional subdivision occurs in macrophages. Thus M1 and M2 cells, when acting as antigen-presenting cells, also promote different helper cell responses.

It may also be that the same dendritic cell can promote a type 1, type 2, or Th17 response, depending on the dose and type of antigen it encounters. The response may also depend on its location. For example, DCs from the intestine or airways seem to preferentially secrete IL-10 and IL-4 and thus promote type 2 responses. In such cases, the intestinal microbiota may provide the cDC2–polarizing signals.

pDCs increase dramatically during inflammation and infection. They are relatively inefficient in promoting helper cell proliferation but produce large quantities of IFN-γ and IL-17. They thus promote Th1 polarization.

Interleukin-12

IL-12 is a key cytokine produced by macrophages, DCs, B cells, and neutrophils. Its targets are T cells and NK cells. IL-12 determines Th1/Th2 polarization. Th1 cells develop when it is present. Th2 cells develop when it is absent. IL-12 is a member of a family of related proteins that also includes IL-23, IL-27, IL-35 and IL-39. These are all heterodimeric proteins. For example, IL-12 is formed by two chains, p35 and p40. Some of these chains are shared with other family members. All the members of the family regulate T cell function. Thus IL-12 and IL-27 generate Th1 cells, whereas IL-23 generates Th17 cells.

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Dendritic Cells in Domestic Animals

DCs are found in all the major domestic mammals and do not appear to differ in any significant respect from DCs in humans and mice. In domestic animals, cDCs have been characterized in horses, ruminants, pigs, dogs, and chickens, whereas Langerhans cells have been described in horses, ruminants, pigs, dogs, and cats. pDCs have been characterized in pigs and horses.

Horses: Equine DCs express MHC class II, CD11, EqWC1, and EqWC2. Different subsets have been identified based on their expression of MHC class II and other markers. Equine pDCs have been characterized and shown to produce large amounts of IFN-α on stimulation with TLR9 agonists.

Ruminants: Bovine DCs express MHC II, CD80, CD86, and CD40 (Fig. 10.8). Cattle possess two dendritic cell subpopulations that differ in their ability to stimulate CD4 and CD8 T cells. One population synthesizes more IL-12, whereas the other population produces more IL-1 and IL-10. These may well represent cDC1 and cDC2 subpopulations. Cattle also possess some DCs that produce large amounts of type I interferons and are therefore likely functional equivalents of pDCs.

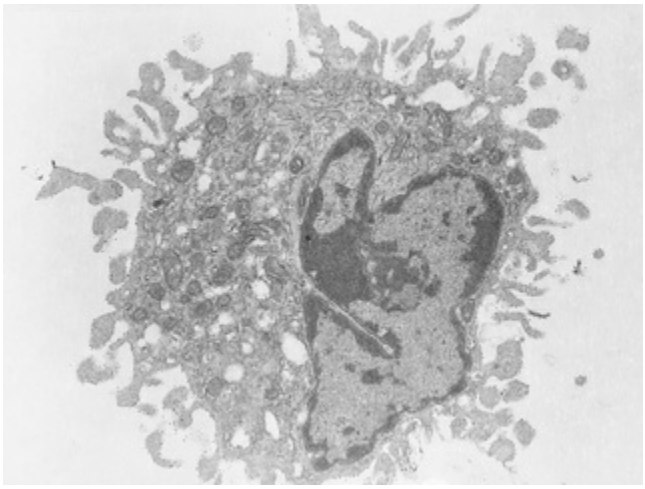


FIG. 10.8 A transmission electron micrograph of a dendritic cell from bovine afferent lymph. It has been stained with a monoclonal antibody specific for bovine CD1b. (The antibody is linked to colloidal gold particles, which are visible as small, electron dense dots around the outside of the cell). (Courtesy Dr. C.J. Howard and Dr. P.

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DCs derived from sheep peripheral blood monocytes express MHC class II, CD11c, and are CD14-negative. pDC, cDC1 and cDC2 cells have been identified in this species.

Pigs: Pigs have both cDCs and pDCs. Pig cDCs are $CD172a^{+}$, $CD11R1^{+}$, $CD1^{+/-}$, and $CD80/86^{+/-}$, whereas their pDCs are $CD172a^{+}$, $CD4^{+}$, $CD1^{+/-}$, and $CD80/86^{+/-}$. Both types secrete IL-10 and IL-12. Porcine DCs also express FcγRII and FcγRIII and thus can be activated by immune-complexes. They express TLRs and are responsive to stimulation by bacterial lipopolysaccharide and CpG dinucleotides. The functions of pig DCs have been characterized based on their transcriptome. Thus pig blood pDCs are the major source of TNF-α, IL-12p40, IFN-α, and some complement components. Pig cDCs are most efficient in antigen presentation and T cell stimulation.

It is of interest to note that pig pDCs produce IFN-α in response to several common viruses, including transmissible gastroenteritis, pseudorabies, and swine flu, but not porcine reproductive and respiratory syndrome virus (PRRSV). This virus impairs antigen presentation by cDCs and enhances their IL-10 production. It is not surprising therefore that PRRSV causes persistent infection and stimulates weak immunity.

Dogs: Dog DCs can be stimulated to differentiate by many different cytokine mixtures, but the most widely employed is GM-CSF plus interleukin-4. They resemble DCs in other species. There are two main populations of canine DCs. One is MHC class II^{+}, $CD11c^{+}$, $CD34^{+}$, and $CD14^{-}$; the other is MHC class II^{+}, $CD34^{+}$, and $CD14^{+}$. CD40 is expressed on canine DCs but not on monocytes. They produce a diverse array of cytokines. The precise mixture produced depends on the stimulus employed but in general resembles those produced in humans and mice. One unusual feature is, however, the production of large amounts of IL-10, IL-12, IL-13 and IFN-α by LPS-stimulated canine DCs. Canine DCs also express functional CD1 molecules that can bind and present lipid antigens to lymphocytes.

Cats: As in other species, these are readily induced by exposure to GM-CSF and IL-4. Feline Langerhans cells are $CD18^{+}$, MHC class II^{+}, $CD1a^{+}$, and $CD4^{+}$. DCs from feline blood mononuclear cells are

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$CD1^+$, $CD14^+$, and MHC class I and II^+ (Fig. 10.9).

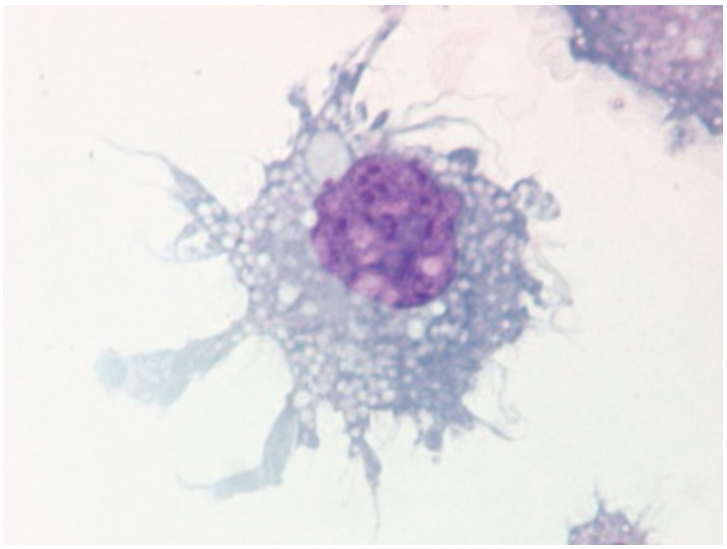


FIG. 10.9 A feline dendritic cell cultured in the presence of recombinant human IL-4 and GM-CSF. Note the extensive dendrites so characteristic of these cells. Bright field illumination ×100. (From Sprague WS, Pope M, Hoover EA: Culture and comparison of feline myeloid DCs vs macrophages, *J Comp Pathol* 133:139, 2005.)

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Other Antigen-Processing Cells

Naïve T cells require prolonged close interaction with DCs before they can respond to antigens. Once primed, however, these T cells may be further activated by relatively brief interactions with two other major cell types: antigen-presenting macrophages and B cells.

Macrophages

Macrophages are the most accessible and best understood of the antigen-processing cells. Their properties are described in Chapter 6. Once antigens are taken up by macrophages, a portion is processed and presented to sensitized T cells. Macrophages, however, are unable to engage in prolonged interactions with T cells. As a result, they cannot activate naïve T cells. In addition, antigen processing by macrophages is inefficient since much of the ingested antigen is destroyed by lysosomal proteases and oxidants. Indeed, macrophages and B cells can be considered cells with other priorities (some call them "semiprofessional" antigen-processing cells).

B Cells

B cells, like macrophages, cannot undertake prolonged interactions with T cells. They do, however, have antigen receptors that enable them to bind and process large amounts of specific antigen. They ingest and process antigens before presenting them, in association with MHC class II molecules, to sensitized T cells (see Fig. 15.7). B cells probably play a minor role in antigen processing in a primary immune response but a much more significant one in a secondary response when their numbers have greatly increased and T cells are easier to stimulate.

Other Cells

T cells may also be activated by many different "nonprofessional" cell types. These include neutrophils, eosinophils, basophils, T cells,

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endothelial cells, fibroblasts, NK cells, smooth muscle cells, astrocytes, microglial cells, platelets, and some epithelial cells such as thymic epithelial cells and corneal cells. Their effectiveness may depend on the local environment. Thus fibroblasts may be very effective antigen-processing cells when located within granulomas. Presumably, costimulation can come from other nearby cells in this cytokine-rich environment. Vascular endothelial cells can also take up antigens, synthesize IL-1, and under the influence of IFN-γ, express MHC class II molecules. Even skin keratinocytes can secrete cytokines similar to IL-1, express MHC class II molecules, and present antigen to T cells. In pigs, a subpopulation of circulating γ/δ T cells may act as professional antigen-processing cells.

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Antigen Processing

MHC Class II Pathway

The presentation of exogenous antigens is regulated by MHC class II molecules. These are cell surface receptors that bind processed peptide fragments. Although many cells can phagocytose foreign antigens, only those that can express antigen fragments bound to MHC class II molecules will trigger an immune response. As described previously, the most efficient antigen-processing cells are thus mature MHC class II^+ cDCs. Unlike macrophages, dendritic cell lysosomes have limited proteolytic activity and degrade internalized antigens slowly. As a result, antigens may persist within these cells for a long time. MHC class II molecules bind fragments of these ingested antigens and present them to helper cells (Fig. 10.10). T helper cells recognize and respond to antigen fragments only when the fragments are bound to MHC class II molecules. If an antigen is presented to T cells in the absence of MHC class II, the T cells will be turned off or die, and tolerance may result (Chapter 20).

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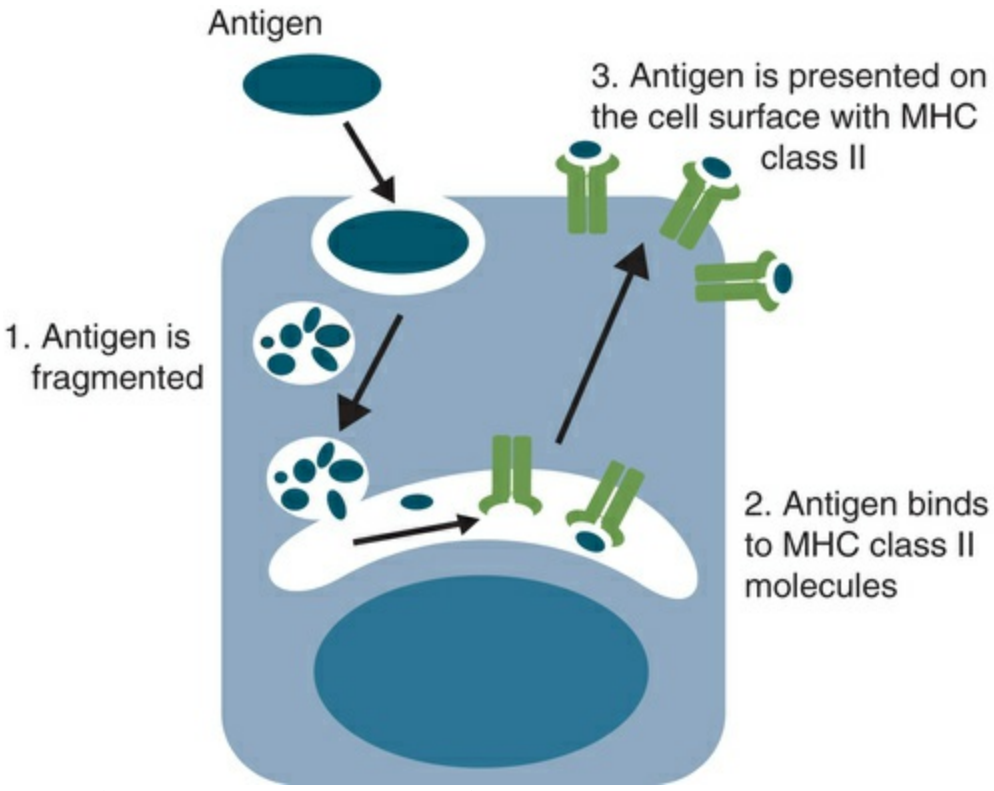


FIG. 10.10 The processing of exogenous antigen by an antigen-presenting cell. Ingested antigens are taken into phagosomes where they are fragmented by proteases. Peptides are then carried to the endosomal compartments where the antigenic peptides are placed in the binding grooves of MHC class II molecules. The antigen-MHC complexes are then carried to the cell surface where they are presented to helper T cells.

Exogenous antigen processing involves multiple steps. First, the antigen must be endocytosed and taken into phagosomes. These phagosomes then fuse with lysosomes. The ingested proteins are broken up by the lysosomal proteases into peptide fragments of varying length. The endosomes containing these peptide fragments then fuse with other endosomes carrying newly synthesized MHC class II molecules to generate the lysosome-MHC class II compartment (MIIC). Endogenous antigens may also enter the MIIC through autophagy (Chapter 5).

Newly synthesized MHC class II chains are translocated to the endosomes, where, together with a peptide called the invariant chain (Ii), they form a protein complex. The Ii occupies the MHC antigen-binding site. This complex travels to the MIIC, where the Ii is digested, leaving a small peptide called the class II-associated Ii peptide (CLIP), filling the MHC antigen-binding groove. When antigen-containing phagosomes fuse with the MHC-containing

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endosomes, the foreign peptide fragments are exchanged for the CLIP chain. An MHC class II antigen-binding groove can hold a peptide of 12 to 24 amino acids as a straight, extended chain that projects out of both ends of the binding groove. Side chains from the peptide bind in pockets on the walls of the binding groove.

The presence of the CLIP chain prevents endosomes containing MHC class II molecules from being prematurely transported to the cell surface Thus, unlike most new transmembrane proteins that are expressed minutes after assembly, MHC class II molecules are retained inside the cell for several hours until they are needed. Once the antigen peptide binds to an MHC molecule, the MIIC vesicle moves toward the cell surface. When it reaches the surface, the vesicle fuses with the cell membrane, and the MHC-peptide complex is exposed and available for inspection by any passing T cell.

It has been calculated that an antigen-processing DC contains about 2×10^5 MHC class II molecules that can present peptide fragments to T cells. If costimulation is provided, a single T cell can be activated by exposure to as few as 200 to 300 of these peptide-MHC complexes. It is therefore possible for a single antigen-processing cell to present many different antigens to different T cells simultaneously.

Since T helper cells must recognize MHC-antigen complexes in order to respond to an antigen, the MHC class II molecules effectively determine whether an animal will mount an adaptive immune response to any antigen. Class II molecules can bind some, but not all, peptides created during antigen processing, and, in effect, they select those antigen fragments that are to be presented to T cells. (Further coverage of MHC molecules is provided in Chapter 11.)

MHC Class I Pathway

One function of T cell–mediated immune responses is the identification and destruction of cells producing abnormal or foreign proteins. The best examples of such cells are those infected by viruses. Viruses take over the protein-synthesizing machinery of infected cells and use it to make new viral proteins (Fig. 10.11). To

control virus infections, cytotoxic T cells must be able to recognize any viral proteins expressed on the surface of infected cells. T cells can indeed recognize and respond to these endogenous antigens, but only if they are processed and bound to MHC class I molecules (Chapter 11).

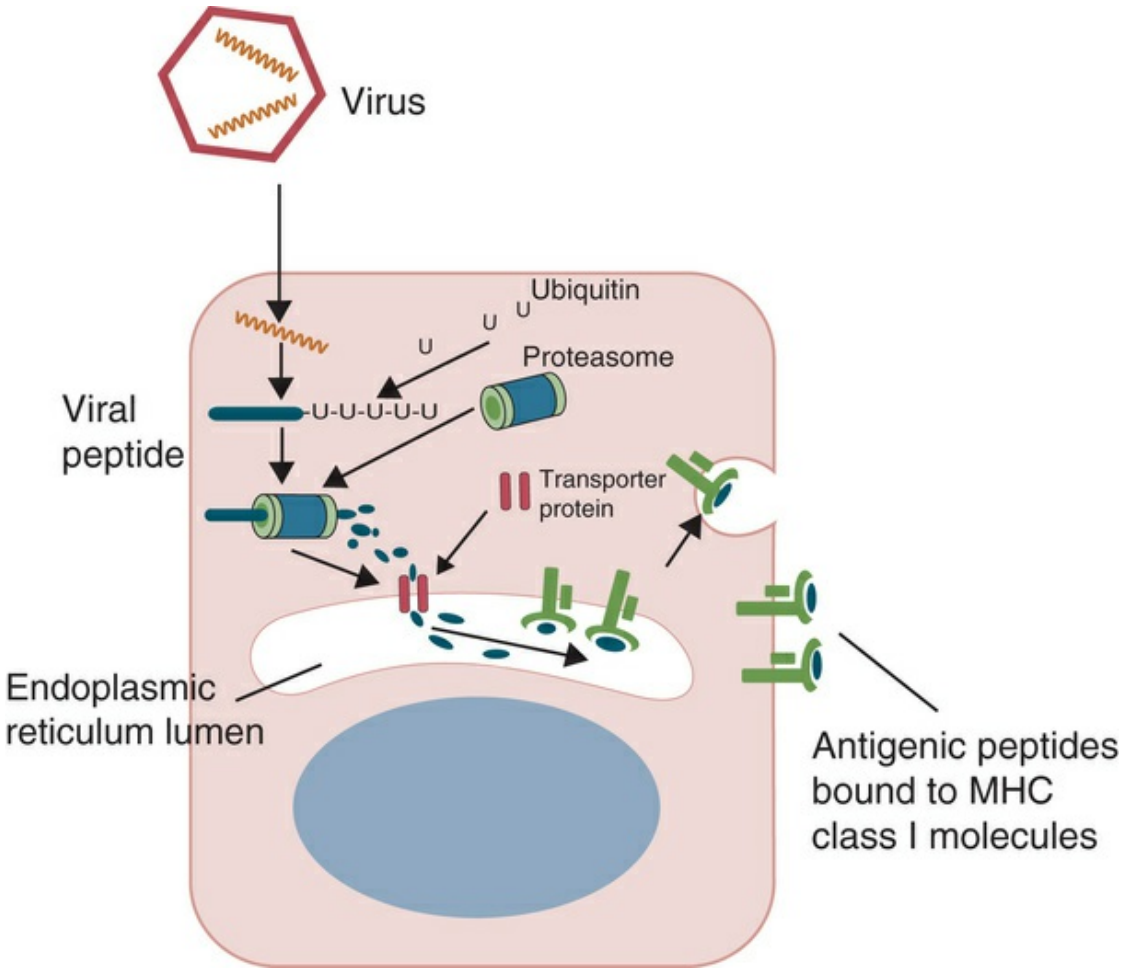


FIG. 10.11 The processing of endogenous antigen. Samples of newly synthesized proteins are ubiquinated before being chopped into peptides by a proteasome. The peptides attach to a transporter protein located in the membrane of the endoplasmic reticulum. They are then carried into the lumen of the endoplasmic reticulum, where they are placed in the antigen-binding groove of MHC class I molecules. The MHC class I-peptide complexes are carried to the cell surface, where they encounter cytotoxic T cells.

The MHC class I molecule is a cell surface receptor folded in such a way that a large antigen-binding site is formed (see Fig. 11.5). This binding site, however, differs from that on MHC class II molecules in that it is closed at each end. As a result, long peptides cannot

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project out of the ends. Because of this, MHC class I molecules only bind peptides containing about nine amino acids. In order to do so, these peptides must bulge out in the middle. Overall, however, the antigen-binding sites on class II and class I molecules function in a similar manner.

The processing of endogenous peptides differs from the processing of exogenous peptides. Living cells continually break up and recycle the proteins they produce. As a result, abnormal proteins are removed, regulatory peptides do not accumulate, and amino acids are recycled. As a first step, the protein must be tagged. To do this, ubiquitin, a small protein found in all eukaryote cells, attaches to lysine residues in target proteins. Additional ubiquitins then attach to the protein-bound ubiquitin so that several ubiquitin molecules are linked like beads on a string. A chain of four ubiquitin molecules appears to be optimal for processing. These polyubiquinated proteins are marked for destruction since they are recognized by enzyme complexes called proteasomes.

Proteasomes are tubular molecular complexes whose function is to degrade ubiquinated proteins. They consist of an inner channel that contains the protease activity and two outer rings that regulate which proteins can enter and be destroyed. Ubiquitinated proteins bind to the outer rings, the tagged protein is unfolded, and the ubiquitin is released and reused. The unfolded protein is inserted into the inner channel, where it is broken into 8- to 15-amino acid long peptides (like a meat grinder). Most of these peptide fragments are recycled into new proteins. For about 1 in 1 million molecules, however, the peptides are rescued from further breakup by attachment to transporter proteins. Two transporter proteins are used: TAP-1 and TAP-2 (*TAP* stands for *t*ransporter for *a*ntigen *p*rocessing). TAP-1 and TAP-2 form a heterodimer that binds peptide fragments and transports them into endosomes. Ideally, an 8- to 10-amino acid peptide precisely fits the binding site on the heterodimer. In this case the peptide is loaded into the TAP dimer, carried to a newly formed MHC, and if it fits the MHC antigen-binding site, it is transferred. Once loaded on the MHC, the MHC-peptide complex is carried to the cell surface by its normal secretory pathway where it is displayed for many hours.

A cell can express about 10^6 MHC-peptide complexes at any one

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time. A minimum of about 200 MHC class I molecules loaded with the same viral peptide is required to activate a cytotoxic T cell. Thus the MHC-peptide complexes can provide passing T cells with fairly complete information on virtually all the proteins being made by a cell. Analyses of peptide binding indicate that the binding groove of a class I protein can bind over a million different peptides with significant affinity. The number is not unlimited, however, because each MHC type usually shows preferences for peptides with certain structures. In fact, out of the great number of peptides generated from the proteome of a pathogen, only a few "immunodominant" peptides are recognized by most of the host's T cells. Thus cytotoxic T cells can then screen these peptides to determine whether any are "foreign" and bind to their TCRs.

Cross-Priming

It must not be assumed that the two antigen-processing pathways function in isolation. In fact, the pathways interact extensively. For example, under some circumstances, exogenous antigens may enter the cytoplasm, join the endogenous antigen pathway, and be presented on MHC class I molecules. Thus in antigen-presenting cells such as macrophages and DCs, endocytosed viral antigen may not be degraded in lysosomes but by proteasomes and so is processed as an endogenous antigen. This antigen thus binds to MHC class I molecules and is recognized by cytotoxic T cells. This may be important in immunity to viruses since it ensures that the antigens from dead virions may still be able to trigger a response by cytotoxic T cells (Chapter 18).

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Histiocytosis and Histiocytomas

Domestic animals suffer from several diseases in which macrophage or DCs proliferate excessively. These are called histiocytomas or histiocytosis. The most common of these, canine cutaneous histiocytoma is a benign epidermal neoplasm of Langerhans cell origin that usually regresses spontaneously. However, it may metastasize in some dogs. Langerhans cell histiocytosis is a nonneoplastic reactive lesion whose trigger is unknown but may be an infectious agent. This condition is not premalignant, and it may occur in cutaneous or systemic forms. Both forms of Langerhans cell histiocytosis present as a lesion or lesions in the skin or subcutis, but systemic histiocytosis also involves other tissues. Cutaneous histiocytosis shows no breed predilection, occurs in adult dogs between 3 and 9 years old, and is characterized by the development of nonpainful solitary or multiple nodules in the skin or subcutis. These lesions tend to occur on the head, neck, extremities, perineum, and scrotum. In contrast, systemic histiocytosis tends to occur in large breeds such as Bernese Mountain dogs, Rottweilers, Golden Retrievers, and Labradors. The age of onset is between 4 and 7 years. The lesions develop in the skin, mucous membranes, eyes, nasal cavity, spleen, lung, liver, bone marrow, and spinal cord. Histologically these lesions contain a mixture of cells. The cells express CD1, CD11c, MHC class II, CD4, and CD90, a phenotype typical of Langerhans cells. The lesions also contain T cells and neutrophils and may be successfully treated with corticosteroids, cyclosporine, or leflunomide (Chapter 41). As many as 30% of cutaneous cases and 10% of systemic cases spontaneously regress following infiltration by $CD4^+$ T cells and the production of Th1 cytokines such as IL-2, TNF-α, and IFN-γ, as well as NOS2, and subsequent recruitment of antitumor effector cells. Feline progressive histiocytosis is a skin disease presenting as solitary or multiple nonpruritic nodules on the feet, legs, and face. These histiocytes express CD1a, CD1c, CD18, and MHC class II molecules. About 10% of cases express E-cadherin, a characteristic of Langerhans cells. This is a slowly progressive disease that may involve internal organs in terminal cases.

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The Major Histocompatibility Complex

CHAPTER OUTLINE

Major Histocompatibility Complex, 100
MHC Class Ia Molecules, 101
Structure, 101
Gene Arrangement, 102
Polymorphism, 102
Nonpolymorphic MHC Class I Molecules, 102
MHC Class II Molecules, 103
Structure, 103
Gene Arrangement, 104
Polymorphism, 104
MHC Class III Molecules, 104
MHC of Domestic Animals, 104
MHC Molecules and Disease, 104
MHC and Body Odors, 107

LEARNING OBJECTIVES

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After reading this chapter, you should be able to:

- Describe the differences between the structure and functions of the major histocompatibility complex (MHC) class I and class II molecules.
- Explain MHC polymorphism and its significance in binding exogenous and endogenous antigens.
- Explain the different functions of polymorphic and nonpolymorphic MHC molecules.
- Understand the mechanisms involved and the reasons behind the maintenance of MHC polymorphism.
- Describe how MHC class I molecules present endogenous antigens to $CD8^+$ T cells.
- Describe how MHC class II molecules present exogenous antigens to $CD4^+$ T cells.
- Describe the contents of the class III region of the MHC.
- Describe the structural basis of MHC-antigen binding.
- Define major histocompatibility complex, exogenous and endogenous antigens, MHC polymorphism, and haplotype.
- Describe the role of the MHC in odor recognition.
- Explain the relationship between MHC haplotype and disease susceptibility.
- Understand the two strategies mammals use to generate MHC polymorphism; allelic polymorphisms, and variations in gene content.

Resistance to infectious diseases is one of the most strongly inherited disease traits. After all, a lethal infection effectively removes susceptible individuals from the gene pool. "Survival of the fittest" applies most strongly to individuals required to defend themselves against infectious agents. As a result, many genes can influence resistance to infection. The most important of these genes are those encoding cell surface glycoproteins that constitute the major histocompatibility complex (MHC).

In order to trigger adaptive immunity, antigen molecules must first be processed. They are broken up inside cells, and the fragments generated are bound to appropriate antigen-presenting receptors (Fig. 11.1). These antigen-presenting receptors are cell surface glycoproteins encoded by genes clustered together to form

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the major histocompatibility complex (MHC). The receptors are therefore called MHC molecules. Antigens can only trigger an adaptive immune response after binding to MHC molecules. These antigen-MHC complexes are then presented to T cell antigen receptors and trigger a response. Since each MHC molecule acts as an antigen receptor, the genes encoding them effectively determine which antigens can or cannot trigger adaptive immunity. Thus the MHC can be considered a cluster of genes that control antigen presentation and so determine resistance to infectious diseases. The ability of pathogens to evade, escape, or subvert immune defenses places strong selection pressures on MHC genes and results in their rapid evolution.

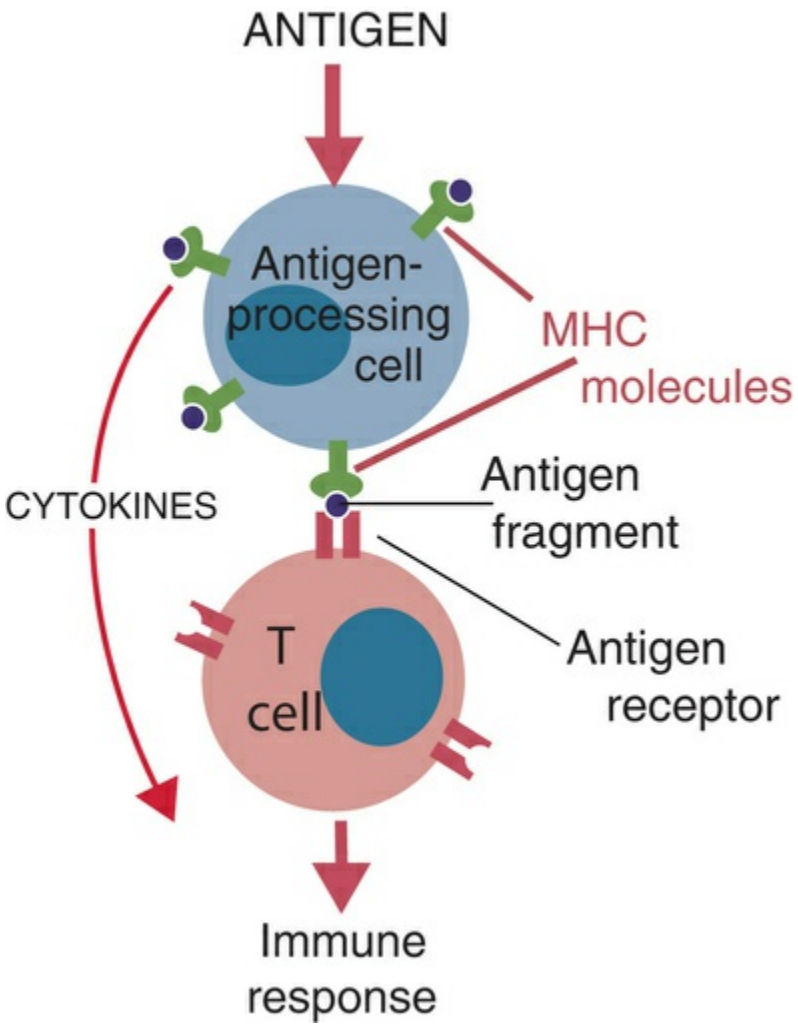


FIG. 11.1 The key initial step in any immune response is the presentation of antigens by antigen-processing cells to antigen-sensitive cells. This step is mediated by MHC molecules located on the surface of antigen-processing cells.

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Major Histocompatibility Complex

All vertebrates possess an MHC. Each mammalian MHC contains about 200 expressed genes divided into three regions (I, II and III) (Fig. 11.2). The class I region contain genes coding for MHC molecules expressed on most nucleated cells. Class I genes can be subdivided into those that are highly polymorphic (class Ia genes) and those that show very little polymorphism (class Ib, Ic, or Id genes). (Polymorphism refers to structural variations between proteins.) Class Id genes are located outside the MHC on a different chromosome. Genes in class II regions encode polymorphic MHC molecules usually restricted to professional antigen-presenting cells (dendritic cells, macrophages, and B cells) (Table 11.1). Genes within the MHC class III region code for a mixture of proteins, many of which are important in innate immunity such as complement. Although each MHC contains all three gene regions, their gene content and arrangement vary among species.

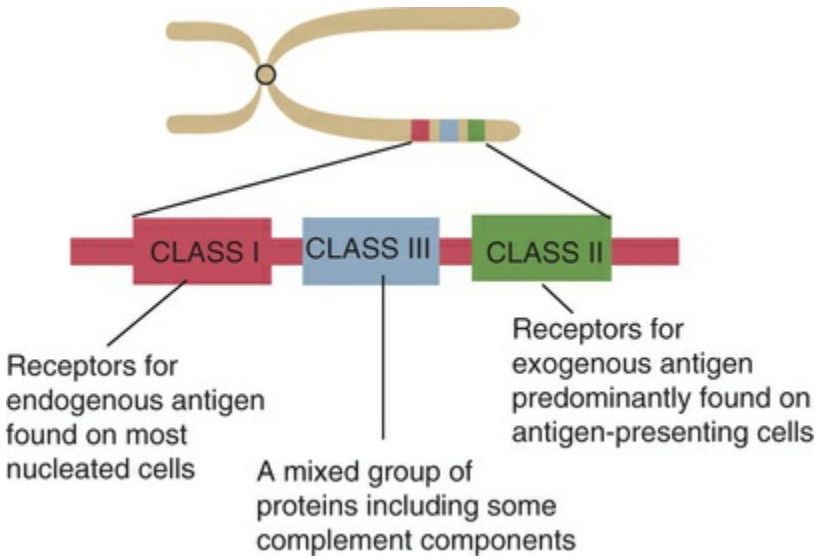


FIG. 11.2 The three major classes of genes located within the major histocompatibility complex are grouped together in regions. The products of each class have a unique distribution and function.

TABLE 11.1

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Comparison of MHC Class I and Class II Structure

	Class I	Class II
Loci include	Typically A, B, and C	DP, DQ, and DR
Distribution	Most nucleated cells	B cells, macrophages, and dendritic cells
Function	Present antigen to cytotoxic T cells	Present antigen to T helper cells
Result	T-cell-mediated toxicity	T-cell-mediated help

The collective name given to the proteins encoded by MHC genes depends on the species. In humans, these molecules are called human leukocyte antigens (HLA); in dogs, they are called DLA; in rabbits, RLA; in cattle (bovines), BoLA; in horses, ELA; in swine, SLA; and so forth. In some species, MHC molecules were identified as transplantation antigens before their true function was recognized, and their nomenclature is anomalous. Thus, in the mouse the MHC is called H-2, and in chickens it is called B. The complete set of alleles found within an individual animal's MHC is called its MHC haplotype.

While the human and mouse MHC class I genes exhibit extreme allelic polymorphism, this is not the case in other mammals. In these, MHC diversity is generated by variations in the number of MHC class I genes expressed. If some MHC genes are expressed in some MHC haplotypes but not in others, the effect will be to generate even more diversity than alternative combinations of alleles of a fixed gene. Gene content variation and allelic polymorphism can therefore be considered as two alternative strategies to diversify MHC haplotypes. Nonhuman primates, rodents, horses, pigs, and ruminants all rely on variations in MHC gene content. In contrast, humans, mice, dogs, and cats have relatively few functional MHC class I genes and rely on allelic polymorphism.

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MHC Class Ia Molecules

Class Ia molecules are expressed on most nucleated cells. In pigs, for example, class I molecules have been detected on lymphocytes, platelets, granulocytes, hepatocytes, kidney cells, and sperm. They are not usually found on mammalian red cells, gametes, neurons, or trophoblast cells. Some cells, such as myocardium and skeletal muscle, may express very few class Ia molecules.

Structure

Class Ia molecules consist of two linked glycoprotein chains. An α chain (45 kDa) is associated with a much smaller chain called β_2-microglobulin (β_2M) (12 kDa) (Fig. 11.3). The α chain is inserted in the cell membrane. It consists of five domains: three extracellular domains called α_1, α_2, and α_3, each about 100 amino acids long; a transmembrane domain; and a cytoplasmic domain. The antigen-binding site is formed by the α_1 and α_2 domains. The β_2M chain consists of a single domain and stabilizes the structure.

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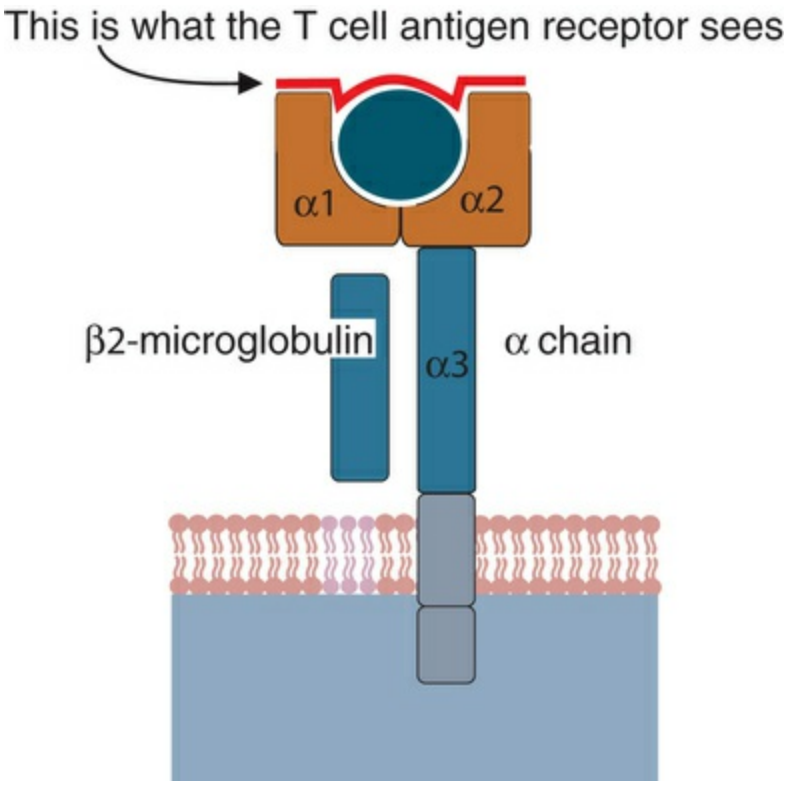


FIG. 11.3 The structure of a class Ia MHC molecule on a cell membrane. Its antigen-binding site is formed by the folding of both its α_1 and α_2 domains.

Gene Arrangement

The size of the MHC class I region varies. Humans and rodents have the largest, and pigs have the smallest. Each MHC class I region has a common framework of non-MHC genes, and size differences are mainly due to variations in these framework genes.

The number of class Ia genes also varies between mammals ranging from rats with more than 60 to pigs with 11. Not all these genes are functional. In mice, only two or three class I genes are expressed. The remainder are pseudogenes (defective genes that cannot be expressed). In humans the functional polymorphic genes are called *A*, *B*, and *C*. In mice they are called *K* and *D* (and in some strains, *L*). In other mammals they are usually numbered (Fig. 11.4).

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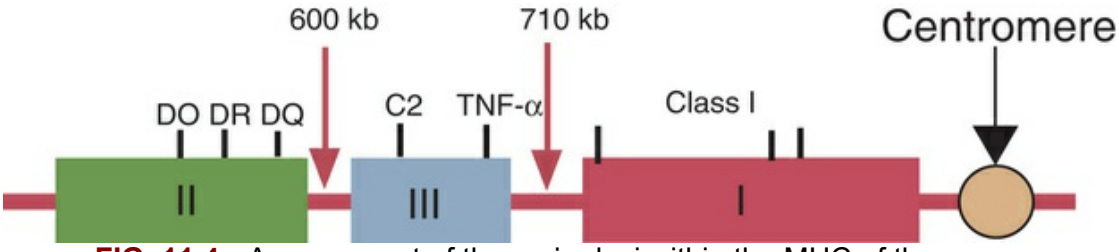


FIG. 11.4 Arrangement of the major loci within the MHC of the horse—a typical mammalian MHC.

Polymorphism

In mice and humans, the class Ia genes show extreme polymorphism. This is a result of variations in the amino acid sequences in their α_1 and α_2 domains. The most extreme polymorphism is restricted to three to four small regions within the α_1 and α_2 domains. In these variable regions, two or three alternative amino acids can occur at each position. The other domains of MHC class Ia molecules show little variation.

The α_1 and α_2 domains of MHC class I molecules fold together to form an open-ended groove. A flat β sheet forms the floor of this groove, and its walls are formed by two α helices (Fig. 11.5). This groove can bind antigenic peptides that are 8 to 10 amino acids long. The variable regions located along the walls of this groove determine its shape. The shape of the groove in turn determines which peptides can be bound and thus trigger immune responses.

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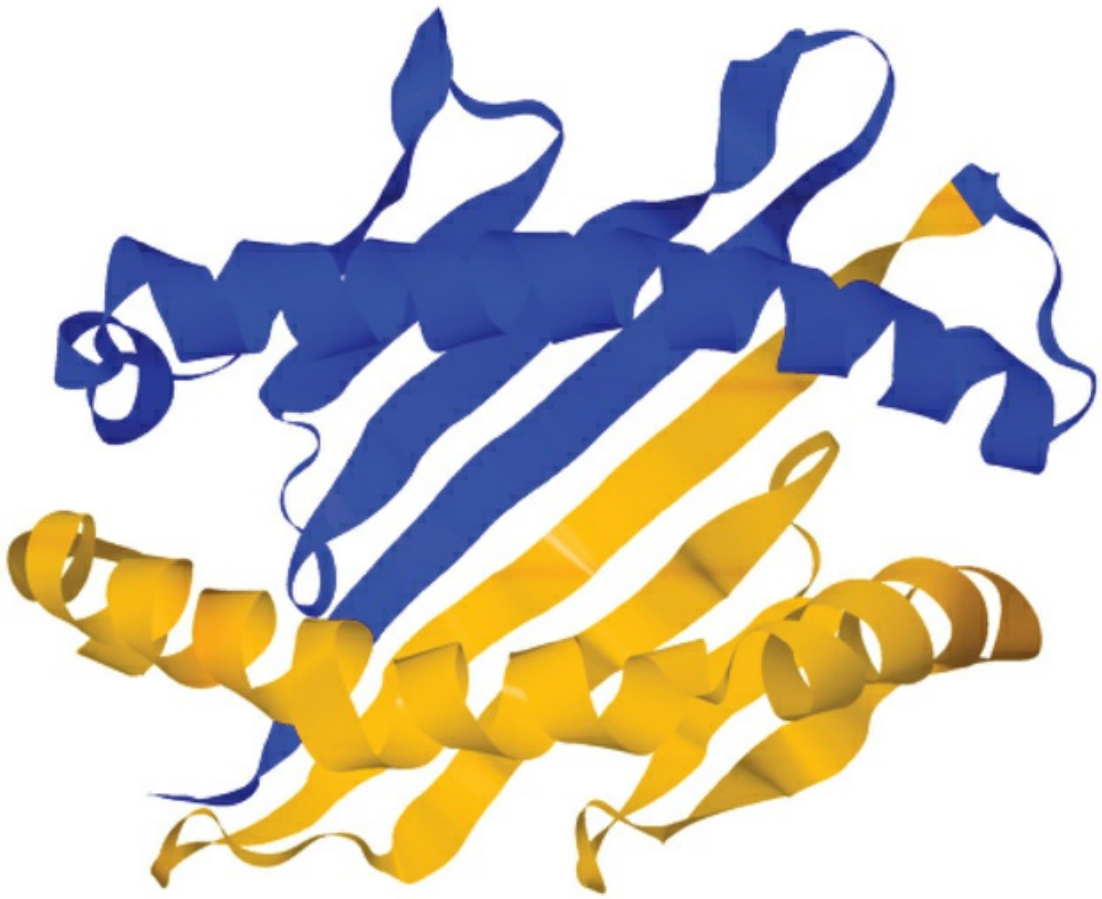


FIG. 11.5 A ribbon diagram showing a view (from above) of the antigen-binding groove on an MHC class I molecule. The floor of the groove is formed by an extensive β-pleated sheet. The walls of the groove are formed by two parallel α helices. This structure is formed by the folding of the α_1 *(blue)* and α_2 *(yellow)* domains of the α chain. (Courtesy Dr. B. Breaux.)

Polymorphism in the α_1 and α_2 domains results from variations in the nucleotide sequences between MHC alleles. These sequence variations result from point mutations, reciprocal recombination, and gene conversion. Point mutations are simply changes in individual nucleotides. Reciprocal recombination involves crossing over between two chromosomes. In gene conversion, small blocks of DNA are exchanged between different class I genes in a nonreciprocal fashion. The donated DNA blocks may come from nearby nonpolymorphic class I genes, from nonfunctional pseudogenes, or from other polymorphic class I genes. Class I MHC genes have the highest mutation rate of any germline genes yet studied (10^{-3} mutations per gene per generation in mice). This high mutation rate implies that there are significant advantages to be gained by having very polymorphic MHC genes.

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Mammals use two distinct strategies for maintaining high levels of MHC class I diversity. Mice and humans simply use a small number of highly polymorphic genes. In other primates, herbivores, and rats, however, MHC diversity is generated by varying the number and combinations of their class I gene loci.

Nonpolymorphic MHC Class I Molecules

Mammalian cells also express many nonpolymorphic class I molecules. Some are encoded by genes within the MHC class I region, others by genes on other chromosomes. They are classified according to their evolutionary origin.

Class Ib molecules show reduced expression and tissue distribution compared with class Ia molecules but are part of the MHC complex. They have limited polymorphism and their genes probably originated from class Ia precursors by duplication. For example, the class Ib genes in mice are found in three loci called *Q*, *T*, and *M*. They code for proteins on the surface of regulatory and immature lymphocytes and on hematopoietic cells. These also consist of a membrane-bound α chain associated with β_2-microglobulin, so their overall shape and antigen-binding groove are similar to those in MHC class Ia molecules. Since they are not polymorphic, however, MHC class Ib molecules bind a limited range of ligands. They act as pattern recognition receptors for commonly encountered, microbial PAMPs.

Class Ic genes have limited polymorphism and are found within the MHC. Their products include MIC-A and MIC-B, specialized proteins that are involved in signaling to natural killer (NK) cells, but do not bind antigenic peptides (Chapter 19).

Class Id genes are nonpolymorphic class I-related genes not located on the MHC chromosome. Many of their products contribute to innate immunity since they bind PAMPs. For example, CD1 molecules are antigen-presenting receptors that bind lipid antigens (Chapter 19). FcRn is a class Id MHC molecule that acts as an antibody (Fc) receptor on epithelial cells. It is expressed on mammary gland epithelium and on the enterocytes of newborns (Chapter 23).

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MHC Class II Molecules

Mammals also differ in their expression of MHC class II molecules. In rodents, these are restricted to the professional antigen-presenting cells (dendritic cells, macrophages, and B cells) but can be induced on T cells, keratinocytes, and vascular endothelial cells. In pigs, dogs, cats, mink, and horses, MHC class II molecules are constitutively expressed on nearly all resting adult T cells. In cattle, MHC class II molecules are expressed only on B cells and activated T cells. In pigs, resting T cells express MHC class II molecules at about the same level as macrophages. In humans and pigs, MHC class II molecules are expressed on renal vascular endothelium and glomeruli—a fact of significance in kidney graft rejection. The expression of class II molecules is enhanced in rapidly dividing cells and in cells treated with interferon-γ (IFN-γ) (Chapter 34).

Structure

MHC class II molecules consist of two chains called α and β. Each chain has two extracellular domains (one constant and one variable), a connecting peptide, a transmembrane domain, and a cytoplasmic domain (Fig. 11.6). A third invariant chain, called the Ii or γ chain, is associated with the assembly of class II molecules within cells and was discussed in Chapter 10.

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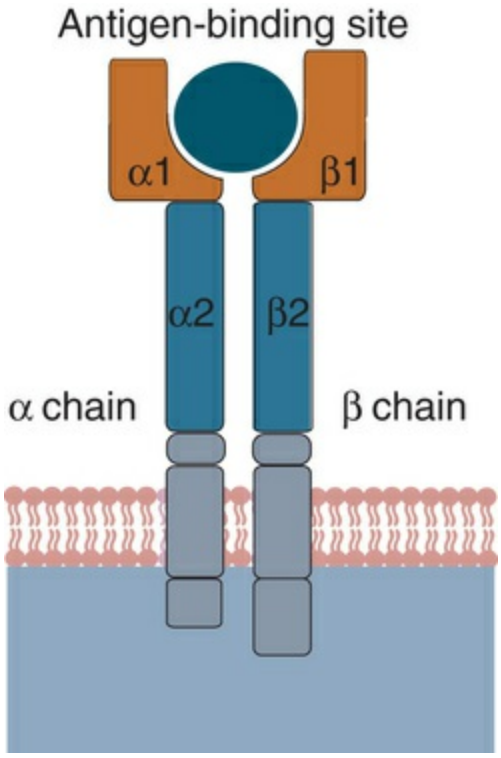


FIG. 11.6 Diagram showing the structure of a MHC class II antigen located on a cell surface. Note that the antigen-binding site is formed by the variable domains from both peptide chains.

Gene Arrangement

A "complete" MHC class II region contains three paired loci. In primates, these are DPA and DPB, DQA and DQB, and DRA and DRB. (The genes for the α chains are designated A, and the genes for the β chains are called B.) Some of these genes are polymorphic. There may also be additional non-polymorphic loci present such as DM and DO in humans. The DM and DO gene products regulate the loading of antigen fragments into the MHC groove. Not all mammals possess a complete set of class II genes since nonprimates lack DPA and DPB. Not all loci contain genes for both chains, and some contain many pseudogenes. These pseudogenes serve as DNA donors that can be used to generate class II polymorphism by gene conversion.

Polymorphism

MHC class II proteins have an antigen-binding groove formed by

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their α_1 and β_1 domains. Its walls are formed by two parallel α-helices, and its floor consists of a β sheet. Polymorphism results in variations in the amino acids forming the sides of the groove. These variations are generated in the same way as in class Ia molecules. Other genes within the class II region code for molecules involved in antigen-processing. These include the transporter proteins TAP1 and TAP2 and some proteasome components.

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MHC Class III Molecules

The remaining genes within the MHC are located within the class III region (Fig. 11.7). They code for proteins with many diverse functions. Some are important in the defense of the body such as the genes for the complement components C4, FB, and C2 (Chapter 4). They also include genes that encode tumor necrosis factor-α (TNF-α), several lymphotoxins, and some NK cell receptors.

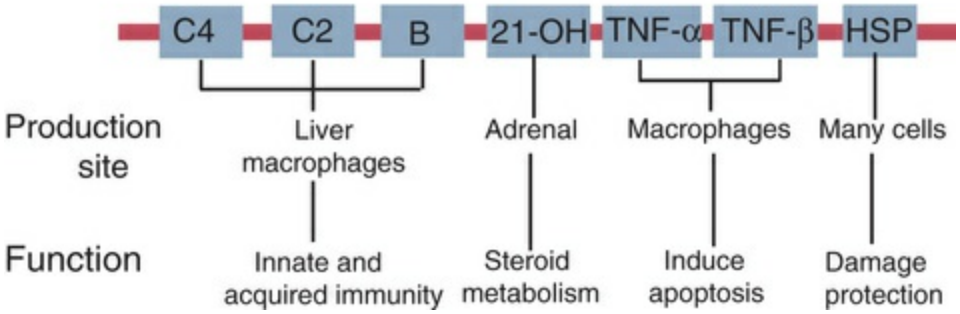


FIG. 11.7 The arrangement of selected genes within the MHC class III region. These genes all play a role in innate and adaptive immunity. There are many other genes within this region that have no apparent role in immunity.

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MHC of Domestic Animals

Every mammalian MHC contains class I, class II, and class III regions. When the MHCs of different mammals are compared, some regions such as class III are conserved, whereas others are highly diverse. Likewise, the precise arrangement and number of loci varies among species (Fig. 11.8). In general, genes within the class II and class III regions possess obvious orthologs in all species. That is, they are clearly derived from a single ancestor and have not usually been subjected to major rearrangements during evolution (ruminant class II genes are an exception). Class I genes, in contrast, have been reorganized so many different times by deletion and duplication that their amino acid sequences differ widely, and it is very difficult to compare class I genes in different species. They are said to be paralogous.

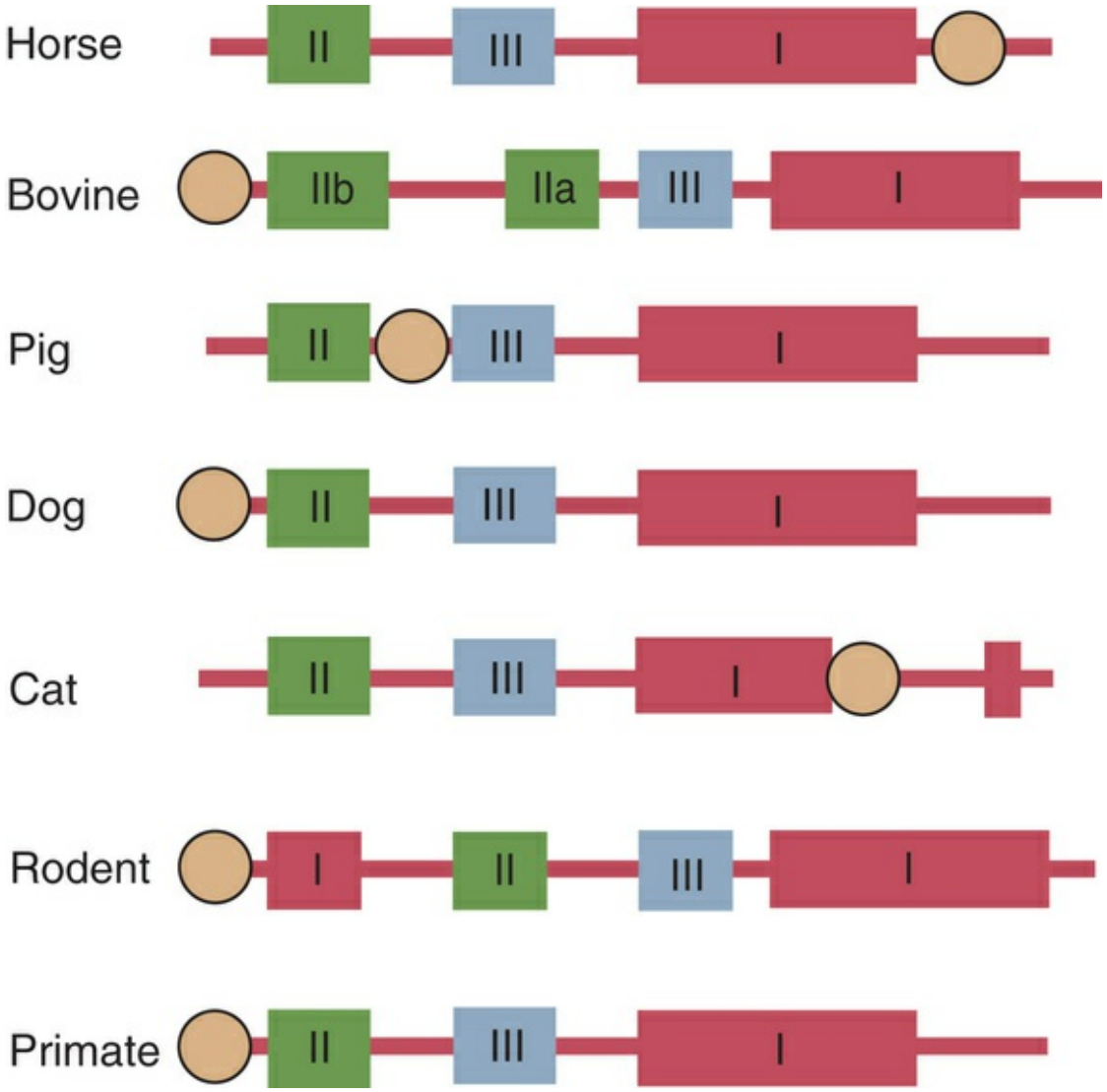


FIG. 11.8 The arrangement of gene regions within the MHC in different domestic mammal species. The *circle* denotes the location of the centromere.

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MHC Molecules and Disease

Since the function of MHC molecules is to present antigens to the cells of the immune system, MHC genes regulate immune responses. A foreign molecule that cannot be bound to at least one MHC molecule will not trigger an adaptive immune response (Fig. 11.9). Thus, expression of specific MHC alleles determines resistance to infectious and autoimmune diseases. Because class Ia and class II MHC molecules are structurally diverse, each MHC allele can bind and present a different set of antigenic peptides. The more diversity within an animal's MHC, the more antigens it can respond to. Thus an MHC heterozygous animal will express many more alleles and respond to a greater diversity of antigens than can a homozygous animal (Fig. 11.10).

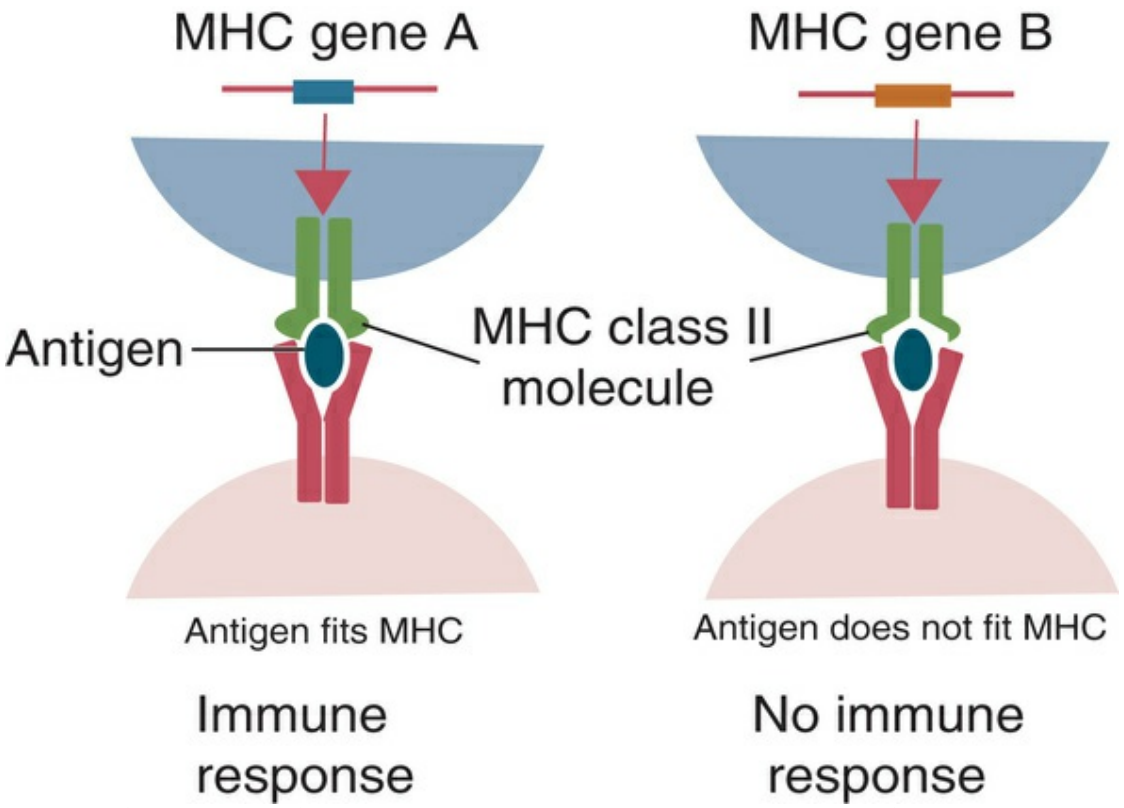


FIG. 11.9 MHC molecules regulate the immune response. Only antigen fragments that can bind in the groove of a MHC molecule will trigger an immune response. This is called MHC restriction. Thus the genes that code for these MHC molecules will also regulate immune responsiveness.

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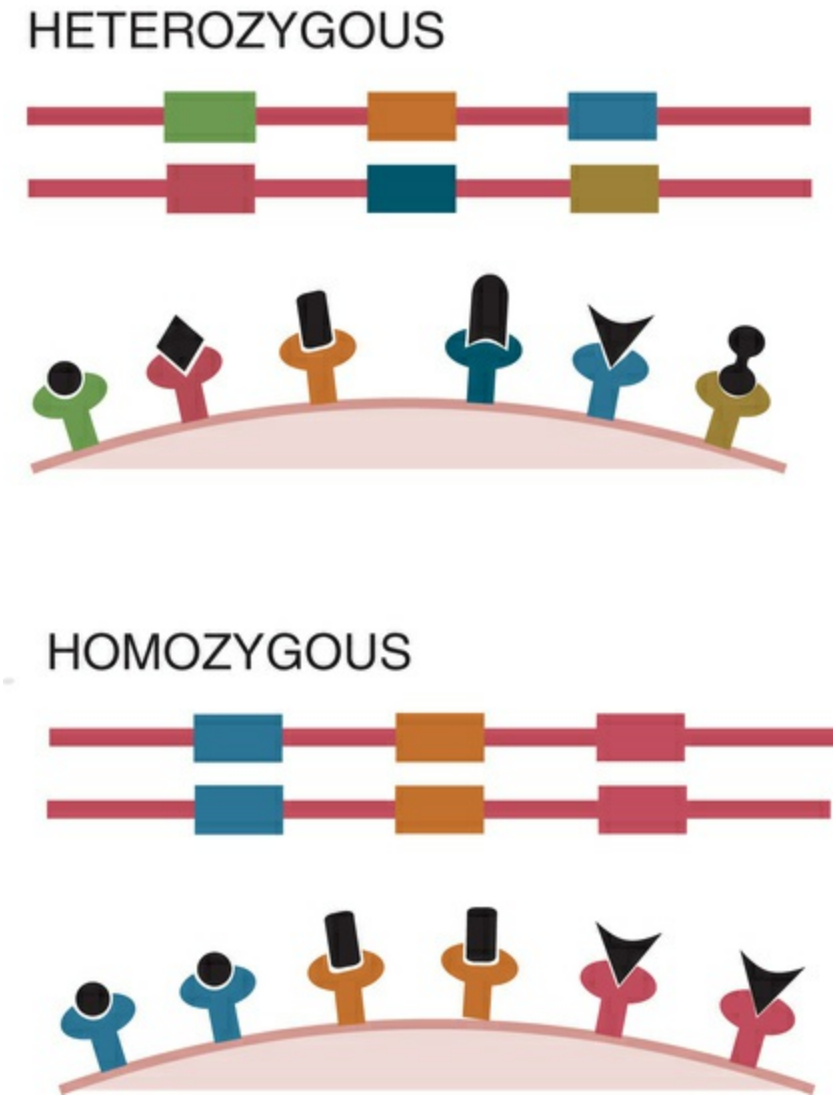


FIG. 11.10 Heterozygous animals with two MHC alleles at each locus express six different antigen-presenting molecules on the cell surface. Therefore, they generate a more diverse and effective immune response than homozygous animals with only a single allele, at each locus. An example of heterozygote advantage.

MHC polymorphism is maintained in populations by a process called overdominant selection or heterozygote advantage. Simply put, MHC heterozygotes are at an advantage because they can respond to a greater range of microbial antigens and so are best fitted to survive infectious diseases. The antigen-binding sites of MHC class Ia or II molecules are also very nonspecific, and it has been estimated that an average MHC molecule can bind about 2500 different peptides. This is because the MHC groove binds to the

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peptide backbone rather than to its amino acid side chains. Nevertheless, structural constraints limit the efficiency of binding of each allele. As a result, it is likely that only one or two peptides from an average antigenic protein can bind to any given MHC molecule. The ability of MHC molecules to bind antigens must be a limiting factor in generating adaptive immunity and resistance to infectious agents. Increasing the diversity of MHC molecules increases the diversity of antigens that can be bound and so increases resistance to infectious diseases. Because most individuals are MHC heterozygotes, each individual normally expresses at most six different class Ia molecules (in humans, for example, two each are coded for by the HLA-A, -B, and C loci). The number of expressed MHC molecules is not greater because that would increase the risk that the MHC molecules could bind and present more self-antigens. This would require the elimination of many more self-reactive T cells during development (Chapter 20). Thus the presence of six different MHC class Ia molecules appears to be a reasonable compromise between maximizing the recognition of foreign antigens while at the same time minimizing the chances of recognizing self-antigens, at least in humans (Fig. 11.11).

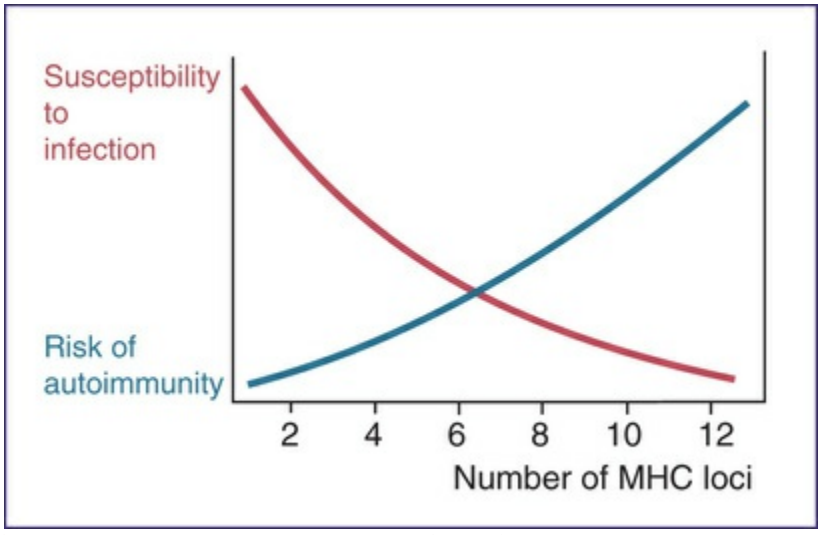


FIG. 11.11 The optimal number of MHC loci is a balance between the need to respond to as many different microbial antigens as possible and the need to avoid autoimmune responses. Computer modeling suggests that the optimal number of MHC loci in humans is six.

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MHC class Ia loci encode very polymorphic genes. For example, the H-2K locus in the mouse codes for more than 100 alleles. Since there can never be more than two alleles per locus in any individual animal, it appears that this number of alleles has evolved to maximize polymorphism. This may protect the population as a whole from complete destruction. Because of MHC polymorphism, most individuals in a population carry a unique set of class Ia alleles, and each individual can therefore respond to a unique mixture of antigens. When a new infectious disease strikes such a population, it is likely that at least some individuals will possess MHC molecules that can bind the new antigens and trigger immunity. Those that can respond will mount an immune response and live. Those that lack these molecules cannot respond and will die.

When large populations of humans or mice are examined, no single MHC haplotype predominates. In other words, no single MHC haplotype confers major survival advantages on individual animals. This reflects the futility of the host attempting to bind all the antigens in a population of invading microorganisms. Microbes will always be able to mutate and evade the immune response faster than we mammals can develop resistance. Any changes in an MHC allele may increase resistance to one organism but at the same time decrease resistance to another. It is more advantageous therefore for the members of a population to possess many highly diverse MHC alleles so that any pathogen spreading through a population will have to adapt anew to each individual.

Highly adaptable social animals, such as humans or mice, with large populations through which disease can spread rapidly, usually show extensive MHC polymorphism (Fig. 11.12). In contrast, low-density solitary species such as the marine mammals (whales and elephant seals), moose, or Tasmanian devils have much less polymorphism. It is also of interest to note the case of the cheetah, where some wild populations have reduced MHC class II polymorphism as a result of recent population bottlenecks. Because of this low MHC diversity, some cheetahs will accept allografts from other, unrelated cheetahs. Likewise an infectious disease such as feline infectious peritonitis causes 60% mortality in captive cheetahs compared with 1% to 2% mortality in domestic cats.

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However, there is little evidence to suggest that wild cheetah populations have reduced immune competence.

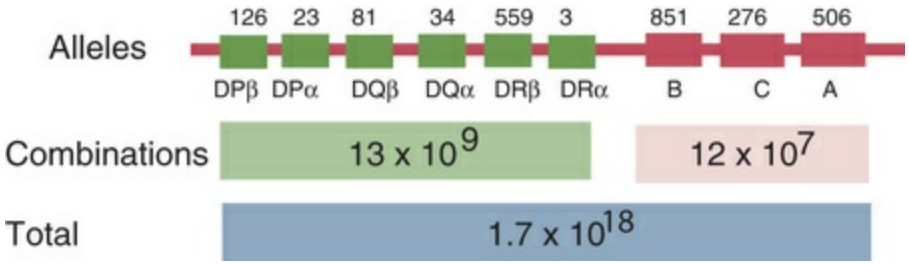


FIG. 11.12 An example of how MHC polymorphism can generate an enormous number of different MHC haplotypes. The numbers above each locus are the number of identified alleles in the human MHC. The number of different combinations can be determined by multiplying all of them together. Thus there are 13×10^9 class II combinations, 12×10^7 class I combinations, and 1.7×10^{18} total possible combinations; more than sufficient to give every human a unique haplotype.

There are many examples of links between MHC haplotype and resistance to infectious disease. For example, in cattle there is an association between possession of certain BoLA alleles and resistance to bovine leukosis, squamous cell eye carcinoma, and trypanosomiasis; responsiveness to foot-and-mouth disease virus; and susceptibility to the tick *Boophilus microplus*. In sheep, there is an association of the class I allele SY1 with resistance to *Trichostrongylus colubriformis*. Resistance to scrapie and caseous lymphadenitis is also associated with certain MHC class I alleles. In goats, the class I allele Be7 is associated with resistance, and Be1 and Be14 are associated with susceptibility, to caprine arthritis-encephalitis. In horses, an allergic response to the bites of *Culicoides* midges is linked to ELA-Aw7. In pigs, the SLA complex has an influence on major reproduction parameters such as ovulation rate, litter size, and piglet viability.

Selection for specific MHC haplotypes has potential for use in developing disease-resistant strains of domestic animals. However, it must be pointed out that by selecting for a specific gene locus, one may also inadvertently select for susceptibility at closely linked loci. This may outweigh the benefits of a resistant allele at one locus. An animal cannot be resistant to all possible infectious diseases.

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MHC and Body Odors

Mammals use odors to detect information about another individual's gender, status, and individuality. The molecules that carry this information are small volatile peptides found in urine. These peptides can bind to the antigen-binding grooves of MHC class I molecules. Thus, peptides known to bind to two mouse MHC class I molecules of different haplotypes were shown to induce responses (field potentials) in mouse vomeronasal organs. The responses were not haplotype specific, but different peptides induced different activation patterns. This finding may well explain how mammals such as mice can recognize the MHC of other mice by smell.

The class I region of mice, cattle, and pigs contains numerous genes coding for pheromone olfactory receptors. As a result, the MHC haplotype affects the recognition of peptide ligands causing individual odors in an allele-specific fashion and thus influences the mating preferences of mammals. Thus under controlled conditions, mice prefer to mate with MHC-incompatible individuals. Such matings preferentially generate heterozygote advantage, resulting in optimized disease resistance (Box 11.1). However, this type of mating could also prevent genome-wide inbreeding. Inbreeding avoidance may be the most important function of MHC-based mating preferences and therefore the fundamental selective force diversifying MHC genes in species with such mating patterns. Despite not having a vomeronasal organ, humans also have the ability to sense MHC peptides in body odor, and this may influence human mate choice.

Box 11.1

MHC and Sperm Counts in Horses

The MHC haplotype is closely linked to body odors. Mate choice appears to be determined in some species by odor. For example, 12 stallions were exposed to a MHC-similar mare and then to a MHC-dissimilar mare or vice versa for four weeks. Blood testosterone levels were determined weekly. Ejaculates were collected at the

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end of the experiment. Testosterone levels were higher in the stallions exposed to the MHC-dissimilar mare than to the MHC-similar mare. Sperm numbers were correlated to mean testosterone levels and were higher if MHC-dissimilar mares were presented last than if MHC-similar mares were presented last. Thus MHC-linked olfactory signals influence testosterone levels and sperm counts.

Burger D, Dolivo G, Marti E, Sieme H, Wedekind C: Female major histocompatibility complex type affects male testosterone levels and sperm number in the horse (*Equus caballus*), *Proc Biol Sci* 282:20150407, 2015.

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12

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Organs of the Immune System

CHAPTER OUTLINE

Sources of Lymphocytes, 109
Primary Lymphoid Organs, 109
Thymus, 109
Structure, 109
Function, 110
Thymic Hormones, 111
Bursa of Fabricius, 111
Structure, 111
Function, 111
Peyer's Patches, 112
Structure, 112
Function, 113
Lymphoglandular Complexes, 113
Bone Marrow, 113
Secondary Lymphoid Organs, 113
Lymph Nodes, 113
Structure, 113
Function, 115

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Lymphocyte Circulation, 117
Species Differences, 117
Hemolymph Nodes, 118
Spleen, 118
Structure of White Pulp, 118
Function, 119
Other Secondary Lymphoid Organs, 120
Tertiary Lymphoid Organs, 121

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Understand that adaptive immunity is mediated by cells called lymphocytes found mainly within lymphoid organs.
- Recognize that lymphocytes arise from lymphoid stem cells in the bone marrow.
- Understand the different roles of primary and secondary lymphoid organs.
- Understand and explain the functions of the primary lymphoid organs.
- Describe the structure and development of the thymus and understand that T cells mature within the thymus.
- Describe the structure and function of the bursa of Fabricius.
- Explain the different effects of neonatal and adult thymectomy.
- Describe how B cells mature within gastrointestinal lymphoid tissues, the bone marrow, or the bursa of Fabricius.
- Understand why, if newly developed lymphocytes have receptors that can bind self-antigens, they are killed before they can leave primary lymphoid organs.
- Understand the functions of the secondary lymphoid organs, the lymph nodes, and spleen.
- Describe the structure of lymph nodes and the spleen.
- Describe the circulation of lymphocytes.
- Understand the different functions of ileocecal and jejunal Peyer's patches.

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• Define red pulp, white pulp, germinal center, high endothelial venule, Peyer's patch, Hassall's corpuscles, and tertiary lymphoid organ.

Although antigens are trapped and processed by dendritic cells, macrophages, and B cells, adaptive immune responses are actually mounted by cells called lymphocytes. Lymphocytes are the small unspectacular round cells that predominate in organs such as the spleen, lymph nodes, and thymus (Fig. 12.1). These are called lymphoid organs. Lymphocytes have antigen receptors on their surface and can recognize and respond to foreign antigens. Lymphocytes are eventually responsible for the production of antibodies and for cell-mediated immune responses. The lymphoid organs must therefore provide an environment for efficient interaction among lymphocytes, antigen-presenting cells, and foreign antigens, as well as sites where lymphocytes can respond optimally to processed antigens.

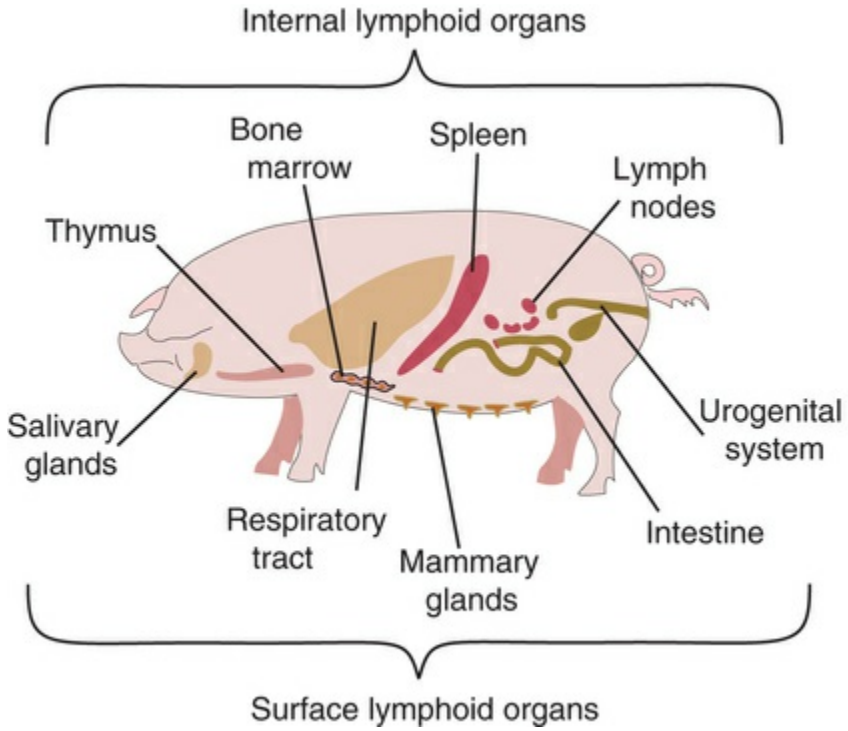


FIG. 12.1 The major lymphoid tissues of the pig, a typical mammal.

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Immune responses must be carefully controlled. Lymphocytes must be selected so that their receptors will only bind foreign antigens, and the response of each lymphocyte must be regulated so that it is sufficient but not excessive for the body's requirements. The lymphoid organs may therefore be classified on the basis of their roles in generating lymphocytes, in regulating the production of lymphocytes, and in providing an environment for trapping foreign antigens, processing them, and maximizing the opportunity for lymphocytes to encounter and interact with these foreign antigens (Fig. 12.2).

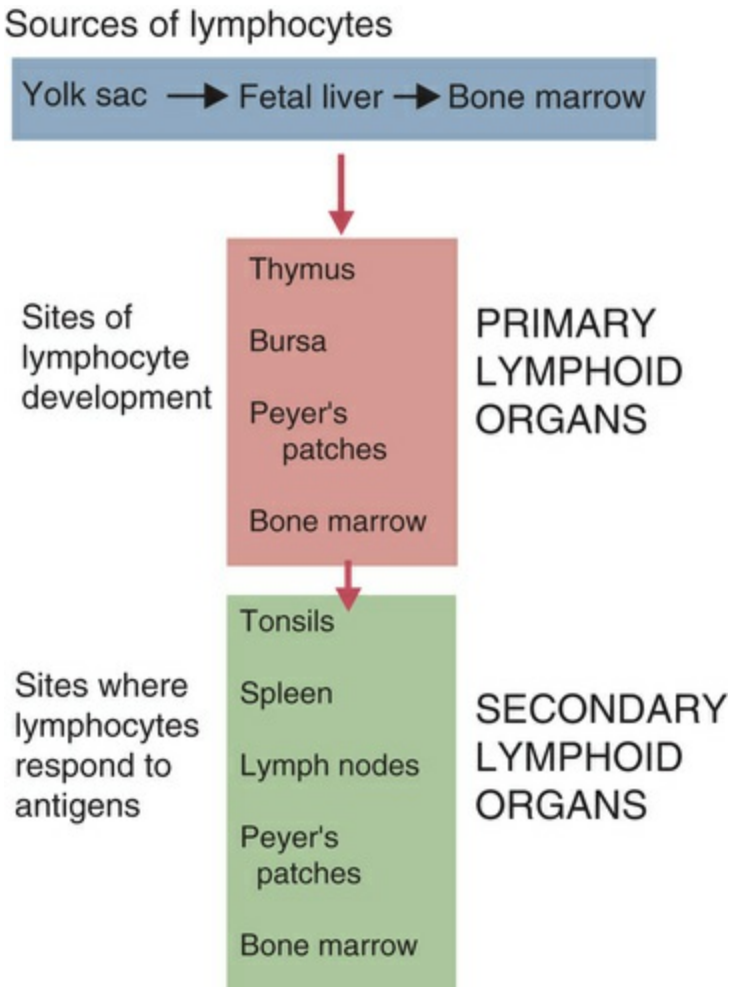


FIG. 12.2 The lymphoid organs can conveniently be divided into three groups based on their role in the development and functioning of lymphocyte populations.

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Sources of Lymphocytes

Lymphoid stem cells are first found in the fetal omentum, liver, and yolk sac. In older fetuses and in adults, these stem cells are mainly found in the bone marrow. The bone marrow has multiple functions in adult mammals. It is a hematopoietic organ containing the precursors of all blood cells, including lymphocytes. In some mammals, such as primates, it is also a primary lymphoid organ (a site where newly produced lymphocytes can mature). Like the spleen, liver, and lymph nodes, the bone marrow is also a secondary lymphoid organ. It contains many dendritic cells and macrophages and thus removes foreign material from the blood. It contains large numbers of antibody-producing cells and is therefore a major source of antibodies. Because of these multiple functions, the bone marrow is divided into a hematopoietic compartment and a vascular compartment. These compartments alternate, like slices of cake, in wedge-shaped areas within long bones. The hematopoietic compartment contains stem cells for all the blood cells as well as macrophages, dendritic cells, and lymphocytes, and is enclosed by a layer of adventitial cells. In older animals, these adventitial cells may become so loaded with fat that the marrow may have a fatty yellow appearance. The vascular compartment, where antigens are mainly trapped, consists of blood sinuses lined by endothelial cells and crossed by a network of reticular cells and macrophages.

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Primary Lymphoid Organs

The organs that regulate the development of lymphocytes are called primary lymphoid organs. Lymphocytes fall into two major populations called T cells and B cells, based on the primary organ in which they mature. Thus all T cells mature in the thymus. B cells, in contrast, mature within different organs depending on species. These include the bursa of Fabricius in birds, the bone marrow in primates and rodents, and the intestinal lymphoid tissues in rabbits and ruminants. These primary lymphoid organs all develop early in fetal life. As animals develop, newly produced, immature lymphocytes migrate from the bone marrow to the primary lymphoid organs, where they mature (Table 12.1). The primary lymphoid organs are not sites where lymphocytes encounter foreign antigens, and they do not enlarge in response to antigenic stimulation.

TABLE 12.1

Comparison of Primary and Secondary Lymphoid Organs

	Primary	Secondary
Origin	Ectoendodermal junction or endoderm	Mesoderm
Time of development	Early in embryonic life	Late in fetal life
Persistence	Involutes after puberty	Persists in adults
Effect of removal	Loss of lymphocytes	No or minor effects
Response to antigen	Unresponsive	Fully reactive
Examples	Thymus, bursa, some Peyer's patches	Spleen, lymph nodes

Thymus

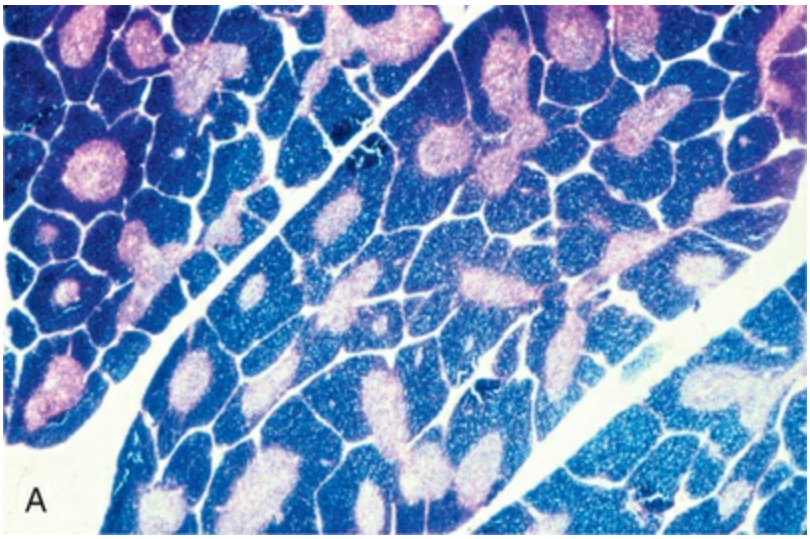
The thymus is located in the thoracic cavity in front of and below the heart. In horses, cattle, sheep, pigs, and chickens, it also extends up the neck as far as the thyroid gland. The size of the thymus varies, its relative size being greatest in the newborn animal and its absolute size being greatest before puberty. It may be very small and difficult to find in adult animals.

Structure

The thymus consists of lobules of loosely packed epithelial cells,

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each covered by a connective tissue capsule. The outer part of each lobule, the cortex, is densely infiltrated with lymphocytes (or thymocytes), but the inner medulla contains fewer lymphocytes, and the epithelial cells are clearly visible (Fig. 12.3). Within the medulla are also found round, layered bodies called thymic or Hassall's corpuscles. These contain keratin, and the remains of a small blood vessel may be found at their center. In cattle, these corpuscles may contain immunoglobulin A (Chapter 16). An abnormally thick basement membrane and a continuous layer of epithelial cells surround the capillaries that supply the thymic cortex. This barrier prevents circulating foreign antigens from entering the cortex. No lymphatic vessels leave the thymus. As an animal ages, the thymus shrinks and is gradually replaced by fat. However, the aged thymus still contains small amounts of lymphoid tissue and remains functionally active.



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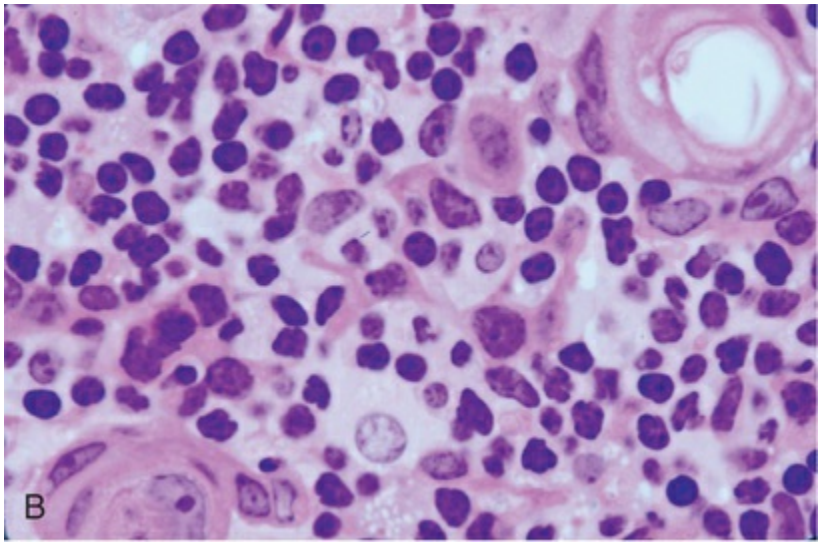


FIG. 12.3 **A,** A section of a monkey thymus. Each lobule is divided into a cortex rich in lymphocytes, hence staining darkly, and a paler medulla consisting mainly of epithelial cells. Original magnification ×10. **B,** A high-power view of the medulla of a monkey thymus showing several pale-staining epithelial cells with cytoplasmic processes and many dark-staining, round lymphocytes. Original magnification ×1000.

Function

The functions of the thymus are best demonstrated by studying the effects of its removal in rodents of different ages. For example, mice thymectomized within a day of birth become susceptible to infections and may fail to grow. These animals have very few circulating lymphocytes and cannot reject foreign organ grafts because they lose the ability to mount cell-mediated immune responses (Table 12.2). In contrast, adult thymectomy has no immediate obvious effect. But if these mice are monitored for several months, their blood lymphocytes and their ability to mount cell-mediated immune responses gradually decline. This suggests that the thymus remains functional in adults, but there is a reservoir of long-lived thymus-derived cells that must be exhausted before the effects of adult thymectomy become apparent.

TABLE 12.2

Effects of Neonatal Thymectomy and Bursectomy

Function	Thymectomy	Bursectomy
Numbers of circulating lymphocytes	Disappear	No effect
Presence of lymphocytes in T-dependent areas	Disappear	No effect

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Graft rejection	Suppressed	No effect
Presence of lymphocytes in T-independent areas	Minor depletion	Disappear
Plasma cells in lymphoid tissues	Minor drop	Disappear
Serum immunoglobulins	Minor drop	Major drop
Antibody formation	Minor effects	Major drop

The results of thymectomy indicate that the neonatal thymus is the source of most blood lymphocytes and that these lymphocytes are mainly responsible for mounting cell-mediated immune responses. They are called thymus-derived lymphocytes or T cells. T-cell precursors originate in the bone marrow but then enter the thymus. Once within the thymus, the cells (called thymocytes) divide rapidly. Of the new cells produced, most die by apoptosis, whereas the survivors (about 5% of the total in rodents and about 25% in calves) remain in the thymus for 4 to 5 days before leaving and colonizing the secondary lymphoid organs.

T cells that enter the thymus have two conflicting tasks. They must recognize foreign antigens, but, at the same time, must not respond strongly to normal body constituents (self-antigens). A two-stage selection process in the thymic medulla accomplishes this feat. Thus thymocytes with receptors that bind self-antigens strongly and that could therefore cause autoimmunity are killed by apoptosis. Thymocytes with receptors that cannot bind any major histocompatibility complex (MHC) class II molecules and thus cannot react to any processed antigen are also killed.

On the other hand, those thymocytes that survive this "negative selection" process but can still recognize specific MHC class II-antigen complexes with moderate affinity are stimulated to grow—a process called positive selection. These surviving cells eventually leave the thymus as mature T cells, circulate in the bloodstream, and colonize the secondary lymphoid organs.

Thymic epithelial cells are unusual since they express more than 400 antigens normally expressed in other tissues. In addition, these cells have a very high level of autophagy. As a result, their intracellular antigens are bound to MHC class II molecules and expressed in large amounts on the epithelial cell surfaces. This "promiscuous" antigen presentation ensures that developing thymocytes are presented with an unusually diverse array of normal tissue antigens. Since T cells with receptors that bind and respond to these antigens will die, the system ensures that those T cells leaving the thymus lack receptors for most self-antigens and,

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as a result, cannot respond to normal body components.

Thymic Hormones

Within the thymus, cells are regulated by a complex mixture of cytokines and small peptides collectively known as thymic hormones. These include peptides variously called thymosins, thymopoietins, thymic humoral factor, thymulin, and the thymostimulins. Thymulin is especially interesting because it is a zinc-containing peptide secreted by the thymic epithelial cells, and it can partially restore T cell function in thymectomized animals. Zinc is an essential mineral for the development of T cells. Consequently, zinc-deficient animals have defective cell-mediated immune responses (Chapter 40). Hassall's corpuscles play a functional role in regulating thymic activity since they produce a growth factor called thymic stromal lymphopoietin (TSLP, Chapter 21). TSLP activates thymic dendritic cells that can stimulate regulatory T cells and so controls the positive selection process.

Bursa of Fabricius

The bursa of Fabricius is found only in birds. It is a round sac located just above the cloaca (Fig. 12.4). Like the thymus, the bursa reaches its greatest size in the chick about 1 to 2 weeks after hatching and then shrinks as the bird ages. It is very difficult to identify in older birds.

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FIG. 12.4 The bursa of Fabricius obtained from a 1-week-old chicken. It has been cut open to reveal the folds inside.

Structure

Like the thymus, the bursa consists of lymphocytes embedded in epithelial tissue. This epithelial tissue lines a hollow sac connected to the cloaca by a duct. Inside the sac, folds of epithelium extend into the lumen, and scattered through the folds are round masses of lymphocytes called lymphoid follicles (Fig. 12.5). Each follicle is divided into a cortex and a medulla. The cortex contains lymphocytes, plasma cells, and macrophages. At the corticomedullary junction there is a basement membrane and capillary network on the inside of which are epithelial cells. These medullary epithelial cells are replaced by lymphoblasts and lymphocytes in the center of the follicle. Specialized neuroendocrine dendritic cells of unknown function surround each follicle.

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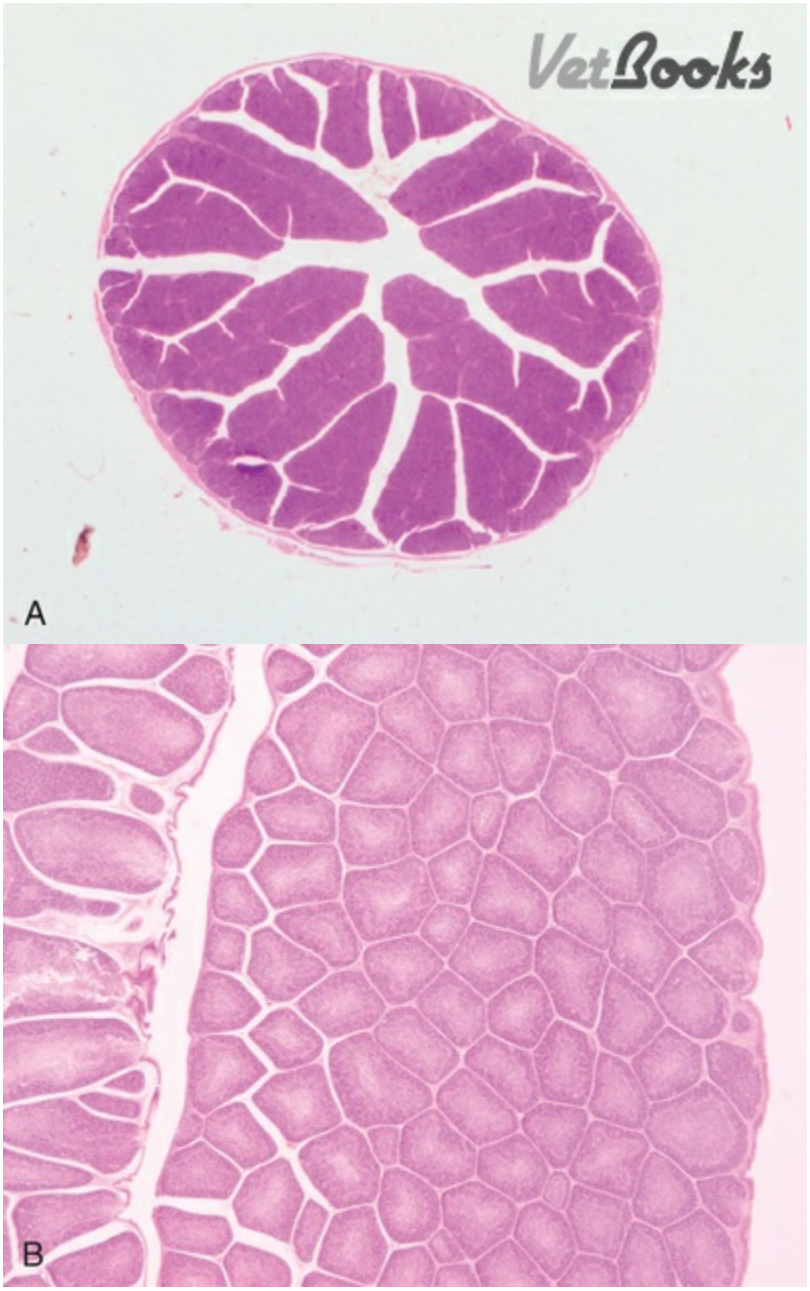


FIG. 12.5 Photomicrographs showing the structure of the bursa of Fabricius. **A,** Low-power micrograph showing the bursa of a 13-day-old chick. Original magnification ×5. **B,** A high-power view. Original magnification ×360. (From a specimen provided by Drs. N.H. McArthur and L.C. Abbott.)

Function

The bursa may be removed either surgically or by infecting newborn chicks with a virus that destroys the bursa (infectious bursal disease virus). Since the bursa shrinks when chicks become sexually mature, bursal atrophy can also be provoked by

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administration of testosterone. Bursectomized birds have very low levels of antibodies in their blood, and antibody-producing cells disappear from lymphoid organs. However, they still possess circulating T cells and can reject foreign skin grafts. Thus, bursectomy has little effect on the cell-mediated immune response. Bursectomized birds are more susceptible than normal to leptospirosis and salmonellosis but not to intracellular bacteria such as *Mycobacterium avium.*

Thus the bursa is a primary lymphoid organ that functions as a maturation and differentiation site for the cells of the antibody-forming system. Lymphocytes originating in the bursa are therefore called B cells. The bursa acts like the thymus insofar as immature cells produced in the bone marrow migrate to the bursa. These cells then proliferate rapidly, but 90% to 95% of these eventually die by apoptosis; the negative selection of self-reactive B cells. Once their maturation is completed, the surviving B cells emigrate to secondary lymphoid organs.

Close examination shows that the bursa is not a pure primary lymphoid organ because it can also trap antigens and undertake some antibody synthesis. It also contains a small focus of T cells just above the bursal duct opening. Several different hormones have been extracted from the bursa. The most important of these is a tripeptide (Lys-His-glycylamide) called bursin that activates B cells but not T cells.

Peyer's Patches

Structure

Peyer's patches (PPs) are lymphoid organs located in the walls of the small intestine. Their structure and functions vary among species. Thus in ruminants, pigs, horses, dogs, and humans (group I), 80% to 90% of the PPs are found in the ileum, where they form a single continuous structure that extends forward from the ileocecal junction. In young ruminants and pigs, the ileal PPs may be as long as 2 m. Ileal PPs consist of densely packed lymphoid follicles, each separated by a connective tissue sheath, and contain only B cells (Fig. 12.6).

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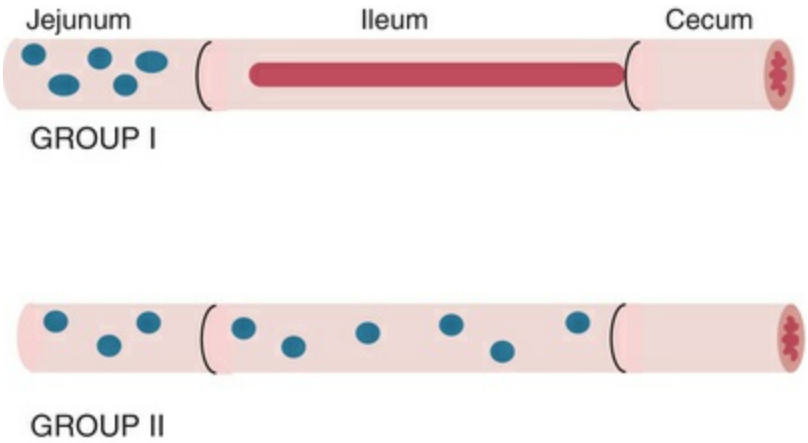


FIG. 12.6 Schematic diagram showing the differences between the arrangement of Peyer's patches in group I and group II mammals. The large ileal Peyer's patch in group I mammals *(red)* is a primary lymphoid organ that regresses at about a year of age. The small jejunal Peyer's patches *(blue)* are secondary lymphoid organs involved in the defense of the intestinal tract and control of the microbiota.

The ileal PPs reach maximal size and maturity before birth at a time when they are shielded from foreign antigens and collectively form the largest lymphoid tissue in 6-week-old lambs. (They constitute about 1% of total body weight, like the thymus.) They disappear by 15 months of age and cannot be detected in adult sheep.

The group I species also have a second type of PP that consists of multiple discrete accumulations of follicles in the jejunum. These jejunal PPs persist for the life of the animal. They consist of pear-shaped follicles separated by extensive interfollicular tissue and contain mainly B cells with up to 30% T cells.

The pig is also a group I species. Pigs have about 30 jejunal PPs that are of conventional structure and a single, large ileal PP. Their ileal PP lacks T cells and has a structure similar to that seen in sheep. It regresses within the first year of life, but it does not appear to be a primary lymphoid organ since it is not required for B cell development. It appears to be a secondary lymphoid organ that plays a role in the immune response to the intestinal microbiota. Dogs also belong to group I. They have two types of PPs, including a single ileal PP that involutes early and contains predominantly immature B cells.

In other mammals, such as primates, rabbits, and rodents (group II), PPs are located at random intervals in the ileum and jejunum. In

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these mammals, the PPs do not develop until 2 to 4 weeks after birth and persist into old age. The development of the PPs in some group II animals appears to depend entirely on stimulation by the normal intestinal microbiota, since they remain small and poorly developed in germ-free animals.

Function

The ileal PPs of some group I species such as sheep function in a manner similar to the avian bursa. Thus, ileal PPs are sites of rapid B cell proliferation, although most cells then undergo apoptosis, and the survivors are released into the circulation. If their ileal PPs are surgically removed, lambs become B cell deficient and fail to produce antibodies. The bone marrow of lambs contains many fewer lymphocytes than the bone marrow of laboratory rodents, and the ileal PPs are therefore their most significant source of B cells.

Lymphoglandular Complexes

Lymphoglandular complexes are present in the wall of the large intestine and cecum in horses, ruminants, dogs, and pigs. They consist of submucosal masses of lymphoid tissue penetrated by radially branching extensions of mucosal glands. These glands penetrate both the submucosa and the lymphoid nodule. They are lined by intestinal columnar epithelium containing goblet cells, intraepithelial lymphocytes, and M cells (Chapter 22). Their function is unknown, but they contain many plasma cells, suggesting that they are sites of antibody production.

Bone Marrow

The specialized ileal PP is the primary lymphoid organ for B cells only in group I mammals such as ruminants. In group II mammals the bone marrow probably serves this function. There is no exclusive B cell development site in the bone marrow, although it is suggested that precursor B cells develop at the outer edge of the marrow and migrate to the center as they mature and multiply. Negative selection occurs within the bone marrow so that, as in

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other primary lymphoid organs, most pre-B cells generated are destroyed.

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Secondary Lymphoid Organs

The cells of the immune system must be able to respond to a huge diversity of pathogens that an animal may encounter. It is especially important that antigen-specific lymphocytes can encounter their target antigens. To maximize the probability of such encounters, the body employs secondary lymphoid organs. In contrast to the primary lymphoid organs, the secondary lymphoid organs arise late in fetal life and persist in adults. Unlike primary lymphoid organs, they enlarge in response to antigenic stimulation. Surgical removal of one of them does not significantly reduce immune capability. Examples of secondary lymphoid organs include the spleen, the lymph nodes, the tonsils, and other lymphoid tissues in the intestinal, respiratory, and urogenital tracts. These organs contain dendritic cells that trap and process antigens and lymphocytes that mediate the immune responses. The overall anatomical structure of these organs therefore facilitates antigen trapping and provides the optimal environment for the initiation of immune responses. Secondary lymphoid organs are connected to both the blood and lymphoid systems, thus allowing them to continuously sample and concentrate circulating antigens.

Lymph Nodes

Structure

Lymph nodes are round or bean-shaped filters strategically placed on lymphatic vessels in such a way that they can sample antigens carried in the lymph (Fig. 12.7). Lymph nodes consist of a capsule beneath which is a reticular network filled with lymphocytes, macrophages, and dendritic cells, and through which lymphatic sinuses penetrate (Fig. 12.8). The lymph node thus acts as a filter for lymph fluid. A subcapsular sinus is located immediately under the connective tissue capsule. Other sinuses pass through the body of the node but are most prominent in the medulla. Afferent lymphatics enter the node around its circumference, and efferent lymphatics leave from a depression or hilus on one side. The blood vessels supplying a lymph node also enter and leave through the

hilus.

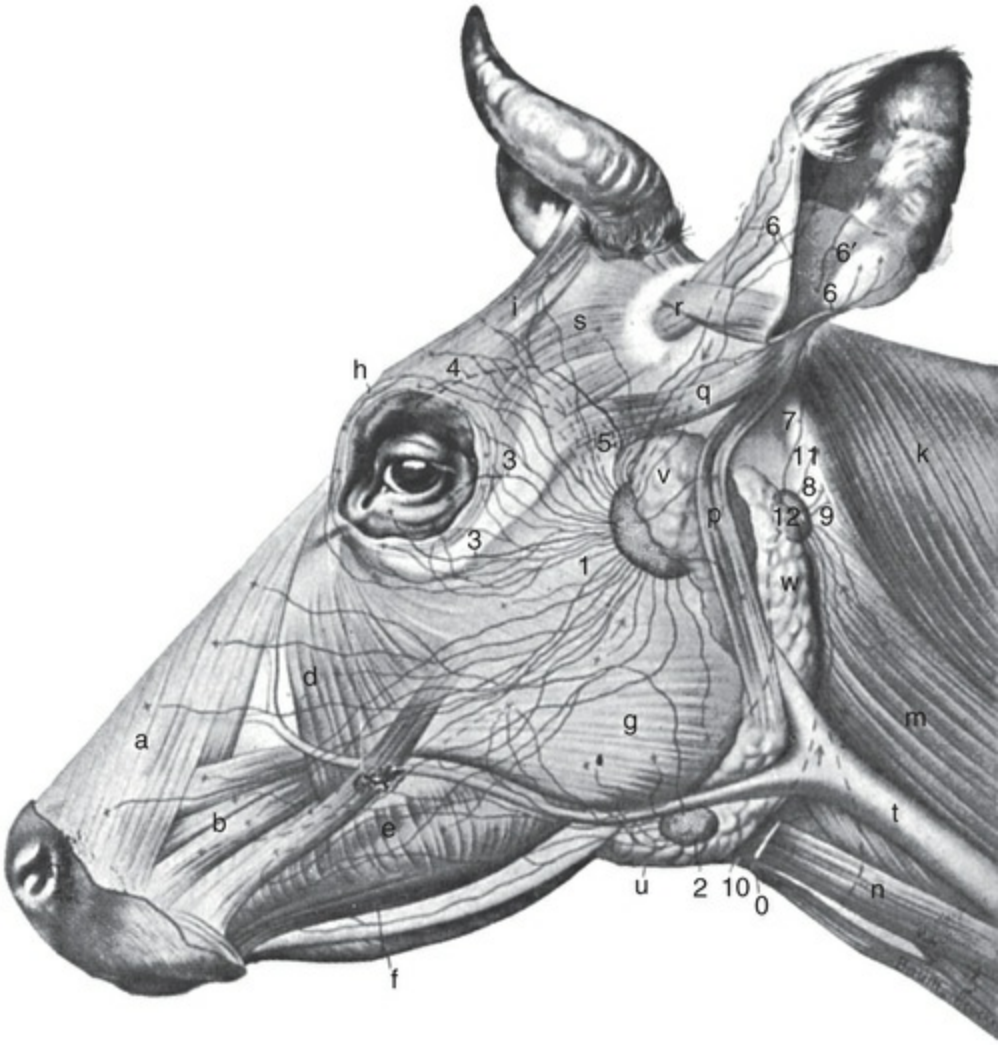


FIG. 12.7 Lateral view of the head of a bovine showing the way in which the lymphatics drain to the parotid lymph node. (From Sisson S [revised by Grossman JD]: *Anatomy of the domestic animals,* ed 4, Philadelphia, 1953, Saunders.)

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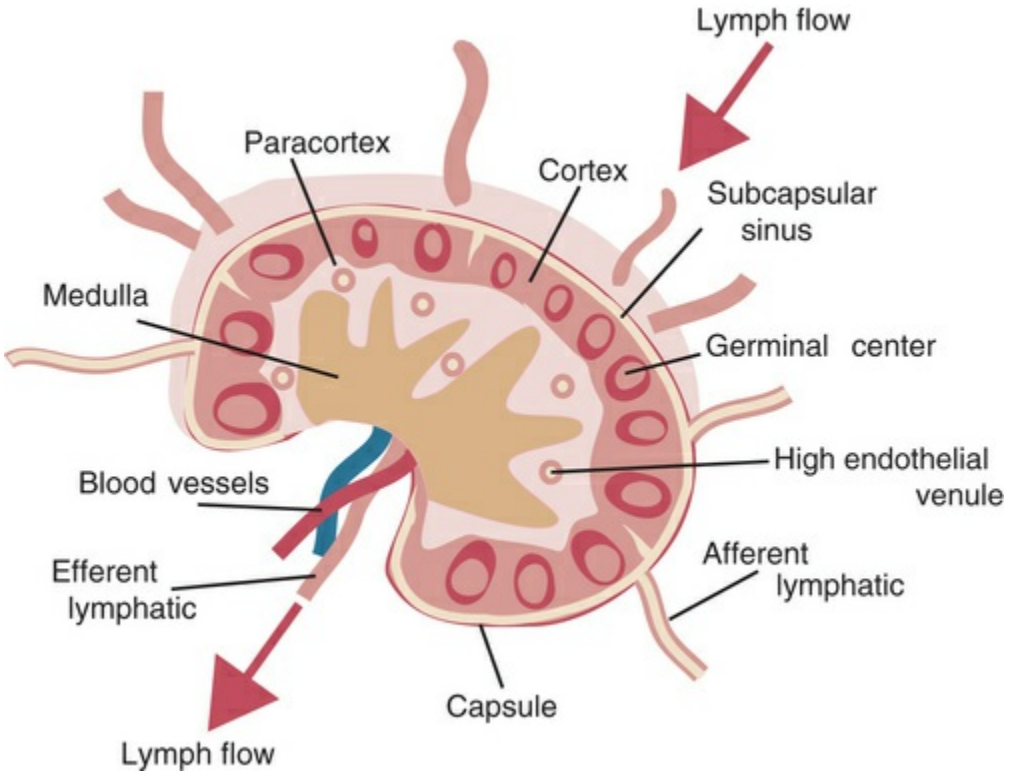


FIG. 12.8 The major structural features of a typical mammalian lymph node.

The interior of lymph nodes is divided into three regions: a peripheral cortex, a central medulla, and an ill-defined region in between, called the paracortex (Fig. 12.9). B cells predominate in the cortex, where they are arranged in aggregates called follicles. In lymph nodes that have been stimulated by antigen, some of these follicles form specialized structures called germinal centers (Fig. 12.10).

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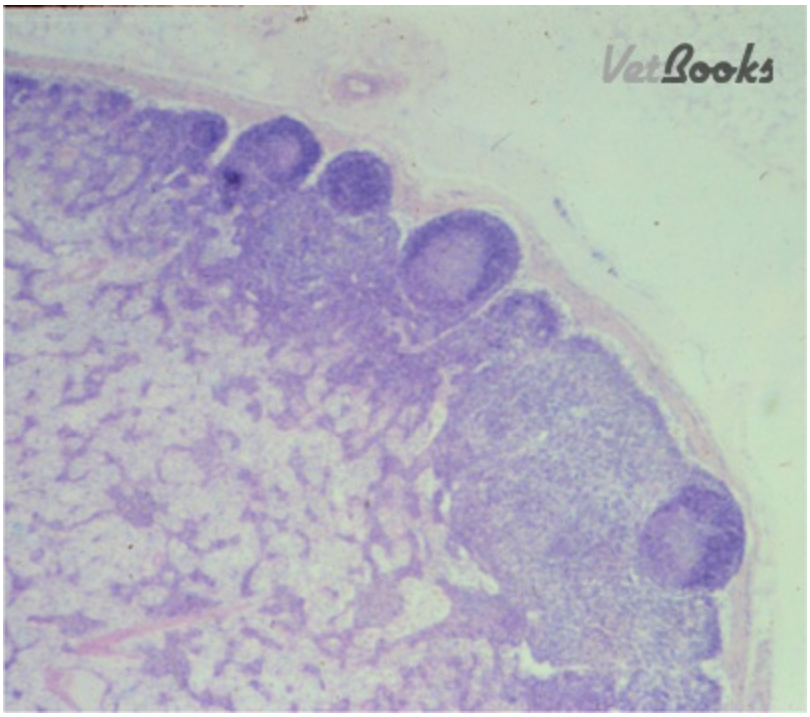


FIG. 12.9 A section of bovine lymph node. Note the presence of several germinal centers in the cortex. Original magnification ×12. (From a specimen provided by Dr. W.E. Haensly.)

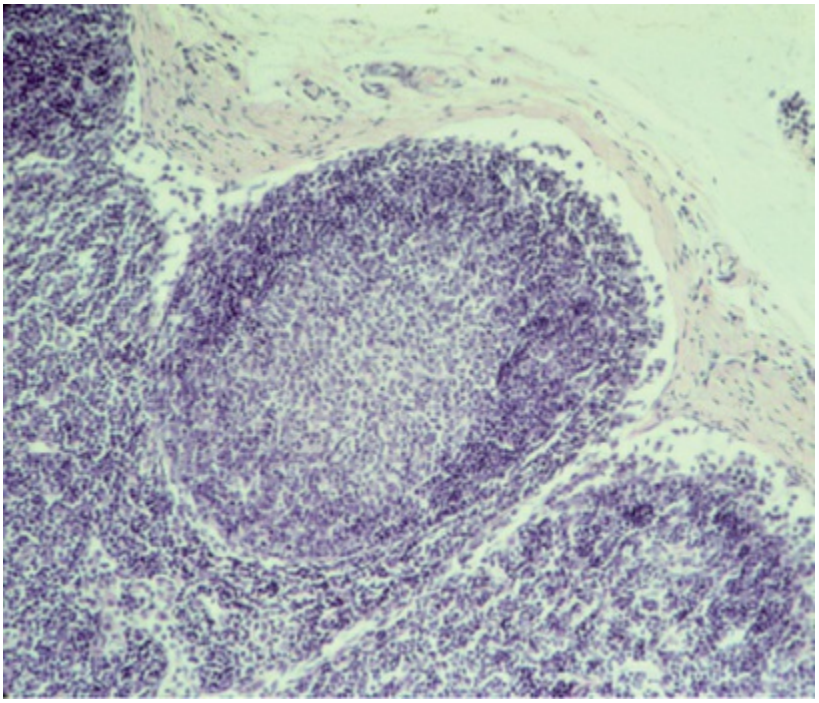


FIG. 12.10 A germinal center in the cortex of a cat's lymph node. Note the obvious dark and light zones. B cells undergo mutation in the dark zone, while memory cells develop in the central light zone. Original magnification ×120. (From a specimen provided by Dr. W.E. Haensly.)

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Germinal centers are sites where B cells grow, mutate, and mature. They are ovoid clusters of cells divided into a light and dark zone. Germinal centers originate when a few antigen-specific B cells enter a follicle and then divide rapidly to become the centroblasts that form the dark zone. This is the site where B cells proliferate and undergo a process called somatic mutation (Chapter 15). The centroblasts eventually produce non-dividing centrocytes that migrate to the light zone. The light zone is the site where immunoglobulin class switching and memory B cell formation occurs (Chapter 15). Light zones are rich in antigen-trapping follicular dendritic cells (fDCs) and $CD4^+$ T cells (Fig. 12.11).

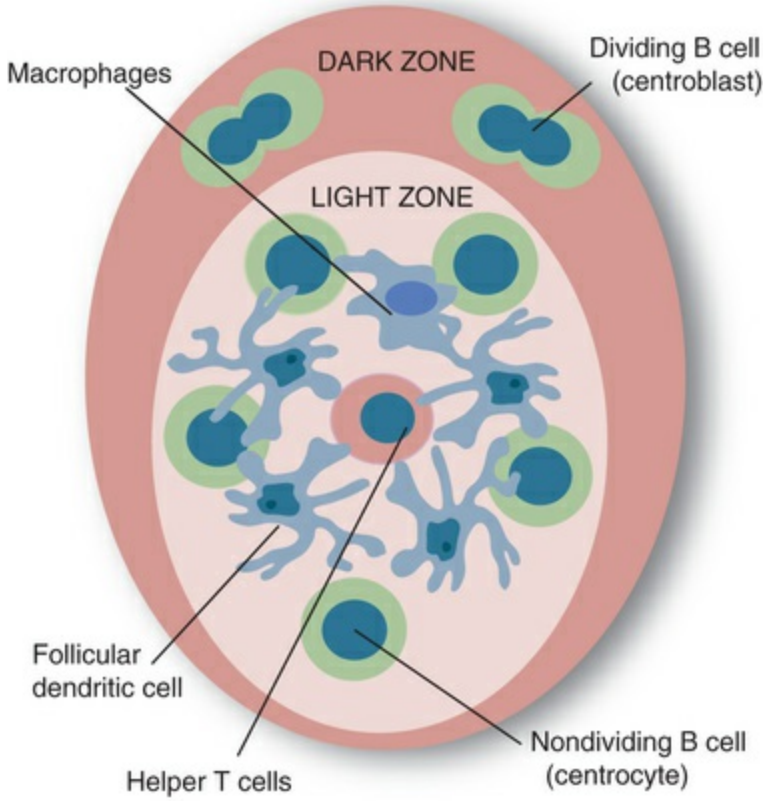


FIG. 12.11 A schematic diagram showing the structure of a germinal center. The outer dark zone contains dividing and mutating B cells. The central pale zone is a location where antigen-presenting dendritic cells, helper T cells, and B cells interact, and where memory cells develop.

T cells and dendritic cells predominate in the paracortex. The cells are arranged in cords between the lymphatic sinuses. At the

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center of each paracortical cord is a high endothelial venule (HEV). These vessels are lined with tall, rounded endothelial cells quite unlike the flattened endothelium found in other blood vessels (Fig. 12.12). HEVs are surrounded by concentric layers of fibroblastic reticular cells and a narrow space called the perivenular channel.

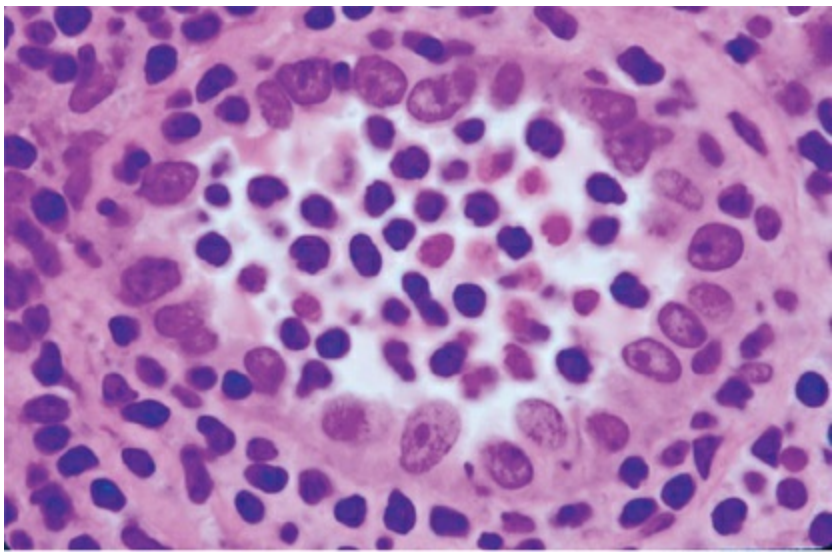


FIG. 12.12 A section of human tonsil showing a high endothelial venule with its characteristic high, rounded endothelial cells. Note the lymphocytes emigrating between the endothelial cells.

The lymph node medulla contains lymph-draining sinuses separated by medullary cords containing many plasma cells, macrophages, and memory T cells.

Lymph nodes are very busy places with cells coming and going in response to a multitude of chemical signals. These signals are delivered through the reticular fibers that provide the structural scaffolding of the lymph node. These fibers are hollow and serve as conduits for the rapid transmission of signaling molecules (Fig. 12.13). The conduits consist of bundles of collagen fibers ensheathed by fibroreticular cells. The fibroreticular cell wall is not continuous, so that follicular B cells and dendritic cells can insert their processes through tiny gaps and sample the antigens within the lymphatic fluid (Fig. 12.14). A similar network of conduits occurs within the T cell zones where antigens are sampled by dendritic cells. The conduits provide for the rapid delivery of soluble antigens from the afferent lymph to the lumen of HEVs and

enable these antigens to reach deep into a node.

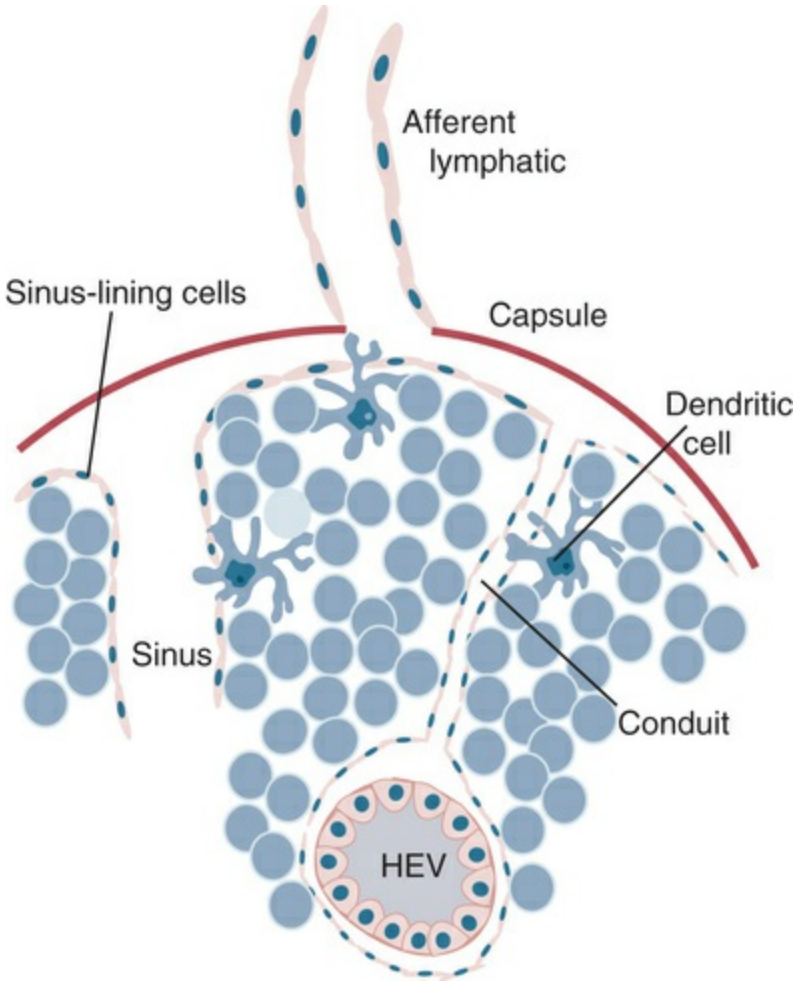


FIG. 12.13 Conduits connect the subcapsular sinus directly to the perivenular space around high endothelial venules. As soluble antigens flow through these conduits, they can be sampled by dendritic cells.

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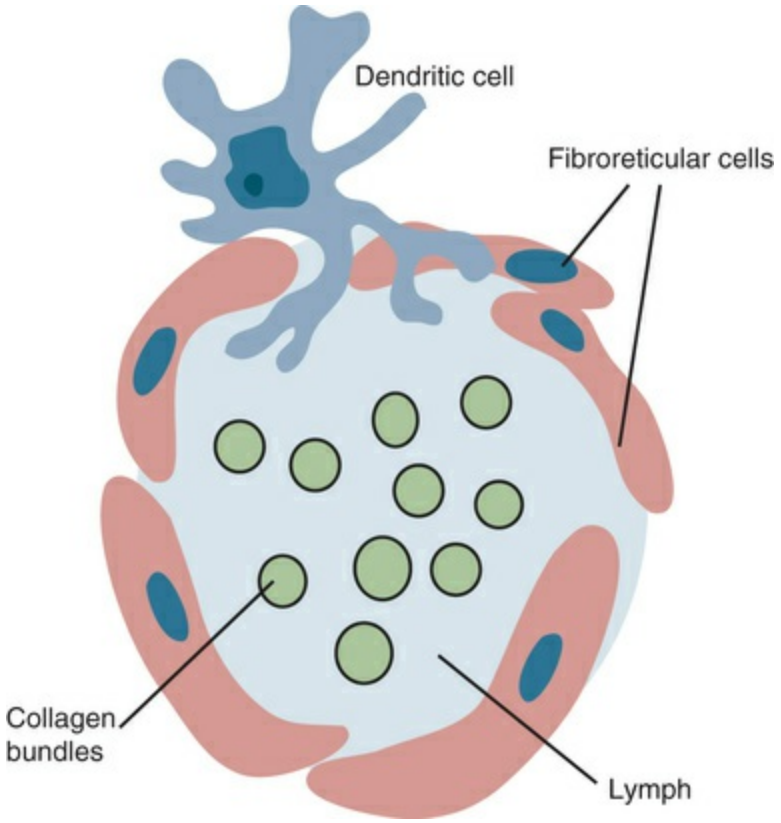


FIG. 12.14 Conduits consist of loosely attached fibroreticular cells surrounding collagen bundles. Dendritic cell processes can reach into the conduits to sample their antigen content.

Function

The principle function of secondary lymphoid organs such as lymph nodes is to facilitate the interactions between antigen-presenting cells and antigen-sensitive T and B cells. Each cell must be guided to its appropriate contacts with great precision. A complex mixture of chemokines directs these cells. Thus chemokines drive the emigration of lymphocytes from HEVs into the lymph node. Once they enter the lymph node, the T and B cells are guided to their respective regions by chemokines secreted by stromal cells and follicular dendritic cells. Immature dendritic cells, once they encounter antigen, are also guided into lymph nodes by chemokines. For example, dendritic cells are attracted to the paracortex, where they present their antigen to T cells. Once this is accomplished, the dendritic cells change their chemokine receptors and then leave the node.

Lymph nodes also contain innate lymphoid cells that are located close to sentinel macrophages lining the lymphatic sinuses. They

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thus are exposed to cytokines, such as IL-18, that are released when the macrophages encounter invaders. The innate lymphocytes in turn rapidly increase IFN-γ secretion that activates the macrophages still further and enhances their antimicrobial activities.

An interesting feature of secondary lymphoid organs is the fact that both B and T cells are highly active and motile. The T cells in the paracortex and B cells in the cortex are guided by follicular dendritic cells. Chemokines control the relocation and recirculation of lymphocytes and ensure that they end up in the right place. For example, T cells are attracted to the perifollicular area of the cortex. B cells, on the other hand, are attracted to the interior of germinal centers. When T cells are activated, they too may enter germinal centers where they "help" B cells respond to antigens. Other secondary lymphoid organs employ different homing receptors. For example, MAdCAM-1 is a homing receptor found in blood vessels in PPs. Lymphocytes that recirculate to the intestine express high levels of the MAdCAM-1 ligand.

Soluble antigens entering the node through its afferent lymphatics first pass into the subcapsular sinus. From there they enter the conduit network and are carried into the cortex. Larger antigens such as viruses are captured by macrophages within the subcapsular sinus. These macrophages carry the viral particles through the sinus floor and directly present them to B cells in the underlying follicles. The B cells then migrate to the T cell–B cell boundary, where they receive specific T cell help. B cells can also enter the paracortex directly from HEVs. There is a specialized population of follicular dendritic cells clustered around these blood vessels so that immigrating B cells can survey any antigens they may be carrying. This is also a perfect location to receive T cell help.

When bacteria invade tissues, the resident dendritic cells are activated and migrate to the draining lymph node, where they accumulate in the paracortex and cortex. These dendritic cells form a web through which antigens must pass. Captured antigens are presented by the dendritic cells to T cells. T cells are initially activated in the paracortex, whereas the B cells remain randomly dispersed in the primary follicles. Both cell populations then migrate to the edges of the follicles where they interact. Once

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antibody production is stimulated, the progeny of these B cells move to the medulla and begin to secrete antibodies. Some of these antibody-producing cells may escape into the efferent lymph and colonize downstream lymph nodes. Several days after antibody production is first observed in the medulla, germinal centers appear in the cortex.

Some T cell–dendritic cell interactions are long-lasting, and in the presence of antigen, T cells and dendritic cells form stable complexes for many hours. However, before selecting its partner, a dendritic cell might sample as many as 500 different T cells/hour and can interact with up to 10 simultaneously (see Fig. 10.6).

Adherence to follicular dendritic cells is the predominant means of antigen trapping once an animal has been sensitized by previous exposure to an antigen. In a secondary response the germinal centers become less obvious as activated memory cells emigrate in the efferent lymph. Once this stage is completed the germinal centers redevelop.

Antigen-stimulated lymph nodes also trap lymphocytes. Interactions between infectious agents and mast cells result in the production of tumor necrosis factor-α (TNF-α). The TNF-α blocks the passage of lymphocytes through these organs, the lymphocytes accumulate, and the lymph nodes swell. This trapping concentrates lymphocytes close to sites of antigen accumulation. After about 24 hours the lymph nodes release their trapped cells and their cellular output is increased for several days.

Lymphocyte Circulation

In adult animals, most cell types reside in stable tissues and do not move a lot. The many different cells of the immune system are, in contrast, highly mobile. Cells move from the bone marrow to the thymus and secondary lymphoid organs; cells migrate around the body looking for invaders and also move from lymphoid organs to sites of microbial invasion. T cells, for example, constantly circulate around the body in the blood and tissue fluid and are the predominant lymphocytes in blood (Fig. 12.15). As they travel, they survey the body for foreign antigens and preferentially home to sites of microbial invasion and inflammation.

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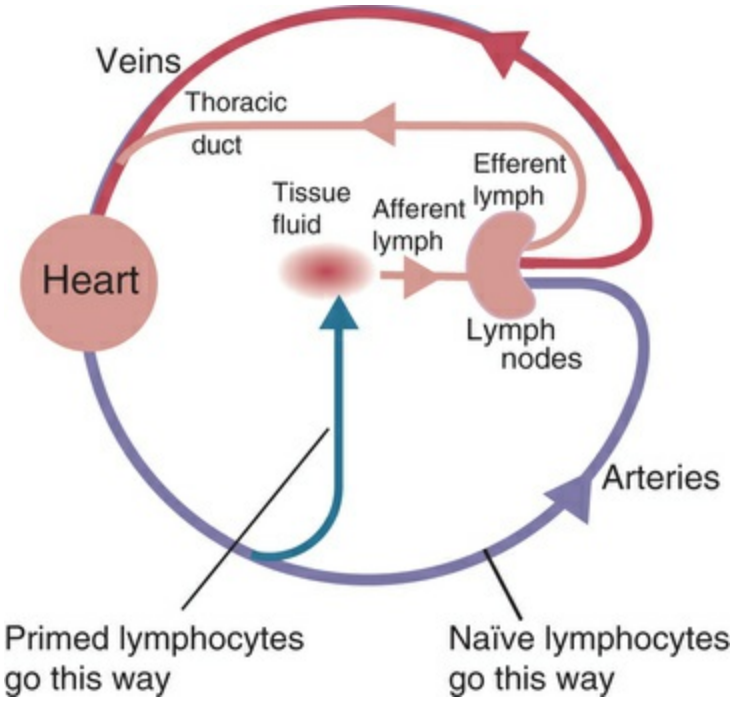


FIG. 12.15 The circulation of lymphocytes. T cells circulate in both the bloodstream and the lymphatic fluid. Their precise route through a lymph node depends on whether they are naïve or primed. Thus naïve lymphocytes enter lymph nodes through the bloodstream and the high endothelial venules. Primed lymphocytes, in contrast, migrate through the tissues and enter through afferent lymphatics. They all leave through efferent lymphatics.

Circulating T cells leave the bloodstream by two routes. T cells that have not previously encountered antigens ("naïve" T cells) bind to HEV in lymph nodes. The high endothelial cells in these vessels are not joined by tight junctions but are linked by discontinuous "spot-welded" junctions. This means that lymphocytes can pass easily between the high endothelial cells. Circulating lymphocytes can adhere to these high endothelial cells and then migrate into the paracortex. The emigration of lymphocytes from HEVs resembles that of neutrophils in inflamed blood vessels. Thus, the cells first roll along the endothelial surface binding to selectins. As they roll, they become activated and express integrins. This results in their complete arrest and emigration. The number and length of HEVs are variable and controlled by local activity. Thus, stimulation of a lymph node by the presence of antigens results in a rapid increase in the length of its HEVs. If, however, a lymph node is protected from antigens, its HEVs shorten. Recognizable HEVs are not normally found in ruminant lymph nodes, but paracortical venules serve the same

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function.

In contrast to naïve T cells, memory T cells leave the bloodstream through conventional blood vessels in tissues and are then carried to lymph nodes through afferent lymphatics. They leave the lymph nodes through the efferent lymphatics. Typically, afferent lymph in sheep contains 85% T cells, 5% B cells, and 10% dendritic cells. Efferent lymph contains greater than 98% lymphocytes, of which 75% are T cells and 25% are B cells. The efferent lymphatics eventually join together to form large lymph vessels. The largest of these lymph vessels is the thoracic duct, which drains the lymph from the lower body and intestine and empties it into the anterior vena cava.

Species Differences

Domestic pigs and related swine, hippopotamuses, rhinoceroses, and some dolphins are different. Their lymph nodes consist of several lymphoid "nodules" oriented so that the cortex of each nodule is located toward the center of the node, whereas the medulla is at the periphery (Fig. 12.16). Each nodule is served by a single afferent lymphatic that enters the central cortex as a lymph sinus. Thus afferent lymph is carried deep into the node. A cortex surrounds the lymph sinus. Outside this region are a paracortex and a medulla. This medulla may be shared by adjacent nodules (Fig. 12.17). Lymph passes from the cortex at the center of the node to the medulla at the periphery before leaving through the efferent vessels that drain the region between nodules. The cortex and paracortex have a similar structure to that seen in other mammals. The medulla has very few sinuses but consists of a dense mass of cells that is relatively impermeable to cells in the lymph. As a result, few cells migrate through the medulla. T cells in these species enter the lymph node in the conventional way through HEVs. However, they do not leave the lymph node through the lymphatics but migrate directly back to the bloodstream through the HEVs of the paracortex (Fig. 12.18). Very few lymphocytes are found in pig lymph.

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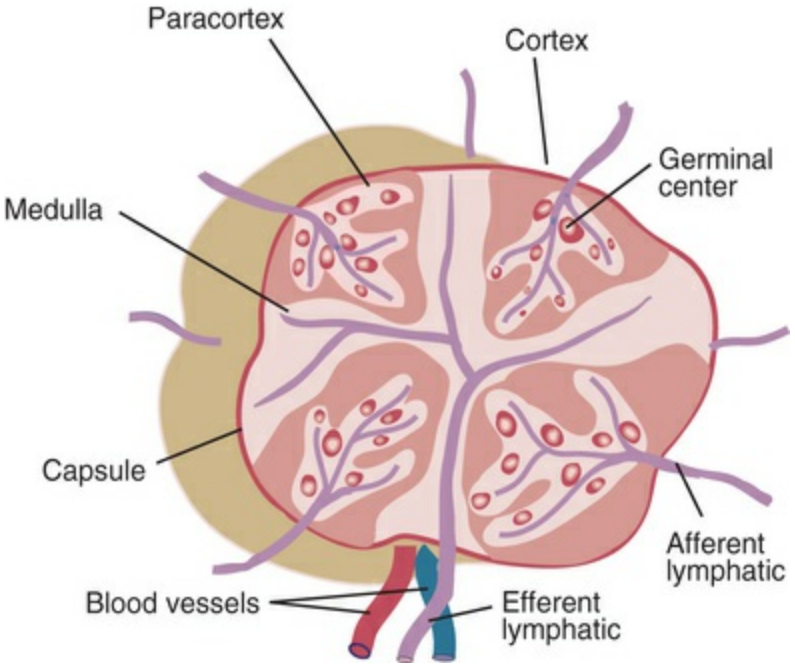


FIG. 12.16 Structure of a pig lymph node. Compare this with Fig. 12.8.

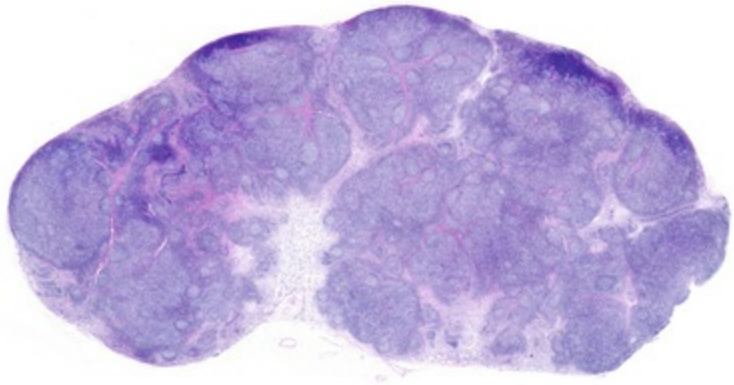


FIG. 12.17 A section of a pig lymph node. Note how the germinal centers are located in the interior of the node. Original magnification ×12. (From a specimen provided by Dr. Brian Porter.)

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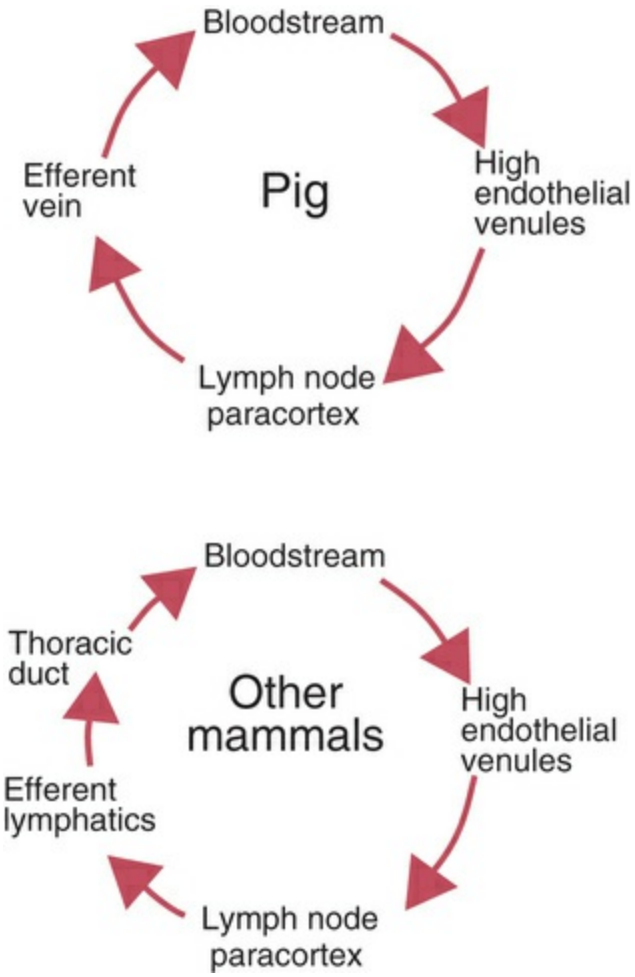


FIG. 12.18 Comparison between the major route of circulation of T cells in the pig and that in other mammals. Note that pig lymphocytes are largely confined to the bloodstream.

In marine mammals, lymph node structure is highly variable. Thus all the lymph nodes of bottlenose dolphins *(Tursiops truncatus)* are of conventional structure. In striped dolphins, *(Stenella coeruleoalba),* on the other hand, some lymph nodes (mesenteric, for example) are of conventional structure, whereas others (mediastinal) have the inverted structure described previously. Thus both forms of lymph node may be present in a single individual.

Hemolymph Nodes

Hemolymph nodes are structures similar to lymph nodes found in association with some blood vessels of ruminants and other mammals. Their function is unclear. They differ from conventional lymph nodes in that their lymphatic sinuses contain numerous red

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cells. They have a cortex containing germinal centers and B cells. T cells predominate at the center in association with lymphatic sinuses. These cells differ, however, from those found in conventional lymph nodes (more γ/δ^+, $WC1^+$ T cells, fewer $CD8^+$ T cells) (Chapter 14). Intravenously injected carbon particles are trapped in the sinusoids of hemolymph nodes, suggesting that they may combine features of both the spleen and lymph nodes.

Spleen

Just as lymph nodes filter antigens from lymph, so the spleen filters blood-borne pathogens and antigens. Indeed, the spleen can be considered a specialized lymph node for blood-borne antigens. The filtering process removes antigenic particles such as blood-borne microorganisms, cellular debris, and aged blood cells. This filtering function, together with highly organized lymphoid tissue, makes the spleen an important component of the immune system. In addition to its immune functions, the spleen also stores red cells and platelets, recycles iron, and undertakes red cell production in the fetus. As a result, the spleen consists of two forms of tissue. One is used predominantly for blood filtering and for red cell storage, called the red pulp. It contains large numbers of antigen-presenting cells, lymphocytes, and plasma cells. Macrophages in the red pulp specialize in removing aged red blood cells and so regulate iron recycling. The other tissue is rich in both B and T cells where immune responses occur and is called the white pulp. The white pulp is separated from the red pulp by a region called the marginal zone. This zone contains numerous macrophages and dendritic cells as well as a large population of B cells. The spleen is not supplied with lymphatic fluid, although it does possess efferent lymphatics.

Structure of White Pulp

Arteries entering the spleen pass through muscular trabeculae before entering the white pulp and branching into arterioles. Immediately on leaving the trabeculae, each arteriole is surrounded by a layer of lymphoid tissue called the periarteriolar lymphoid sheath (Fig. 12.19). The arteriole eventually leaves this sheath and

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branches into penicillary arterioles. In some mammals, these penicillary arterioles are surrounded by ellipsoids (periarteriolar macrophage sheaths). These arterioles then open, either directly or indirectly, into venous sinuses that drain into the splenic venules. Ellipsoids are relatively large and prominent in pigs, mink, dogs, and cats; are small and indistinct in horses and cattle; and are absent in laboratory animals such as mice, rats, guinea pigs, and rabbits. In species that lack ellipsoids, particles are trapped primarily in the marginal zone of the white pulp.

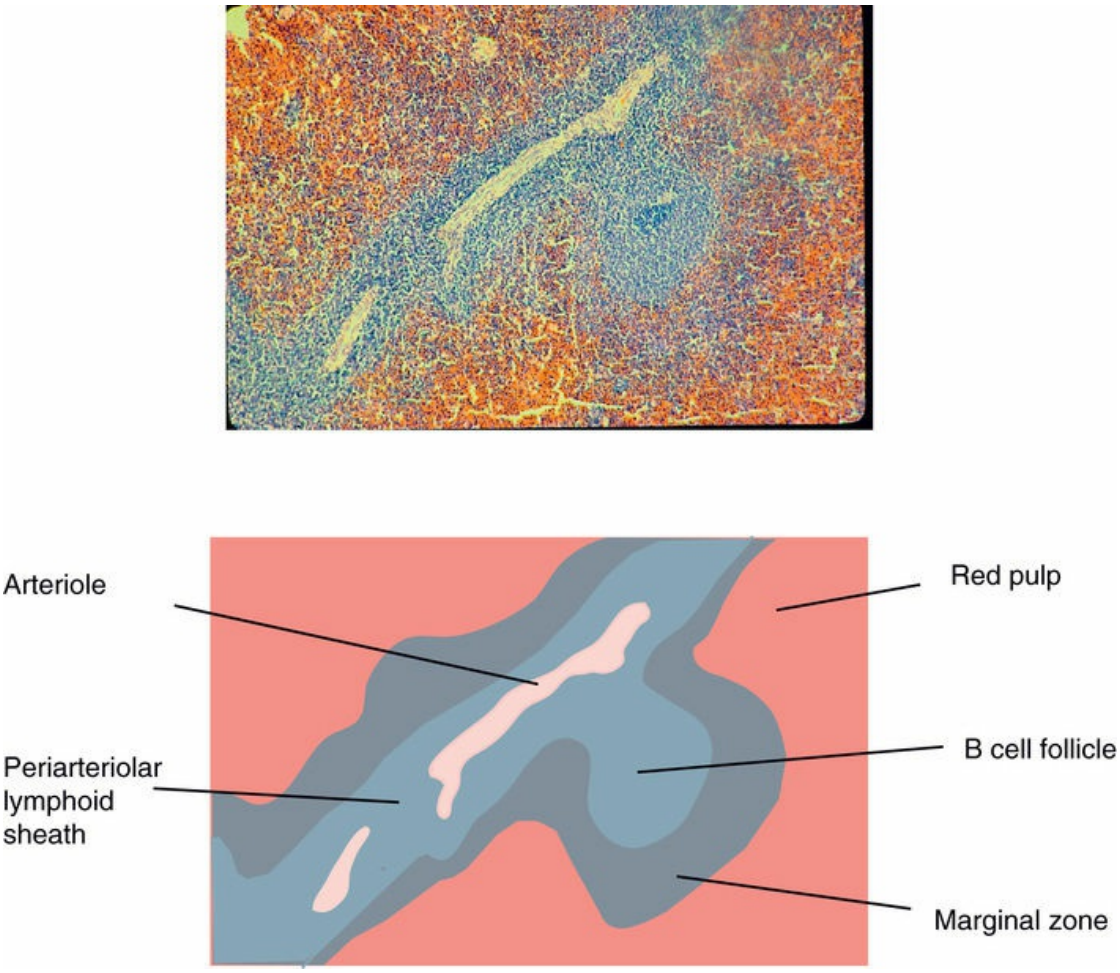


FIG. 12.19 Histological section and diagram showing the structure of the bovine spleen. Original magnification ×50. (From a specimen provided by Dr. J.R. Duncan.)

The white pulp contains both B and T cells, which accumulate in their specific zones under the influence of chemokines. The periarteriolar lymphoid sheaths consist largely of T cells. Within

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these, the T cells interact with dendritic cells and passing B cells. The B cell areas, in contrast, consist of round primary follicles scattered through the sheaths. These follicles are sites where germinal center formation, clonal expansion, isotype switching, and somatic hypermutation occur.

The white pulp is separated from the red pulp by a marginal sinus, a reticulum sheath, and a marginal zone of cells. This marginal zone is an important transit area for white cells moving between the blood and the white pulp. It is also rich in macrophages, dendritic cells, and B cells. Most of the blood that enters the spleen flows into the marginal sinus and through the marginal zone before returning to the circulation through venous sinuses. This flow pattern ensures that these antigen-presenting cells can capture any blood-borne antigens and deliver them to the B cells in the marginal zone. The white pulp is involved in adaptive immune responses, whereas cells of the marginal zone can participate in both innate and adaptive responses. White pulp does not contain HEVs. Instead, lymphocytes enter the white pulp through the marginal zone, although the route by which they leave is unclear.

Function

Intravenously administered antigens are trapped in the spleen. Depending on the species, they are taken up by dendritic cells in the marginal zone or in the periarteriolar macrophage sheaths. These dendritic cells and macrophages carry the antigen to the primary follicles of the white pulp, from which, after a few days, antibody-producing cells migrate. These antibody-producing cells (plasma cells and plasmablasts) colonize the marginal zone and move into the red pulp. Antibodies produced by these cells diffuse rapidly into the bloodstream. Germinal center formation also occurs in the primary follicles. In an animal possessing circulating antibodies, trapping by dendritic cells within the follicles becomes significant. As in a primary immune response, the antibody-producing cells migrate from these follicles into the red pulp and the marginal zone where antibody production occurs.

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Other Secondary Lymphoid Organs

Secondary lymphoid organs include not only the spleen and lymph nodes but also the bone marrow, tonsils, and lymphoid tissues scattered throughout the body, most notably in the digestive, respiratory, and urogenital tracts. The lymphoid tissues of the intestinal tract constitute the largest pool of lymphocytes in the body, but the bone marrow also contains very large numbers of lymphocytes. If antigen is given intravenously, much will be trapped not only in the liver and spleen but also in the bone marrow. During a primary immune response, antibodies are mainly produced in the spleen and lymph nodes (Fig. 12.20). Toward the end of that response, memory cells leave the spleen and colonize the bone marrow. When a second dose of an antigen is given, the bone marrow produces very large quantities of antibody and is the major source of antibodies in adult rodents. Up to 70% of the antibody to some antigens may be produced by cells in the bone marrow (Fig. 12.21). Fat-associated lymphoid clusters are small secondary lymphoid nodules within adipose tissues underlying serosal membranes such as the mesentery.

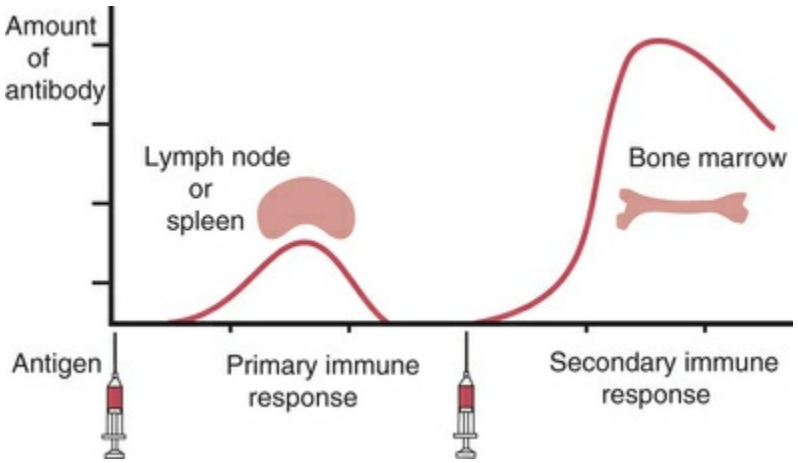


FIG. 12.20 Although the primary immune response to intravenously injected antigen takes place in lymph nodes or spleen, the antibodies produced in a secondary response are largely produced in the bone marrow.

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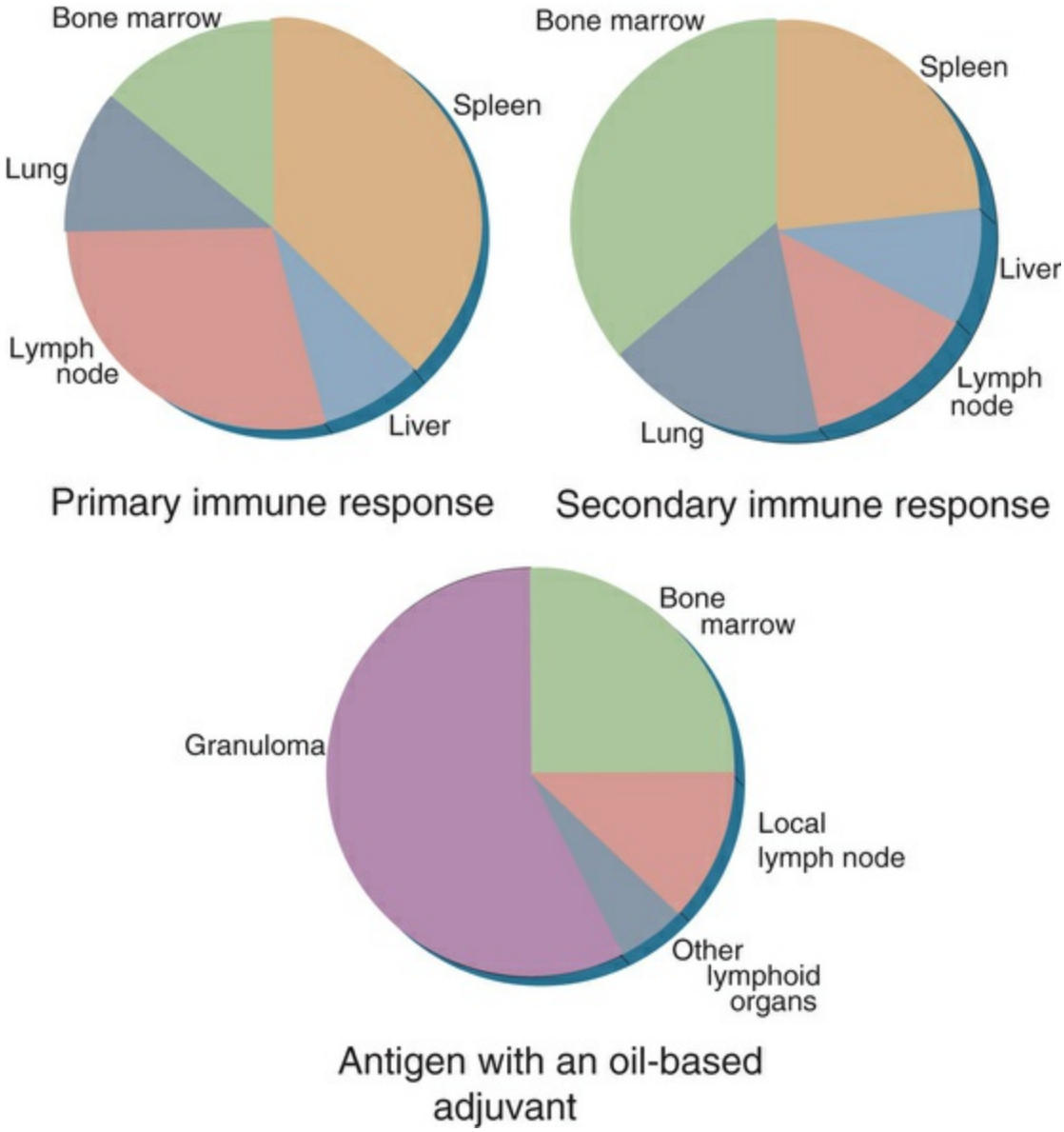


FIG. 12.21 The relative contribution of different organs or tissues to antibody production after administration of antigen either intravenously or intramuscularly with Freund's complete adjuvant. The oil in the adjuvant (Chapter 24) causes the accumulation of lymphocytes and antigen-processing cells. It thus forms a granuloma at the injection site where antibodies are produced.

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Tertiary Lymphoid Organs

Within the body are found lymphoid structures that are structurally similar to secondary lymphoid organs but develop in response to microbial colonization and chronic immune stimulation. These are organized masses of lymphocytes with clearly discrete T and B cell regions that contain germinal centers and other lymphoid tissue components. These can be called tertiary lymphoid organs. Good examples of these develop in the intestinal wall in response to the intestinal microbiota. Other examples are the lymphoid nodules that develop in rheumatoid arthritis joints and in atherosclerotic plaques. The initial trigger for their development probably comes from stimulated fibroblasts that produce appropriate chemokines attracting T cells, B cells and dendritic cells. Angiogenic factors trigger the production of lymphatics and high endothelial venules.

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13

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Lymphocytes

CHAPTER OUTLINE

Lymphocyte Structure, 122
Lymphocyte Populations, 123
Lymphocyte Surface Molecules, 125
Antigen Receptor Complexes, 125
Molecules That Regulate Lymphocyte Function, 127
Cytokine Receptors, 128
Antibody Receptors, 128
Complement Receptors, 128
Adherence Molecules, 128
Integrins, 128
Selectins, 129
Immunoglobulin Superfamily, 129
CD58 and CD2, 129
Other Major Surface Molecules, 129
WC1, 129
Changes in Phenotype, 129
Species Differences, 129

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Horses, 129
Bovine, 129
Sheep, 130
Pigs, 130
Dogs and Cats, 130
Lymphocyte Mitogens, 130

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Identify a lymphocyte based on its morphology.
- Understand the differences between T cells, B cells, and NK cells.
- Understand how lymphocyte populations can be characterized based on their cell surface molecules.
- Recognize that cell surface molecules are classified by the cluster of differentiation (CD) system.
- Understand that lymphocytes express antigen receptor complexes consisting of antigen-binding and signal-transducing components.
- Describe the characteristic features of T and B cells.
- Explain that lymphocytes also possess receptors for cytokines, immunoglobulins, and complement.
- Define cell phenotypes and explain how they are determined.
- List the major antibody-binding receptors expressed on lymphocytes.
- List some cell-adhesion molecules expressed on lymphocytes and understand their functions.
- Understand how mitogens can be used to determine lymphocyte functionality.
- Define mitogen, lymphocyte, T cell, B cell, innate lymphoid cells, phytohemagglutinin, concanavalin A, TCR, and BCR.

Lymphocytes are the cells responsible for adaptive immune

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responses and the defense of the body. There are three major types of lymphocyte. These are: innate lymphoid cells that play a role in innate immunity, T cells that regulate adaptive immunity and are responsible for cell-mediated immune responses, and B cells that are responsible for antibody production. Within these major types are many subpopulations, each with different characteristics and functions. This chapter reviews the structure and properties of these lymphocytes and some important subpopulations.

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Lymphocyte Structure

Lymphocytes are small, round cells, 7 to 15 μm in diameter. Each contains a single large round nucleus that stains intensely and evenly with hematoxylin (Fig. 13.1). This nucleus is surrounded by a thin rim of cytoplasm containing some mitochondria, free ribosomes, and a small Golgi apparatus (Fig. 13.2). Scanning electron microscopy shows that some lymphocytes are smooth surfaced, whereas others are covered by many small projections (Fig. 13.3). Innate lymphoid cells are often larger than T or B cells and may contain obvious cytoplasmic granules. With this exception, lymphocyte structure provides no clue as to their function or complexity (Fig. 13.4).

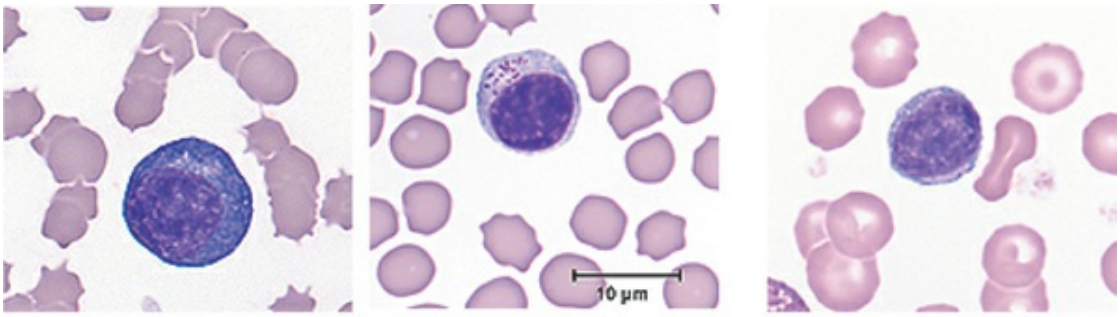


FIG. 13.1 Photomicrographs showing lymphocytes in stained blood smears from horse, cat, and dog. Giemsa stain. (Courtesy Dr. M.C. Johnson.)

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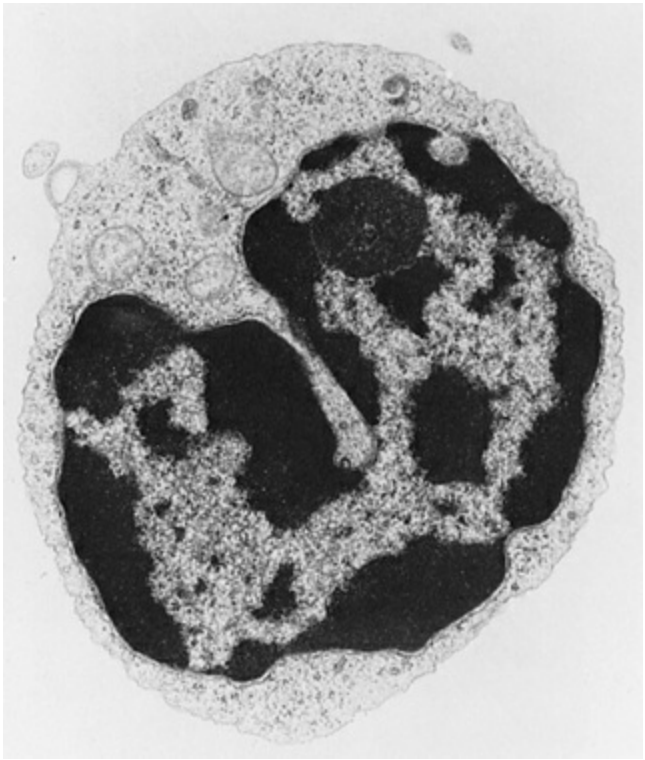


FIG. 13.2 Transmission electron micrograph of a blood lymphocyte from a rabbit. (Courtesy Dr. S. Linthicum.)

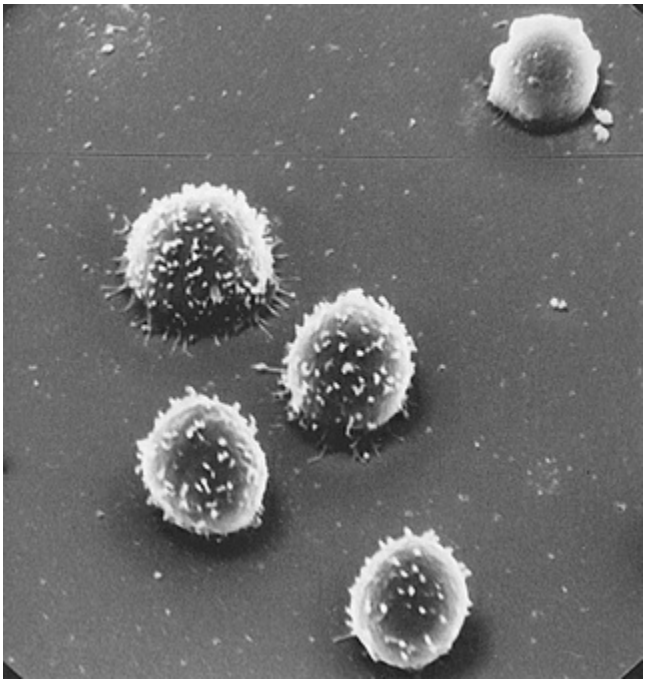


FIG. 13.3 Scanning electron micrograph of lymphocytes from a mouse lymph node. Recent studies have indicated that antigen-binding receptors are concentrated on the tips of the small projections (called microvilli). Original magnification ×1500.

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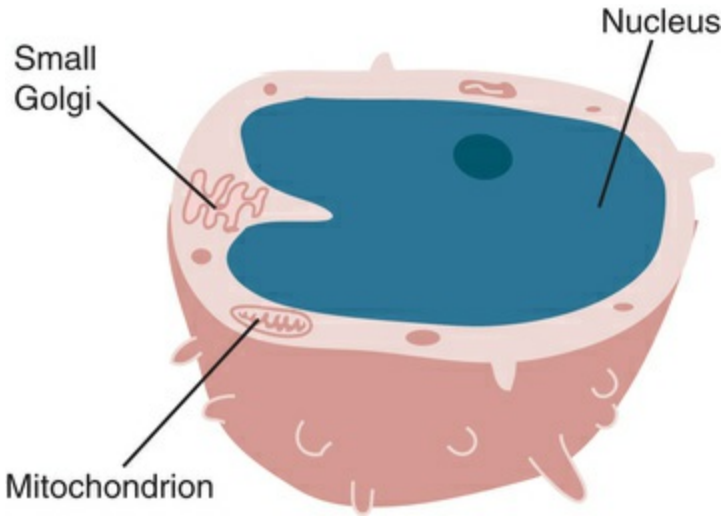


FIG. 13.4 Essential structural features of a lymphocyte. There are actually very few characteristic structural features.

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Lymphocyte Populations

Lymphocytes are found throughout the body in lymphoid organs, in blood, and scattered under body surfaces (Fig. 13.5). Despite their uniform appearance, they are a diverse mixture of distinct populations. Although these populations cannot be identified by structural differences, they can be identified by their characteristic cell surface molecules and by their behavior (Table 13.1). The pattern of cell surface molecules expressed on a cell is called its phenotype. By analyzing cell phenotypes, it is possible to identify and classify many lymphocyte populations.

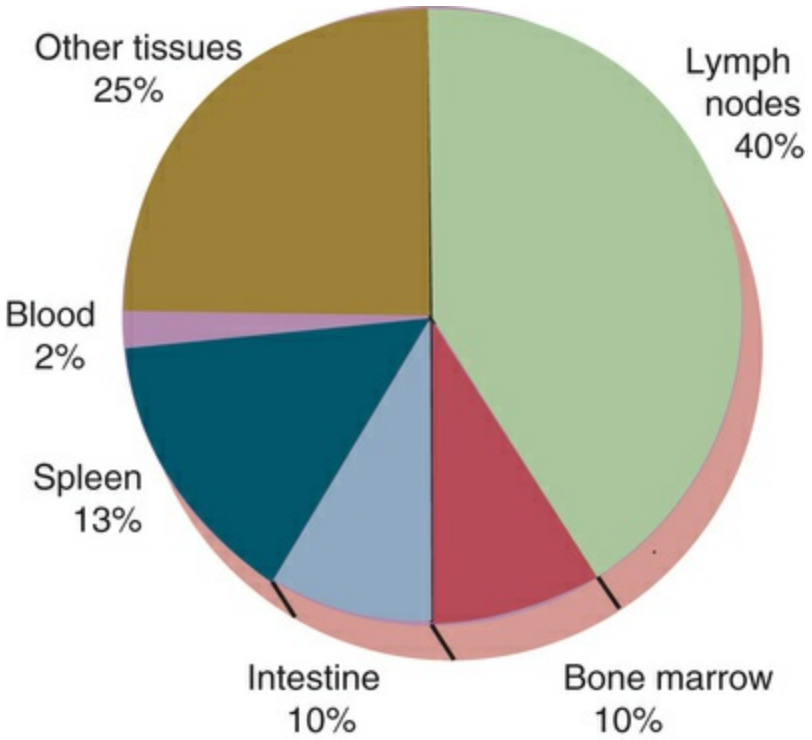


FIG. 13.5 The locations of lymphocytes within the body.

TABLE 13.1

Identifying Features of T and B Cells

Property	B Cells	T Cells
Develop within	Bone marrow, bursa, Peyer's patches	Thymus
Distribution	Lymph node cortex	Lymph node paracortex

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	Splenic follicles	Spleen periarteriolar sheath
Circulate	No	Yes
Antigen receptors	BCR—immunoglobulin	TCR—protein heterodimer Associated with CD3, CD4, or CD8
Important surface antigens	Immunoglobulins	CD2, CD3, CD4, or CD8
Mitogens	Pokeweed, lipopolysaccharide	Phytohemagglutinin, concanavalin A, BCG vaccine, pokeweed
Antigens recognized	Free foreign proteins	Processed foreign proteins in MHC antigens
Tolerance induction	Difficult	Easy
Progeny cells	Plasma cells, memory cells	Effector T cells, memory T cells
Secreted products	Immunoglobulins	Cytokines

The loss of cell-mediated immunity as a result of neonatal thymectomy first demonstrated the existence of T cells (Fig. 13.6). After T cells leave the thymus, they accumulate in the paracortex of lymph nodes, the periarteriolar lymphoid sheaths of the spleen, and the interfollicular areas of the Peyer's patches. T cells also account for 60% to 80% of the lymphocytes in blood (Table 13.2).

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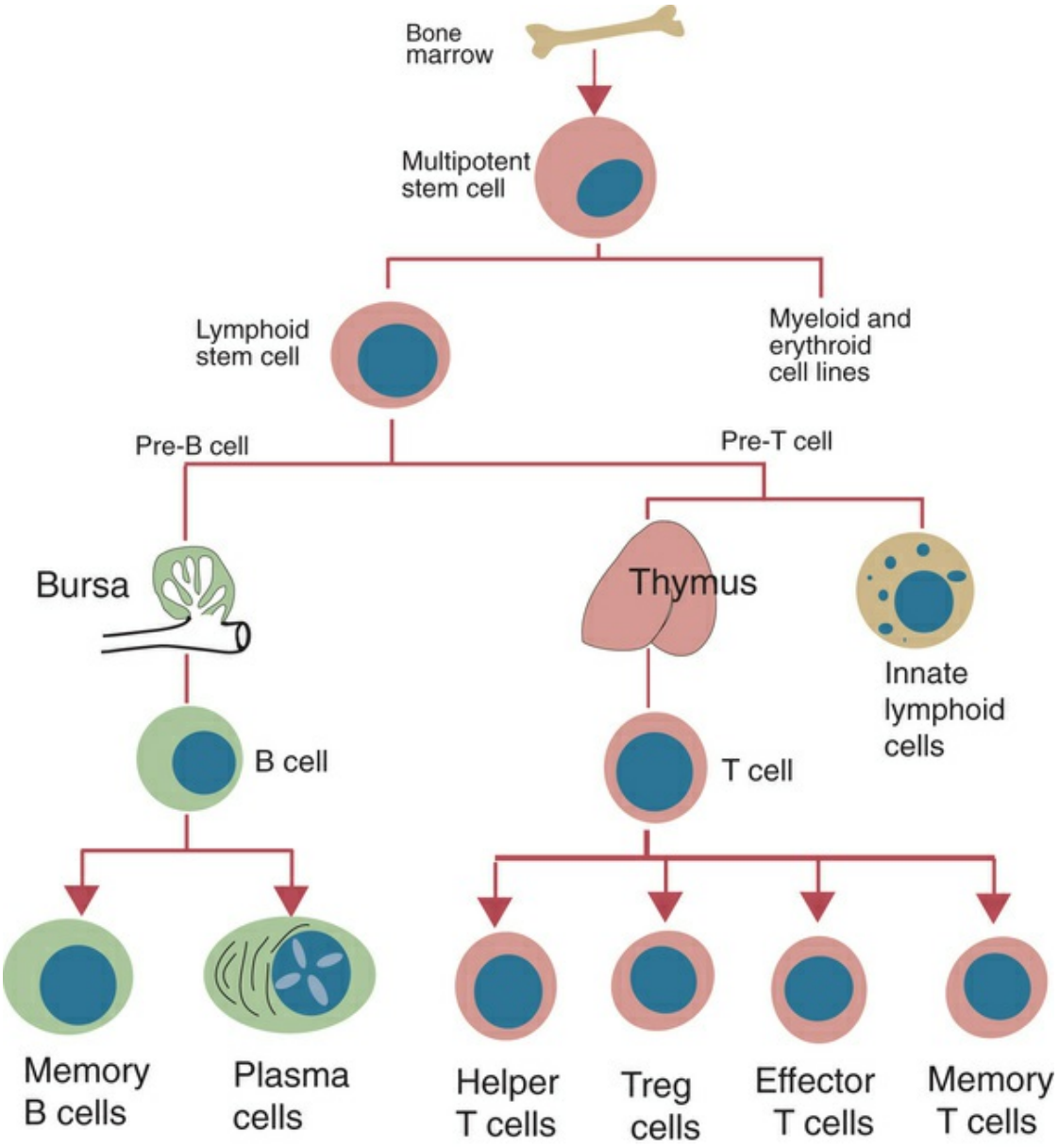


FIG. 13.6 The development of T and B lymphocytes. Both arise from bone marrow precursors. B cells develop in the bursa, Peyer's patches, or bone marrow. T cells develop in the thymus. Innate lymphoid cells are a third population of lymphocytes that are distinct from T and B cells.

TABLE 13.2

Major Peripheral Blood Lymphocyte Populations in Mammals as Percentages of the Total Population

	T Cells	B Cells	$CD4^+$	$CD8^+$	CD4/CD8
Horses	38-66	17-38[g]	56[h]	20-37[g]	4.75[h]
Bovine	45-53[a]	16-21[a]	8-31	10-30	1.53[a]
Sheep	56-64[b]	11-50[c]	8-22[c]	4-22[c]	1.55[b]
Pigs	45-57[d]	13-38[e]	23-43	17-39	1.4[f]

Dogs	46-72	7-30	27-33[i]	17-18[i]	1.7[i]
Cats	31-89[j]	6-50[j]	19-49[j]	6-39[j]	1.9[j]
Humans	70-75	10-15	43-48[k]	22-24[k]	1.9-2.4[k]

[a]Park YH, Fox LK, Hamilton MJ, Davis WC: Bovine mononuclear leukocyte subpopulations in peripheral blood and mammary gland secretions during lactation, *J Dairy Sci* 75:998-1006, 1992.

[b]Thorp BH, Seneque S, Staute K, Kimpton WG: Characterization and distribution of lymphocyte subsets in sheep hemal nodes, *Dev Comp Immunol* 15:393-400, 1991.

[c]Smith HE, Jacobs RM, Smith C: Flow cytometric analysis of ovine peripheral blood lymphocytes, *Can J Vet Res* 58:152-155, 1994.

[d]Pescovitz MD, Sakopoulos AB, Gaddy JA, et al: Porcine peripheral blood $CD4^+/CD8^+$ dual expressing T-cells, *Vet Immunol Immunopathol* 43:53-62, 1994.

[e]Saalmüller A, Bryant J: Characteristics of porcine T lymphocytes and T-cell lines, *Vet Immunol Immunopathol* 43:45-52, 1994.

[f]Joling P, Bianchi AT, Kappe AL, Zwart RJ: Distribution of lymphocyte subpopulations in thymus, spleen, and peripheral blood of specific pathogen free pigs from 1 to 40 weeks of age, *Vet Immunol Immunopathol* 40:105-118, 1994.

[g]McGorum BC, Dixon PM, Halliwell RE: Phenotypic analysis of peripheral blood and bronchoalveolar lavage fluid lymphocytes in control and chronic obstructive pulmonary disease affected horses, before and after “natural (hay and straw) challenges,”*Vet Immunol Immunopathol* 36:207-222, 1993.

[h]Grunig G, Barbis DP, Zhang CH, et al: Correlation between monoclonal antibody reactivity and expression of CD4 and CD8 alpha genes in the horse, *Vet Immunol Immunopathol* 42:61-69, 1994.

[i]Rivas AL, Kimball ES, Quimby FW, Gebhard D: Functional and phenotypic analysis of in vitro stimulated canine peripheral blood mononuclear cells, *Vet Immunol Immunopathol* 45:55-71, 1995.

[j]Walker R, Malik R, Canfield PJ: Analysis of leucocytes and lymphocyte subsets in cats with naturally-occurring cryptococcosis but differing feline immunodeficiency virus status, *Aust Vet J* 72:93-97, 1995.

[k]Bleavins MR, Brott DA, Alvey JD, de la Iglesia FA: Flow cytometric characterization of lymphocyte subpopulations in the cynomolgus monkey *(Macaca fascicularis), Vet Immunol Immunopathol* 37:1-13, 1993.

Similar experiments involving bursectomy in chickens pointed to the existence of B cells. In mammals, B cells originate in the bone marrow but mature within Peyer's patches or the bone marrow before migrating to the secondary lymphoid organs. B cells predominate in the cortex of lymph nodes, in follicles within the Peyer's patches and spleen, and in the marginal zone of the white pulp of the spleen. B cells account for 10% to 40% of blood lymphocytes (see Table 13.2).

Natural killer (NK) cells, a population of innate lymphoid cells, were identified as a result of the detection of cytotoxic lymphocytes

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in unsensitized animals. NK cells probably originate from the same stem cells as T cells but do not undergo thymic processing. They are widely distributed throughout the lymphoid organs. They account for 5% to 10% of blood lymphocytes. Other innate lymphoid cell populations are also found in tissues, especially in the intestine, respiratory tract, and skin (Chapter 19).

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Lymphocyte Surface Molecules

All cells express thousands of different protein molecules on their surface. Hundreds of these surface molecules have been characterized, especially on human and mouse lymphocytes (Box 13.1). As each molecule is characterized it is usually given a functional or chemical name. It is also usually given a cluster of differentiation (CD) designation as well (Figs. 13.7 and 13.8). Currently, the CD system assigns sequential numbers to each molecule: CD1, CD2, CD3, and so on, up to CD371. Since arbitrary numbers are difficult to remember, the basic principle used in this text is that if the molecule's common name is well accepted or describes its function, that name will be used. Examples include the IgA receptor, FcαR (CD89), the interleukin-6 receptor (CD126), and L-selectin (CD62L). CD nomenclature is also used for molecules for which the designation is well accepted, such as CD8 and CD4. A list of the most relevant CD molecules and their functions can be found in Appendix 1.

Box 13.1

A Note on Cell Phenotypes

All the cells of the body arise from a single precursor cell, the fertilized ovum. As the embryo develops and grows, cells differentiate both structurally and biochemically. They do this by activating required genes while turning off unneeded ones. One obvious result is that cells acquire a characteristic morphology. Histologic examination shows these structural differences and has provided much useful guidance regarding a cell's function. Structural differences are limited, however, in what they can tell us. For example, T and B cells look identical but differ significantly in their biochemistry and their function. As a result, biochemical differences must be determined to identify functional cell types. One of the best ways to do this is to examine the proteins expressed on the cell surface. Cells express hundreds of different proteins on their surface, and their identification provides a powerful tool to characterize cells. The CD system of identifying

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cell surface proteins is an organized attempt to catalog these cell surface proteins.

Two otherwise identical cell populations may be distinguished by the set of cell surface molecules they express. In identifying cells in this way, it is possible not only to identify cell subpopulations but also to follow cell development and differentiation as different genes are turned on and off depending on changes in the cell's function.

In many cases it has proven possible to identify cell subpopulations or changes in a cell's phenotype without determining the cell's functional significance. Different phenotypes occur in different domestic animal species. Students will therefore find much of the literature in this area confusing, especially if large numbers of CD molecules are reported for a specific cell phenotype.

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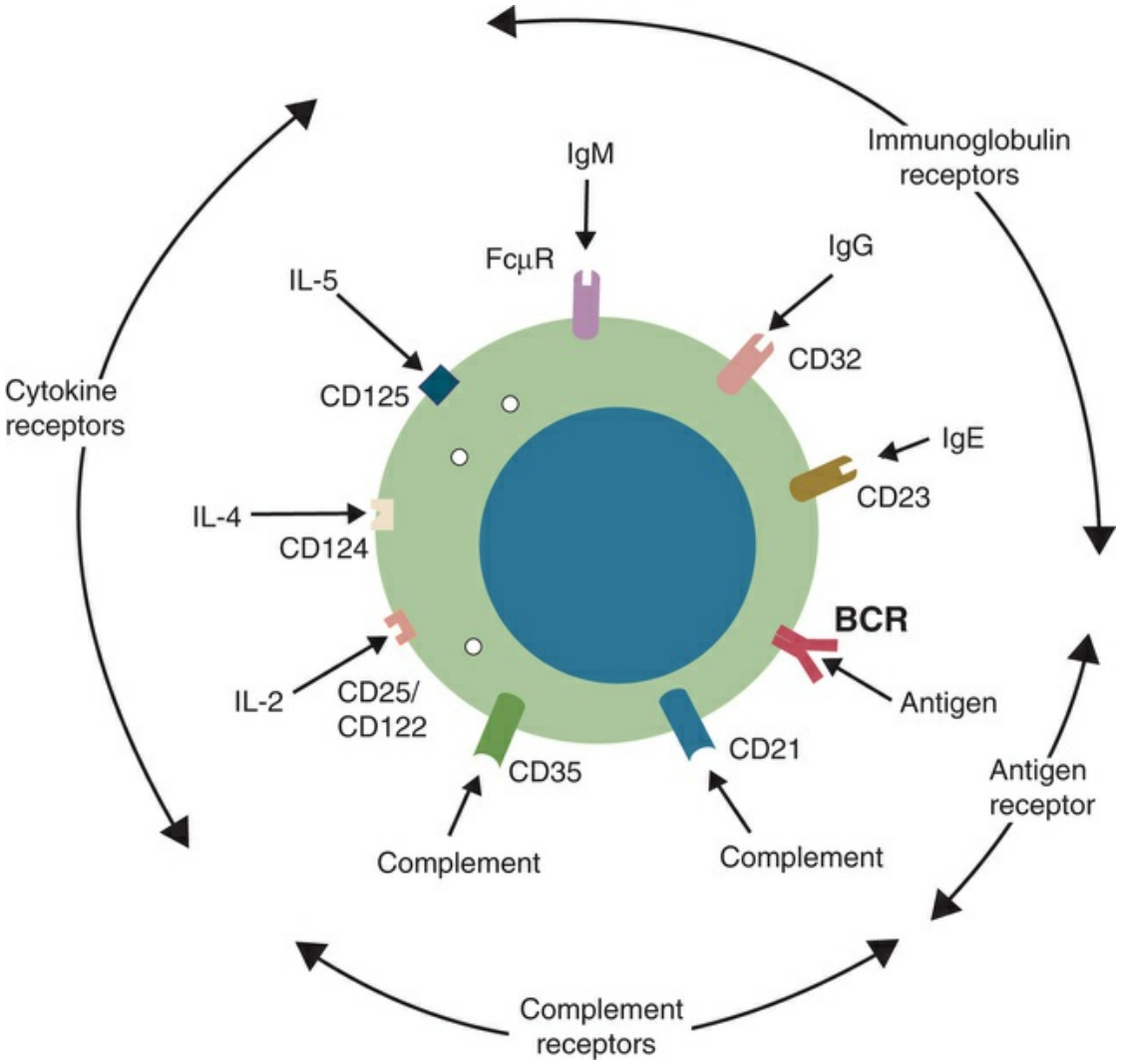


FIG. 13.7 Major surface receptors of B cells, their ligands, and their functions.

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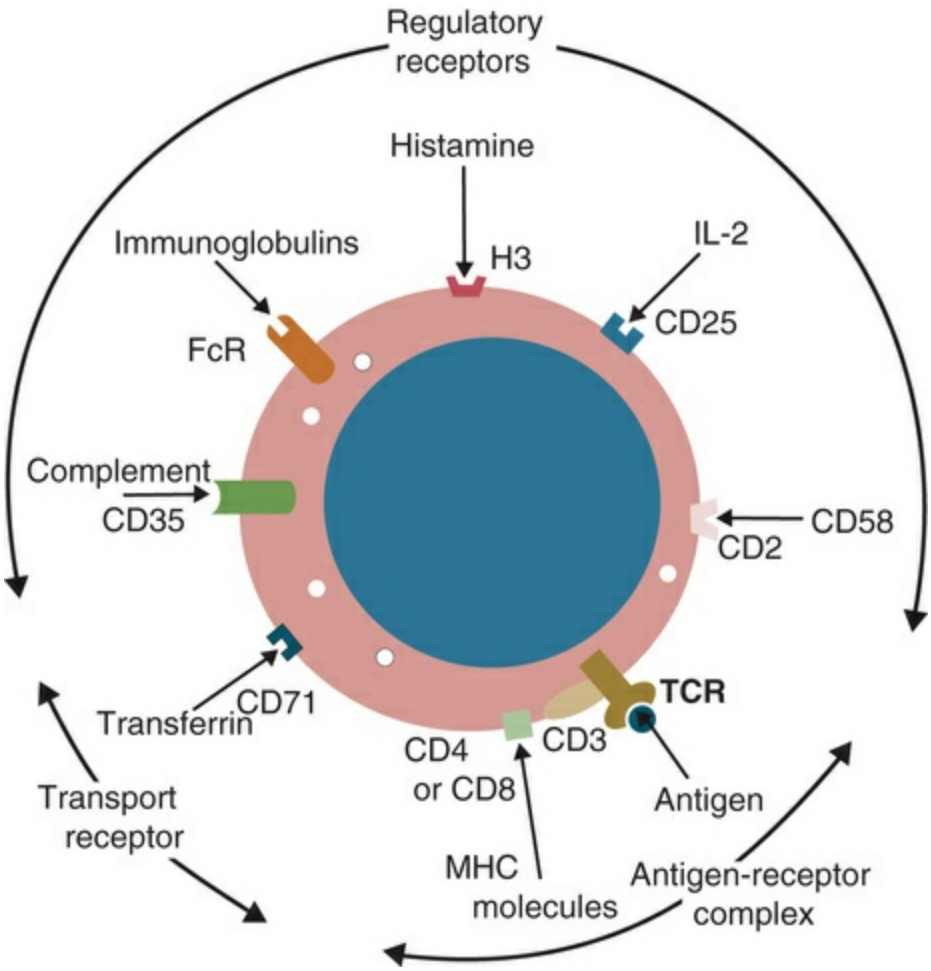


FIG. 13.8 Major surface receptors of T cells, their ligands, and their functions.

CD molecules on the cells of the domestic mammal fall into two categories. Most are also expressed on human and mouse cells (homologs) and thus have the same CD number. There are, however, several cell surface molecules in domestic mammals that have no recognized homolog in human or mouse. These unattributable molecules are given a species abbreviation and the prefix WC (workshop cluster); for example, BoWC1 and BoWC2 are found in cattle. Many of these WC molecules have subsequently been shown to be homologous to previously recognized CD molecules. The phenotypes of cells as expressed by their cell surface molecules are characterized through the use of an instrument called a flow cytometer. This is described in Chapter 42.

Antigen Receptor Complexes

While lymphocytes express thousands of proteins on their surface,

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from an immunological viewpoint, the most important structures are the receptors they use to recognize antigens. These are abbreviated TCR (T cell antigen receptor) or BCR (B cell antigen receptor). Both the TCR and BCR are complex structures assembled from many different protein chains. Some of these protein chains bind antigen molecules, whereas others are used for signal transduction. Additional complexity occurs in lymphocyte subpopulations. For example, there are two populations of T cells differentiated by their TCR antigen-binding chains. One uses paired α and β peptide chains (TCR α/β) and the other uses paired γ and δ chains (TCR γ/δ) (Chapter 14). Subpopulations of B cells use one of five different heavy chains (γ, μ, α, ε, and δ) in their BCRs. BCRs also differ from TCRs in that they are shed from the B cell in large amounts into tissue fluid and the blood, where they are called antibodies. Thus antibodies are simply soluble BCRs (Chapter 15).

Innate lymphoid cells do not have variable antigen receptors like T and B cells. They have germline encoded receptors that can bind to molecules expressed on healthy normal cells but not on diseased, abnormal cells. Innate cells are turned off when they bind to these target molecules on normal cells. On the other hand, innate cells will kill target cells that fail to express major histocompatibility complex (MHC) molecules (Chapter 19).

CD3 is the collective designation given to the set of signal transducing proteins in the TCR. CD3 is therefore found on all T cells. Another protein, CD4, is found only on helper T cells. CD4 molecules are receptors for MHC class II molecules on antigen-presenting cells. A third protein, CD8, is, in contrast, only expressed on T cells that attack and kill abnormal cells, the cytotoxic T cells. CD8 molecules are receptors for MHC class I molecules. Most human and mouse T cells express either CD4 or CD8, but rarely both. For example, about 65% of human T cells are $CD4^+$, $CD8^-$, and 30% are $CD4^-$, $CD8^+$. The remaining T cells express neither ($CD4^-$, $CD8^-$) and are said to be double negative. The ratio of $CD4^+$ to $CD8^+$ cells in blood may be used to estimate lymphocyte function. An elevated CD4 count implies increased lymphocyte reactivity because helper cells predominate, whereas a high CD8 count implies depressed lymphocyte reactivity. The relative proportions of CD4 and CD8 cells differ between humans and other mammals

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(Table 13.3). Neither CD4 nor CD8 are expressed on B cells or NK cells.

TABLE 13.3
Surface Molecules on Peripheral Blood T Cells

Marker	CELL PERCENTAGE			
	Mouse	Bovine	Swine	Sheep
TCRα/β	85-95	5-30	14-34	5-30
TCRγ/δ	5-15	45-50	31-66	22-68
CD2	95	41-60	58-72	10-36
CD4	24	8-28	23-43	8-22
CD8	11	10-30	17-39	4-22
WC1	—	5-44	40	15-70

CD45 denotes a large family of TCR-linked tyrosine phosphatases that are required for T cell signaling. They are expressed in large amounts on all three lymphocyte populations. For example, about 10% of the T cell surface is covered by CD45 molecules. Different forms of CD45 have been identified. For example, naïve T cells express one form of CD45, whereas stimulated and memory T cells express another.

The signal transducing components of the B cell antigen receptor complex are protein heterodimers formed by pairing CD79a (Ig-α) with CD79b (Ig-β). These are discussed in detail in Chapter 15.

Molecules That Regulate Lymphocyte Function

Proteins on cell surfaces serve physiological functions. Some are enzymes, some are transport proteins, and many are receptors. All cells use receptors to receive signals from their environment, including nearby cells. They also need receptors to bind other cells and to receive signals from cytokines, antibodies, and complement.

Cytokine Receptors

Lymphocytes express many different cytokine receptors. Examples include CD25, a part of the interleukin-2 (IL-2) receptor; CD118, an interferon (IFN) receptor; CD120, the tumor necrosis factor (TNF) receptor; and CD210, the IL-10 receptor. (These are discussed in Chapter 8.)

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Antibody Receptors

Lymphocytes receive signals from and hence must have receptors for antibodies. Since these receptors bind to the Fc regions of antibody molecules, they are called Fc receptors (FcR). (The meaning of the term Fc can be found in Chapter 15.) The Fc receptors for immunoglobulin G (IgG) are designated FcγR since they bind the γ chain of IgG. Likewise, those for IgA are designated FcαR and those for IgE are FcεR. Receptors for IgM have been identified on both B and T cells but are not well characterized.

Four different IgG receptors have been described on mouse leukocytes (Table 13.4). They are called FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16), and FcγRIV. All are multichain glycoproteins. One chain usually binds the antibody, whereas the other chains are used for signal transduction. CD64 (FcγRI) is found on dendritic cells, monocytes, and macrophages and to a much lesser extent on neutrophils. (It is not found on lymphocytes but is mentioned here for the sake of completeness.) CD64 binds IgG with high affinity.

TABLE 13.4

Receptors for Immunoglobulin G (FcγR)

Property	FcγRI	FcγRII	FcγRIII	FcγRIV
CD designation	CD64	CD32	CD16	
Molecular weight	75 kDa	39-48 kDa	50-65 kDa	
Cells	Monocytes, macrophages	B cells, macrophages granulocytes eosinophils	NK cells, granulocytes, macrophages	Neutrophils, macrophages, dendritic cells
Affinity	High	Moderate	Low	Intermediate/high
Function	Phagocytosis	B cells: inhibition Macrophages: phagocytosis	NK cells: cytotoxicity Granulocytes: phagocytosis	Proinflammatory

CD32 (FcγRII) is found on B cells, dendritic cells, and myeloid cells. It has a moderate affinity for IgG and will therefore only bind immune complexes (antibody molecules attached to an antigen). There are three subtypes of CD32 called a, b, and c. CD32a is expressed on macrophages and neutrophils, where it is an activating receptor. It promotes phagocytosis and stimulates the release of cytokines. CD32b is found on B cells, where it is an inhibitory receptor and regulates antibody production. The function of CD32c is unclear. All three subtypes are expressed on dendritic

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cells and stimulate dendritic cell maturation and antigen presentation.

CD16 (FcγRIII) binds IgG with low affinity and will therefore only bind immune complexes. It is found on granulocytes, NK cells, and macrophages, but not on B cells. Signaling through CD16 can trigger NK cell activation.

Mice have an additional receptor for IgG called FcγRIV, and related proteins are found in humans, chimpanzees, rats, dogs, cats, pigs, and cattle. This receptor binds IgG2 antibodies with moderate affinity, but it does not bind IgG1 or IgG3. It is expressed exclusively on neutrophils, macrophages, and dendritic cells.

Cattle and sheep also have a unique FcR called Fcγ2R. It is not related to other mammalian FcγRs but belongs to a novel gene family that includes FcαRI (CD89) and the KIRs (Chapter 19). It is expressed on myeloid cells and binds only IgG2. It is important in promoting phagocytosis of antibody-opsonized bacteria in these species.

FcαRI (CD89) is expressed on neutrophils, eosinophils, monocyte-macrophages, and dendritic cells. It binds IgA and mediates its endocytosis and recycling. FcεRI is a high-affinity IgE receptor found on mast cells and discussed in Chapter 29. It plays an important role in allergies. CD23, or FcεRII, in contrast, is a low-affinity IgE receptor expressed on activated B cells, platelets, eosinophils, macrophages, NK cells, dendritic cells, and possibly even T cells. Activated B cells can secrete soluble CD23, which then regulates allergic responses.

PIgR and FcRn are Fc receptors involved in immunoglobulin transport across epithelial surfaces. They are described in Chapters 22 and 23.

Complement Receptors

There are four major complement receptors on lymphocytes (CR1 to CR4). B cells and activated T cells express CR1 (CD35), which binds C3b and C4b, and CR2 (CD21), which binds C3d and C3bi. CR2 is closely associated with the BCR and regulates B cell responses to antigen. NK cells express CR3 and CR4.

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Adherence Molecules

As discussed in Chapter 2, some cell surface molecules bind cells together. They regulate signal network transmission between the cells of the immune system and control the movement of leukocytes in tissues. The cell adhesion molecules found on lymphocytes include integrins, selectins, and members of the immunoglobulin superfamily.

Integrins

Integrins are heterodimeric proteins formed by α and β chains. The β_1-integrins consist of a β_1 chain (CD29) paired with one of several different α chains (CD49). They bind cells to extracellular matrix proteins such as fibronectin, laminin, and collagen. The β_2-integrins consist of a β_2 chain (CD18) paired with one of several α chains (CD11). They control the binding of leukocytes to vascular endothelium and bind T cells to antigen-presenting cells. For example, LFA-1 (CD11a/CD18) on a T cell binds to its ligand, intercellular adhesion molecule-1 (ICAM-1), on the antigen-presenting cell. By prolonging and stabilizing cell interactions, this binding permits successful antigen recognition (Fig. 13.9).

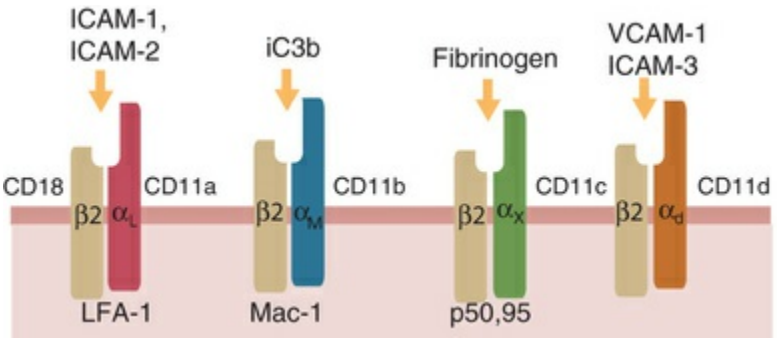


FIG. 13.9 The β_2-integrins act as cell surface adhesion molecules to link cells together so that they can communicate privately. The integrins consist of families of paired peptide chains (heterodimers). These families are based on pairing many different α chains with a limited number of β chains.

Selectins

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The emigration of lymphocytes from the bloodstream into tissues is regulated by P-selectin (CD62P), L-selectin (CD62L), and E-selectin (CD62E). P- and E-selectins are found on vascular endothelial cells. When these cells are activated by inflammation, they express selectins that bind neutrophils, activated T cells, and monocytes. L-selectin binds lymphocytes to high endothelial venules in lymphoid organs (Chapter 12).

Immunoglobulin Superfamily

Some members of the immunoglobulin superfamily (IgSF) are lymphocyte adhesion molecules. For example, ICAM-1 (CD54) binds to the integrin, CD11a/CD18 (see Fig. 13.9). ICAM-1 is normally expressed on dendritic cells and B cells. Inflammation induces ICAM-1 expression on vascular endothelium and permits phagocytic cells to bind and move into inflamed tissues. ICAM-1 is also responsible for the migration of T cells into areas of inflammation (so-called delayed hypersensitivity reactions; Chapter 33). Another IgSF adherence molecule is vascular cell adhesion molecule-1 (VCAM-1), or CD106. VCAM-1 is expressed on inflamed vascular endothelial cells. It binds the β_1-integrin, CD49d/CD29, on lymphocytes and monocytes. (The essential antigen-binding receptors on lymphocytes are also members of the IgSF [see Fig. 14.1].)

CD58 and CD2

CD58 is the ligand for CD2. CD2 is found only on T cells, whereas CD58 is widely distributed on many cell types. When cytotoxic T cells encounter their target cells, the T cell binds them through CD2 and CD58. It is likely that CD58 facilitates T cell binding to any cell undergoing surveillance (Chapter 18). CD58 is found on antigen-presenting cells such as dendritic cells and macrophages. When it binds to T cell CD2, CD58 enhances the recognition of antigen by the T cell and at the same time stimulates the antigen-presenting cell to secrete cytokines.

Other Major Surface Molecules

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B cells can function as antigen-presenting cells and express MHC class II molecules on their surface. In contrast, T cell expression of MHC class II varies between species. Both types of lymphocytes express MHC class Ia and class Ib molecules.

WC1

Lymphocytes of the major domestic mammals express several cell surface proteins not found in either humans or mice. The best defined of these belong to the WC1 family. WC1 are single-chain type I glycoproteins of 220 kDa belonging to the "scavenger receptor cysteine-rich" (SRCR) protein superfamily. They are expressed exclusively on γ/δ T cells. These $WC1^+$ T cells are found in high numbers in the skin and mucous membranes as well as in hemal nodes and the thymus. About 13 WC1 gene family members occur in cattle, whereas 50 to 100 are found in sheep. Three subsets have been defined by serological testing. Homologs of WC1 have been identified in pigs, camels, llamas, deer, elk, platypuses, and chicken. Although their natural ligand is unknown, WC1 proteins probably bind to macrophages and dendritic cells and act as co-stimulatory molecules. Some members of the family may bind directly to bacteria such as *Leptospira* and *Anaplasma.*

Changes in Phenotype

Lymphocytes do not express the same phenotype at all stages in their life cycle. A cell's phenotype depends on its maturity and activation status. For example, immature human T cells carry both CD9 and CD10. As the T cells mature within the thymus, CD9 is lost, and the cells gain CD4 and CD8. Mature thymocytes can then split into two subpopulations; one population becomes $CD4^+$, the other becomes $CD8^+$. The phenotype of lymphocytes also changes after exposure to antigen. For example, naïve T cells express high levels of CD45R and L-selectin and low levels of CD44. Memory T cells show the reverse of this: low levels of CD45R and L-selectin and high levels of CD44.

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Species Differences

Horses

Horse lymphocytes express two species-specific proteins. EqWC1 is found on 70% of equine T cells, 30% of B cells, and 50% of granulocytes and may be a homolog of CD90. EqWC2 is found on granulocytes and most T cells.

Bovine

Cattle lymphocytes express the species-specific cell surface molecules BoWC1 through BoWC15. (BoWC3 is now known to be CD21; BoWC6 is CD205, a C-type lectin expressed on dendritic cells; and BoWC10 is CD26).

In adult cattle, about 10% to 15% of circulating T cells have γ/δ TCRs, whereas the remainder have α/β TCRs. In young calves the proportion of γ/δ T cells may rise to 40%. However, this proportion fluctuates in response to management and stress. The majority of bovine γ/δ T cells also express WC1. Indeed, these cells can be activated either through their TCR or through WC1. In response they produce TNF-α, IL-1, IL-12, and IFN-γ. This mixture suggests that they contribute to both inflammation and a Th1 bias in the bovine immune response and thus link the innate and adaptive immune systems.

CD4 is expressed on 20% to 30% of blood lymphocytes in adult ruminants. Double-negative T cells constitute 15% to 30% of the blood T cells in young ruminants, but this may reach 80% in newborn calves. Most of these double-negative cells are γ/δ^+ and $WC1^+$. Thus the major circulating T cells in ruminants (γ/δ^+, $WC1^+$, $CD4^-$, $CD8^-$) are different from the predominant T cells in the humans and mice (α/β^+, $WC1^-$, $CD4^+$, $CD8^-$).

Sheep

Sheep T cells express OvWC1 (also called T19). The isoform of this molecule expressed on α/β T cells differs from that on γ/δ T cells. In newborn lambs, γ/δ T cells account for 60% of blood T cells, but this

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drops to 30% by 1 year and to 5% by 5 years. The genes encoding these γ/δ T cells show much less diversity than the genes encoding their α/β T cells.

Pigs

Pig leukocytes express nine unique surface proteins (SWC1 to SWC9). SWC1 is expressed on resting T cells, monocytes, and granulocytes but not on B cells. It is homologous to CD52. SWC2 is homologous to CD 27 and is found on T and NK cells. SWC3 is found on monocytes and macrophages. SWC9 is expressed only by mature macrophages. Pigs are a γ/δ–high species. In young pigs, up to 66% of blood T cells are γ/δ positive, but this drops to 25% to 50% in adults. Pigs have two subpopulations of γ/δ T cells. One is $CD2^+$ and the other is $CD2^-$ and has not been identified in other species. Some pig γ/δ T cells can function as antigen-presenting cells using MHC class II molecules. Up to 60% of T cells in pig blood are double positive ($CD4^+$, $CD8^+$). The rest are predominantly double negative ($CD4^-$, $CD8^-$). Some $CD4^+$ T cells also express CD8α/α homodimers and are cytotoxic.

Dogs and Cats

In dogs, CD4 is expressed on neutrophils and macrophages but not on monocytes, whereas in cats, CD4 is found on only a subset of T cells and their precursors.

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Lymphocyte Mitogens

In addition to their surface proteins, lymphocytes can be characterized by the stimulants that make them divide. The most important of these are the lectins that bind to cell surface glycoproteins and trigger cell division (Box 13.2). These lectins are commonly obtained from plants. Examples include phytohemagglutinin (PHA) obtained from the red kidney bean *(Phaseolus vulgaris),* concanavalin A (Con A) obtained from the jack bean *(Canavalia ensiformis),* and pokeweed mitogen (PWM) obtained from the pokeweed plant *(Phytolacca americana).* Lectins bind sugar residues on glycoprotein side chains. For example, PHA binds *N*-acetylgalactosamine, and Con A binds α-mannose and α-glucose. Not all lymphocytes respond equally well to all lectins. Thus PHA primarily stimulates T cells, although it has a slight effect on B cells. Con A is also a T cell mitogen, whereas PWM acts on both T and B cells.

Box 13.2

How to Measure Mitogenicity

To measure the effect of mitogens, lymphocytes are grown in tissue culture. Lymphocytes can be obtained directly from blood. The lymphocytes are cultured for at least 24 hours before the mitogen is added. Once this is done they begin to divide, synthesize new DNA, and take up any available nucleotides from the medium. It is usual to incorporate a small quantity of thymidine labeled with the radioactive isotope of hydrogen, tritium (^{3}H), in the tissue culture fluid. The thymidine is only incorporated into the DNA of cells that are dividing. After about 24 hours, the cultured cells are separated from the tissue culture fluid, either by centrifugation or filtration, and their radioactivity is counted. The amount of radioactivity in the mitogen-treated cells may be compared with that in an untreated lymphocyte culture. This ratio is called the stimulation index (see Fig. 33.8). As an alternative to the use of tritiated thymidine a radiolabeled amino acid such as ^{14}C-leucine can be used. Uptake of this compound indicates increased protein

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synthesis by the cells.

Although the plant lectins are the most efficient lymphocyte mitogens, mitogens may also be found in other unexpected sources. For example, an extract from the snail *Helix pomatia* stimulates T cells, whereas lipopolysaccharide from Gram-negative bacteria stimulates B cells. Other important B cell mitogens include proteases, such as trypsin, and Fc fragments of immunoglobulins. Bacille Calmette-Guérin (BCG) vaccine, an avirulent strain of *Mycobacterium bovis* that is used as a vaccine against tuberculosis, is a T cell mitogen. These mitogens can assist in the differentiation of T and B cells and, by measurement of the response provoked, provide an estimate of lymphocyte responsiveness.

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14

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Helper T Cells and Their Response to Antigens

CHAPTER OUTLINE

Immunoglobulin Superfamily, 132
T Cell Antigen Receptors, 132
The Antigen-Binding Chains, 132
Signal Transduction Component, 134
CD3 Complex, 134
CD4 and CD8, 134
Co-Stimulators, 134
Co-Stimulatory Receptors, 135
CD40-CD154 Signaling, 135
CD28-CD80/CD86 Signaling, 135
Co-Stimulatory Cytokines, 136
Adhesion Molecules, 136
Immunological Synapse Formation, 136
Signal Transduction, 137
Overall Considerations, 137
Superantigens, 138
Helper T Cell Subpopulations, 139
Th1 Cells, 140

VetBooks.ir

Interferon-γ, 140
Interleukin-2, 140
Th2 Cells, 140
Interleukin-4, 140
Th17 Cells, 143
Regulatory T cells (Tregs), 143
Species Differences, 144
γ/δ T Cells, 144
Memory T Cells, 145

LEARNING OBJECTIVES

After you read this chapter, you should be able to:

- Define immunoglobulin superfamily.
- Describe the structure of the T cell antigen-binding receptor (TCR).
- Describe how the TCR recognizes ligands presented by MHC molecules on antigen-presenting cells.
- Understand that the antigen-binding chains of the TCR connect to a complex signal-transducing set of proteins called CD3.
- Explain why each TCR is also associated with either CD4 or CD8.
- Compare the properties and significance of CD4 and CD8, and describe the different roles of $CD4^+$ and $CD8^+$ T cells.
- Describe how T cells must receive additional co-stimulation from cytokines and other molecules.
- Explain how the multiple signals sent by an antigen-presenting cell are communicated to a T cell through an immunological synapse.
- Explain which cytokines induce Th1 cells and which cytokines are produced in response.
- Explain how Th2 cells are induced, what they secrete, and how they promote antibody responses.

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- Explain how Th17 cells are generated and their function.
- Recognize that Treg cells produce suppressive cytokines and regulate the activities of other helper T cells.
- Understand that α/β helper T cells are the predominant T cells in most mammals and that γ/δ helper T cells are the predominant circulating T cells in young ruminants and pigs.
- Identify the major surface proteins characteristic of T cells.
- Define and describe positive and negative selection of T cells.
- Understand and explain the different functions of Th1, Th2, Th17, and T reg cells.
- Discuss the role of γ/δ T cells in domestic mammals.
- Have a basic understanding of how T cell memory develops.
- Understand the roles of IL-4, IL-17, IL-2, and IL-10.
- Define immunoglobulin superfamily, immunological synapse, Th17 cells, and Treg cells.

Unlike the innate immune responses that are triggered by a limited number of molecular patterns on pathogenic microbes, the lymphocytes of the adaptive immune system are able to recognize and respond to "everything," or at least to a large number of very diverse foreign antigens. They can do this because they have receptors that bind these antigens and, under the right conditions, respond by mounting cell-mediated or antibody-mediated immune responses.

There are four major populations of lymphocytes with antigen-binding receptors. These include helper and regulatory T cells that regulate immune responses; effector (or cytotoxic) T cells that destroy abnormal cells; and B cells that produce antibodies. Each of these cell types respond to antigens that bind to their receptors. This chapter describes the first of these major lymphocyte populations, the helper T cells.

Foreign antigens are trapped and processed by antigen-presenting cells and then presented to helper T cells. Each T cell is covered by about 30,000 identical antigen receptors. If these receptors bind sufficient antigen in the correct manner, a helper T cell will respond and initiate an immune response. It does this by

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secreting multiple cytokines, dividing, and differentiating. As you will learn later, the other antigen-responsive cell populations, B cells and cytotoxic T cells, cannot respond to antigens unless they too are stimulated by helper T cells. Because of the central role of helper T cells, they must be carefully regulated by signals from other cells and by the activities of many different cytokines.

It is important to point out that the antigen receptors on T cells are generated randomly. They are not produced in response to specific foreign antigens. As a result, T cell antigen receptors form a large diverse repertoire. Any foreign antigen that enters the body will probably encounter and bind to the receptors on at least one T cell. Because each T cell has a single receptor specificity, the repertoire of receptors is, in effect, the repertoire of the T cells. T cell antigen receptors only recognize antigens associated with MHC molecules. They do not recognize or respond to free antigen molecules.

Given the random way in which these receptors are generated, the strength of binding between an antigen and its receptors (its affinity) will also vary. Thus an antigen may be bound strongly by some receptors and weakly by others. If this binding strength is too weak, this may be insufficient to activate a T cell.

In a newborn animal that has never encountered microbial antigens, the number of T cells that can bind any specific antigen may be very low. To increase the probability of an antigen encountering a T cell with the correct receptor, the T cells are concentrated in secondary lymphoid organs such as lymph nodes, where their chances of a successful encounter with antigen-bearing dendritic cells are maximized. In older animals in which mature T cells are plentiful, they can migrate into other tissues, where they will encounter other antigen-presenting cells, such as macrophages and B cells.

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Immunoglobulin Superfamily

Proteins are constructed by linking multiple peptide domains. Each domain usually has a specialized function. For example, in proteins located on cell surfaces, the membrane-binding domain contains hydrophobic amino acids that can penetrate the cell membrane lipid bilayer. Other domains may be responsible for the structural stability of a protein or for its biological activities. In antibody (immunoglobulin) molecules, one domain is used to bind antigen, and other domains are responsible for cell binding. The presence of similar domains in diverse proteins suggests that they have a common origin, and proteins may be classified into families or superfamilies based on their domain structure.

Proteins belonging to the immunoglobulin superfamily play key roles in immunity. The members of this superfamily all contain at least one immunoglobulin domain. In a typical immunoglobulin domain the peptide chains weave back and forth to form a pleated sheet that folds into a sandwich-like structure. Immunoglobulin domains were first identified in antibody molecules (immunoglobulins). They have since been found in many other proteins. Important proteins with multiple immunoglobulin domains include the B cell antigen receptors (BCRs), the T cell antigen receptors (TCRs), and the MHC class I and II molecules (Fig. 14.1). All of the members of this superfamily are receptors, most are found on cell surfaces, and none has enzymatic activity. Many cellular responses are triggered by interactions between two different members of the superfamily as, for example, between TCR and MHC molecules.

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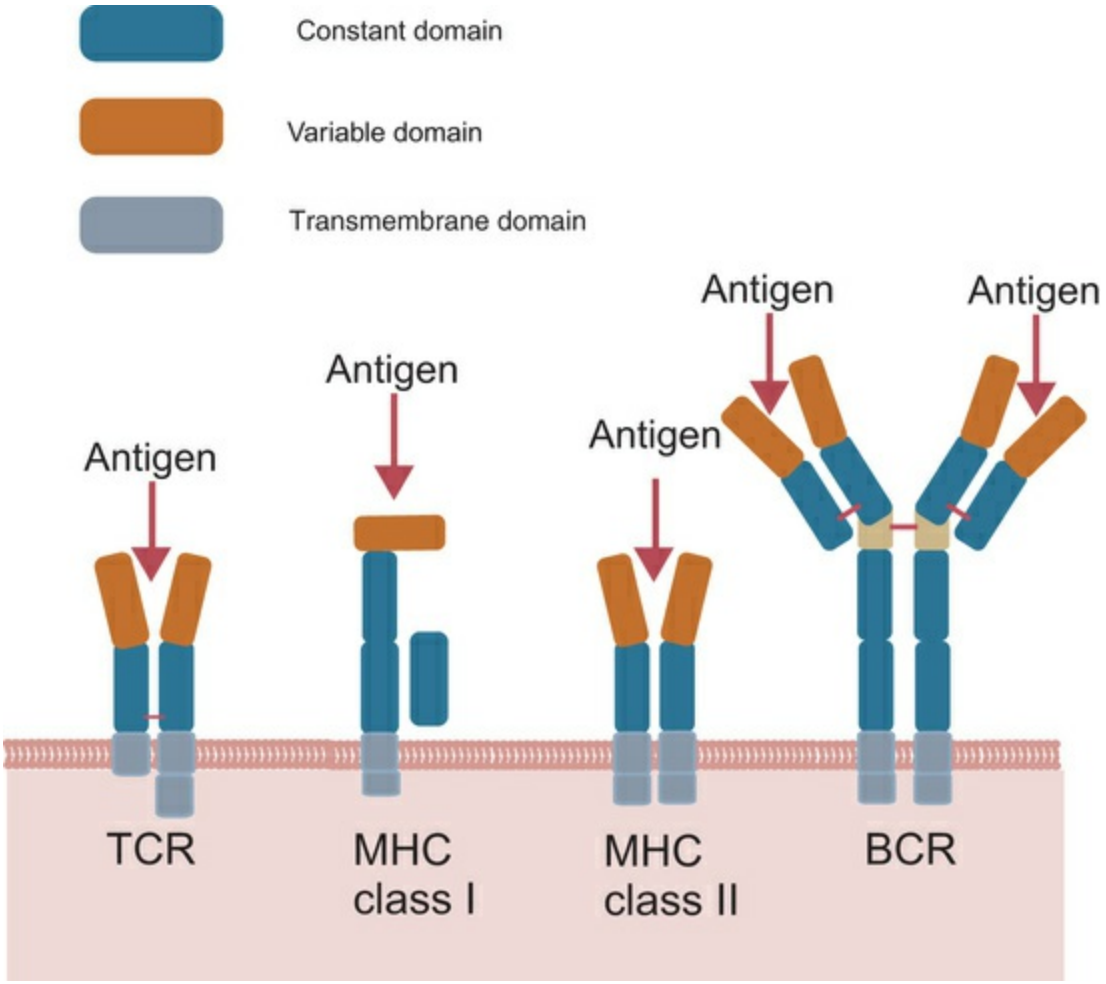


FIG. 14.1 The four key antigen receptors of the immune system—TCR, MHC class I, MHC class II, and BCR—are each constructed using immunoglobulin domains as building blocks. Each binds antigen through the use of variable domains. All are members of the immunoglobulin superfamily.

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T Cell Antigen Receptors

The Antigen-Binding Chains

Each T cell has about 30,000 identical antigen receptors (TCRs) on its surface. Each TCR is constructed from multiple glycoprotein chains. Two of these chains are paired to form the antigen-binding site; the other chains transmit the signal generated by antigen binding to the cell. Two major types of TCR have been identified based on their antigen-binding chains (Fig. 14.2). One type employs chains designated γ and δ (γ/δ) chains. The other employs α and β (α/β) chains. In humans, mice, and probably most nonruminants, 90% to 99% of T cells use α/β chains. In calves, lambs, and piglets, up to 66% of T cells use γ/δ chains.

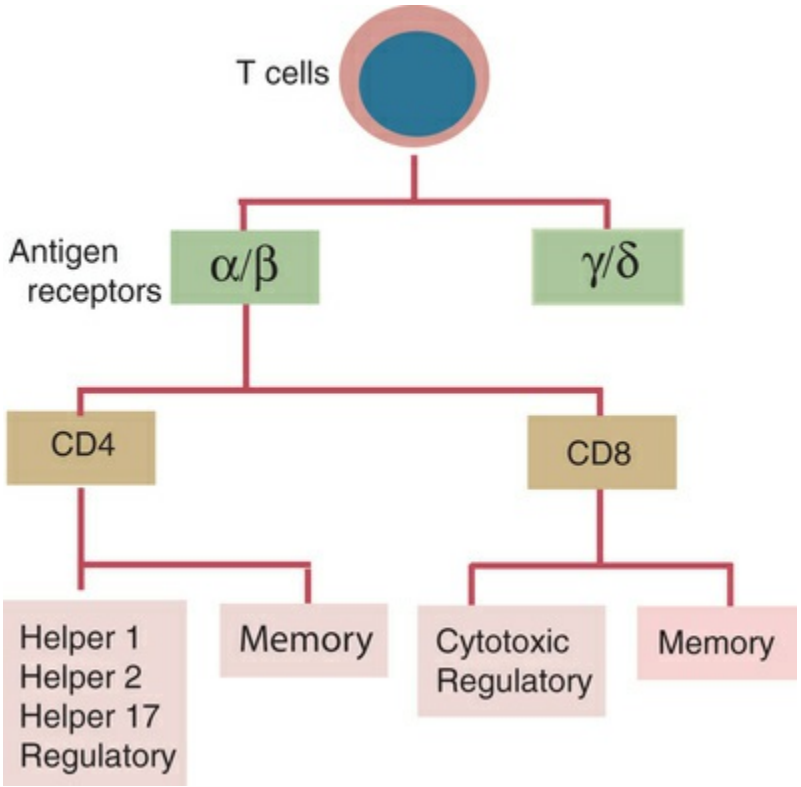


FIG. 14.2 T cells can be divided into many different subpopulations based on the antigen receptors they employ, on the accessory molecules that support their activity, and ultimately on their functions.

The four antigen-binding chains (α, β, γ, δ) are similar in

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structure, although they differ in size as a result of variations in glycosylation. Thus the α chain is 43 to 49 kDa, the β chain is 38 to 44 kDa, the γ chain is 36 to 46 kDa, and the δ chain is 40 kDa. Each chain is constructed from four domains (Fig. 14.3). The N-terminal domain contains about 100 amino acids and their sequence varies greatly among T cells. This is therefore called the variable (V) domain. The second domain contains about 150 amino acids. Its amino acid sequence does not vary, so it is called the constant (C) domain. A third, very small domain consists of 20 hydrophobic amino acids passing through the T cell membrane. The C-terminal domain within the cytoplasm of the T cell is only 5 to 15 amino acids long. The paired chains are linked by a disulfide bond between their constant domains to form a stable heterodimer (Fig. 14.4). As a result, a groove is present between the two V domains that acts as the antigen binding site. The precise shape of this antigen-binding groove varies among different TCRs because of variations in the V domain amino acid sequences. The specificity of the binding between a TCR and an antigen is determined by the shape of this groove.

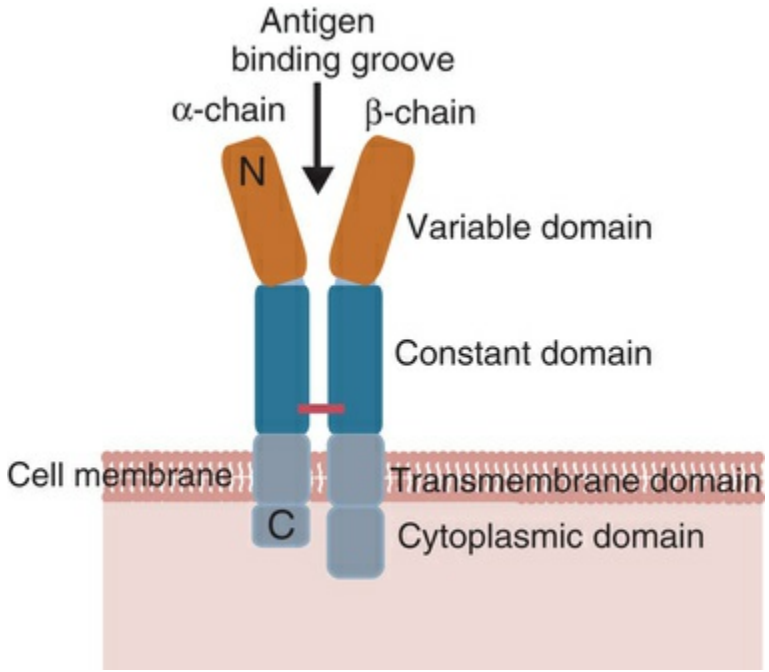


FIG. 14.3 Schematic diagram showing the domain structure of the two peptide chains that make up the antigen-binding component of an α/β TCR.

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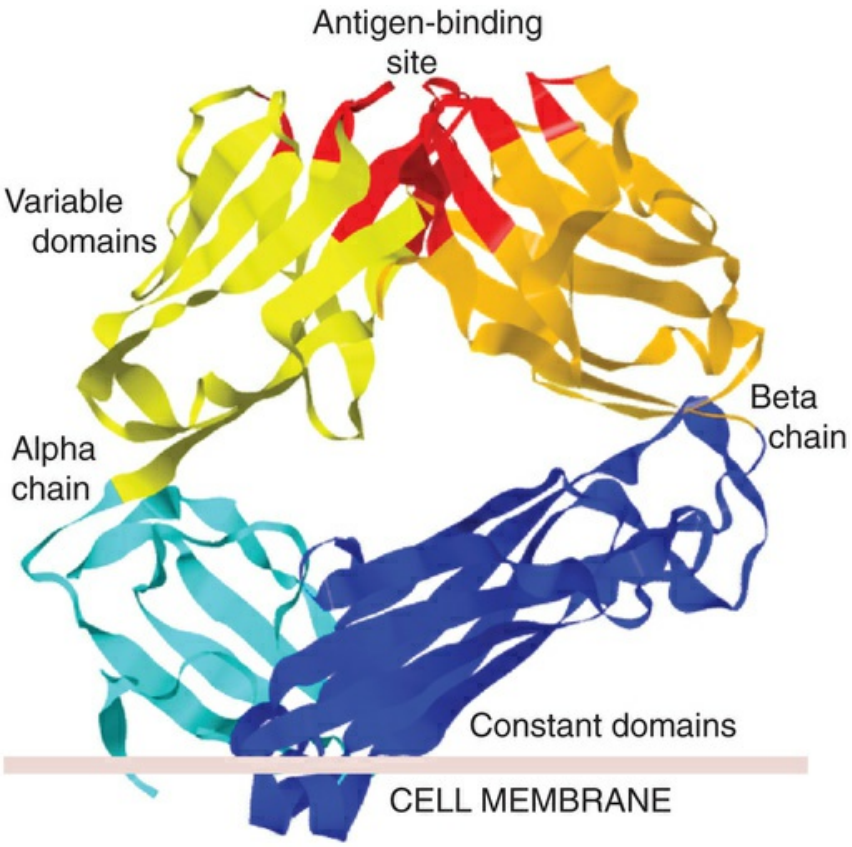


FIG. 14.4 A "Ribbon diagram" showing the three-dimensional structure of a TCR α/β dimer. The antigen-binding site, colored red, is formed by both the alpha and beta chains. (Courtesy Dr. B. Breaux.)

Within each V domain is a region where the amino acid sequence is very highly variable. This is the region that actually comes into contact with the antigen. For this reason, it is called the hypervariable or the complementarity-determining region (CDR). The antigen-binding site of the TCR is formed by the CDRs from each chain that line the groove. The rest of the V domain outside the CDRs has a constant sequence and is called the framework region.

Signal Transduction Component

CD3 Complex

When antigen binds to its TCR, a signal is generated that triggers the T cell response. The paired antigen-binding chains of each TCR are associated with a cluster of signal transducing proteins called the CD3 complex (Fig. 14.5). The CD3 complex consists of five

chains (γ, δ, ε, ζ, and η) arranged as three dimers γ-ε, δ-ε, and either ζ-ζ or ζ-η. The TCR β chain is linked to the γ-ε dimer, and the TCR α chain is linked to the δ-ε dimer.

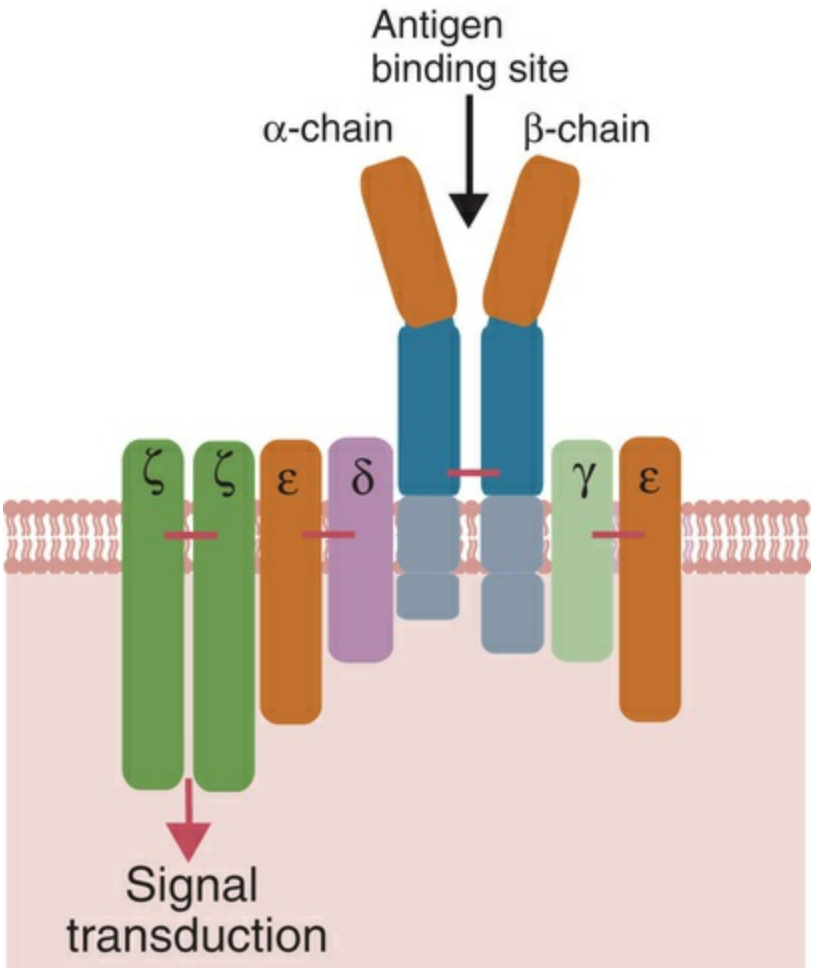


FIG. 14.5 Overall structure of the TCR/CD3 complex. The signal transduction proteins are collectively classified as CD3. About 80% of α/β TCRs use ζζ dimers. The remaining 20% use ηζ heterodimers. Most γ/δ TCRs probably use a completely different signal transduction complex.

CD4 and CD8

Two additional proteins closely associated with the TCR are called CD4 and CD8. CD4 is a single chain of 55 kDa, and CD8 is a dimer of 68 kDa. (One chain of CD8 is called α, the other is β. In humans, pigs, mice, and cats, CD8 is an α-β heterodimer or, less commonly, an α-α homodimer.) CD4 and CD8 determine the class of MHC molecule that can be recognized by the T cell (Fig. 14.6). For

example, CD4, found only on helper T cells, binds MHC class II molecules on antigen-presenting cells. CD8, in contrast, is found only on cytotoxic T cells and binds MHC class Ia molecules on virus-infected or other abnormal cells. Both CD4 and CD8 enhance TCR signal transduction by linking the T cell to an antigen-presenting cell through the MHC.

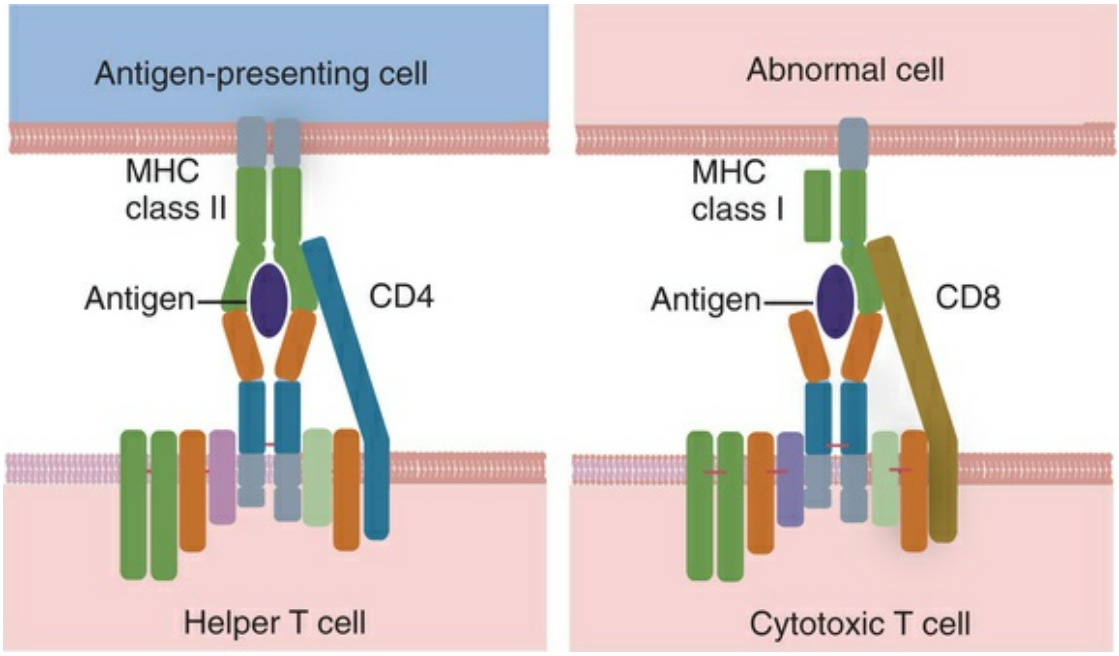


FIG. 14.6 Role of CD4 and CD8 in promoting T cell responses. These molecules link the T cell to the antigen-presenting cell, binding the two cells together and ensuring that an effective signal is transmitted between them. This interaction is seen in Fig. 10.6, *A*.

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Co-Stimulators

The binding of a T cell antigen receptor to an antigen-MHC complex is not sufficient by itself to trigger a helper T cell response. Additional signals are needed for the T cell to respond fully. For example, adhesion molecules must bind the T cells and antigen-presenting cells firmly together and permit prolonged, strong signaling between the cells. TCR-antigen binding then triggers the initial signaling steps. Receptor molecules such as CD40 on antigen-presenting cells bind to their ligands on T cells and amplify these signals. T cells must also be stimulated by cytokines secreted by the antigen-presenting cells. The cytokines determine the way in which a T cell responds to antigen, turning on some pathways and turning off others.

Co-Stimulatory Receptors

Several additional cellular receptors must be stimulated in order to fully activate T cells and determine how they will respond. This is called co-stimulation.

CD40-CD154 Signaling

CD40 is a receptor expressed on antigen-presenting cells. Its ligand is CD154, expressed on helper T cells (Fig. 14.7). When CD154 and CD40 bind, signals are sent in both directions. The signal from the antigen-presenting cell to the T cell causes it to express a receptor called CD28. The signal from the T cell to the antigen-presenting cell stimulates it to express either CD80 or CD86. CD40-CD154 signaling also stimulates the antigen-presenting cell to secrete multiple cytokines, including interleukin-1 (IL-1), IL-6, IL-8, IL-12, CCL3, and tumor necrosis factor-α (TNF-α).

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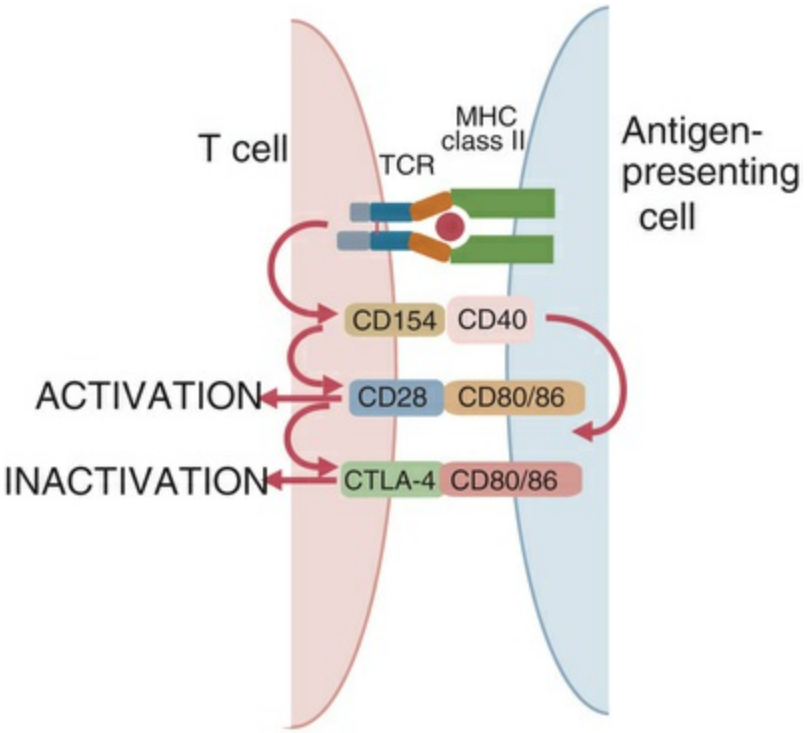


FIG. 14.7 Antigen-presenting cells and helper T cells engage in a dialog. Thus binding of antigen to the TCR causes the T cell to express CD40 ligand (CD154). This engages CD40 on the antigen-presenting cell. As a result, CD28 and CTLA-4 are expressed on the T cell, and CD80 or CD86 is expressed on the antigen-presenting cell. Depending on which receptors are engaged, the T cell may be stimulated or suppressed.

CD28-CD80/CD86 Signaling

CD28, a receptor induced by CD40-CD154 signaling on T cells, has two alternative ligands: either CD80 on dendritic cells, macrophages, and activated B cells, or CD86 on B cells. When CD80 or CD86 bind to CD28, signals are generated that cause the T cell, in turn, to express yet another receptor, CTLA-4 (also called CD152). CTLA-4 may bind to either CD80 or CD86. The binding of CD28 to its ligands amplifies the stimulus to the T cell eight-fold. CD28 stimulation enhances the production of IL-2 and other cytokines, upregulates cell survival genes, promotes energy metabolism, and facilitates T cell division. On the other hand, when CTLA-4 binds to CD80 or CD86, T cell activation is suppressed. The balance between these opposing signals delivered to T cells through these two receptors, CD28 and CTLA-4, regulates the intensity of T cell responses. (Inhibitors of CTLA-4 enhance T cell cytotoxicity and

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have proven to be highly effective anticancer drugs [Chapter 35].)

Resting antigen-presenting cells express neither CD80 nor CD86. It takes 48 to 72 hours after T cell CD154 binds to CD40 before the antigen-presenting cells begin to express CD80/86 and the T cells express CTLA-4. CD80 and CD86 can bind to either CD28 or CTLA-4. However, because CTLA-4 binds these molecules with a higher affinity than does CD28, the inhibitory effect of CTLA-4 gradually predominates. When CTLA-4 binds to CD80 on antigen-presenting cells, it induces the production of indoleamine dioxygenase (IDO), an enzyme that destroys tryptophan. In the absence of this amino acid, T cells cannot respond to antigen, and so the T cell response is terminated (Chapter 20).

Co-Stimulatory Cytokines

Cytokines, as described in Chapter 8, are proteins that regulate immune cell functions. Antigen-presenting cells are major sources of these cytokines. Antigen-presenting cells may be triggered to produce cytokines by many different stimuli. These include microbial PAMPs binding to TLRs, as well as T cells signaling through CD40 and CD154. As described in Chapter 10, different dendritic cell populations secrete different cytokine mixtures. These mixtures in turn determine the nature of the helper T cell response. For example, IL-12 from cDC1 cells promotes the differentiation of Th1 cells. Further differentiation is promoted by IFN-γ and IL-18. In the absence of IL-12, T cells differentiate into Th2 cells and their further differentiation is promoted by IL-33, -25, -4, -13, and TSLP. Classical type 2 dendritic cells (cDC2) and macrophages stimulated through TLR2 secrete IL-23. This cytokine, together with IL-6 and transforming growth factor-β (TGF-β), results in the development of Th17 cells (Fig. 14.8).

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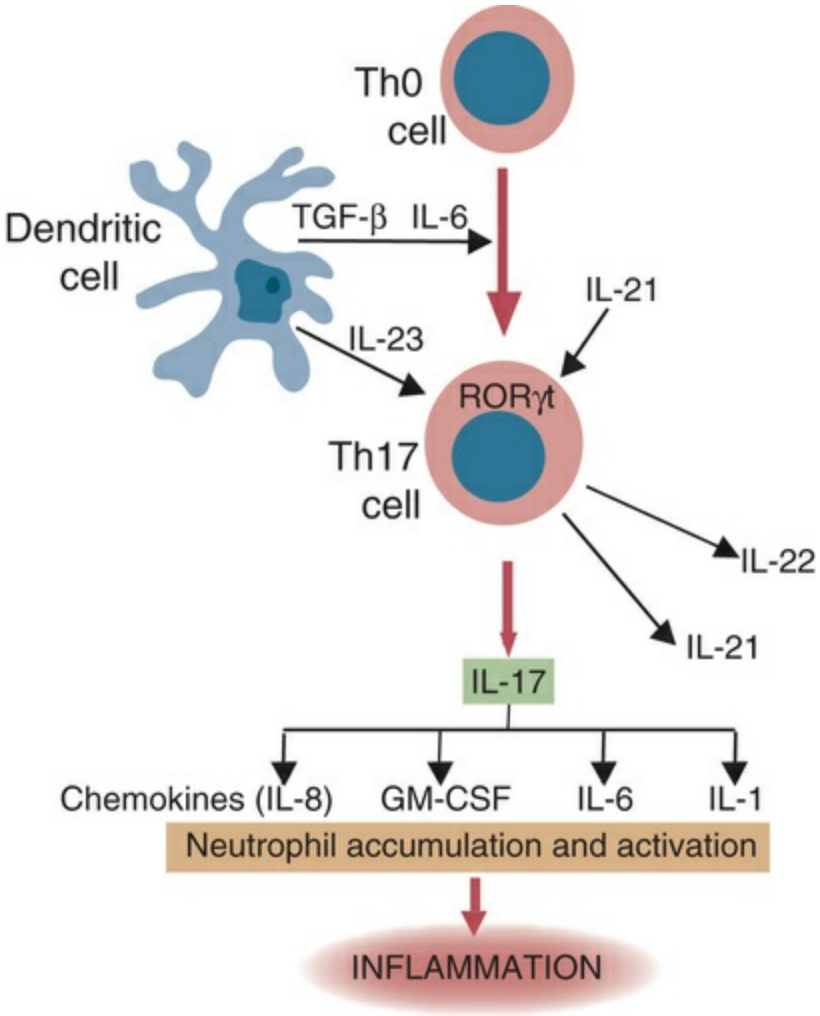


FIG. 14.8 The induction of Th17 cells by exposure to a cytokine mixture containing TGF-β and IL-6. Differentiation is promoted by IL-21 and maintained by IL-23. Th17 cells promote neutrophil accumulation and acute inflammation.

Adhesion Molecules

In addition to the dialog mediated by co-stimulatory molecules, T cells and antigen-presenting cells stimulate each other most effectively if they are linked by adhesion molecules such as the integrins. For example, CD2 and CD11a/CD18 on T cells bind to their ligands CD58 and CD54 on antigen-presenting cells and bind the cells together. Once tightly bound, an immunological synapse forms where the cells come into contact.

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Immunological Synapse Formation

Cell membranes consist of fluid lipid bilayers that contain segregated areas called lipid rafts, where patches in the membrane are enriched in sphingolipids, cholesterol, and proteins. Small rafts are distributed evenly over the surface of resting T cells. When a T cell and an antigen-presenting cell come into contact, their cell membrane rafts aggregate so that the TCR-peptide-MHC complexes and the co-stimulatory receptors cluster together in the area of contact to form an immunological synapse (Fig. 14.9). This synapse consists of concentric rings of molecular complexes called supramolecular activation clusters (SMACs). They form a characteristic "bull's eye structure" consisting of a central (c) SMAC surrounded by a peripheral (p) SMAC and an outer ring. The cSMAC of Th1 cells contains the MHC and TCR molecules as well as CD4, CD3, CD2, CD28, CD80/86, and CD40/154. The pSMAC contains CD45, the adhesion molecule ICAM-1 and leukocyte function-associated antigen-1 (LFA-1). The third, outer ring contains proteins excluded from the central synapse such as CD43. (CD43 is a very large antiadhesive molecule that could interfere with the functioning of the synapse.)

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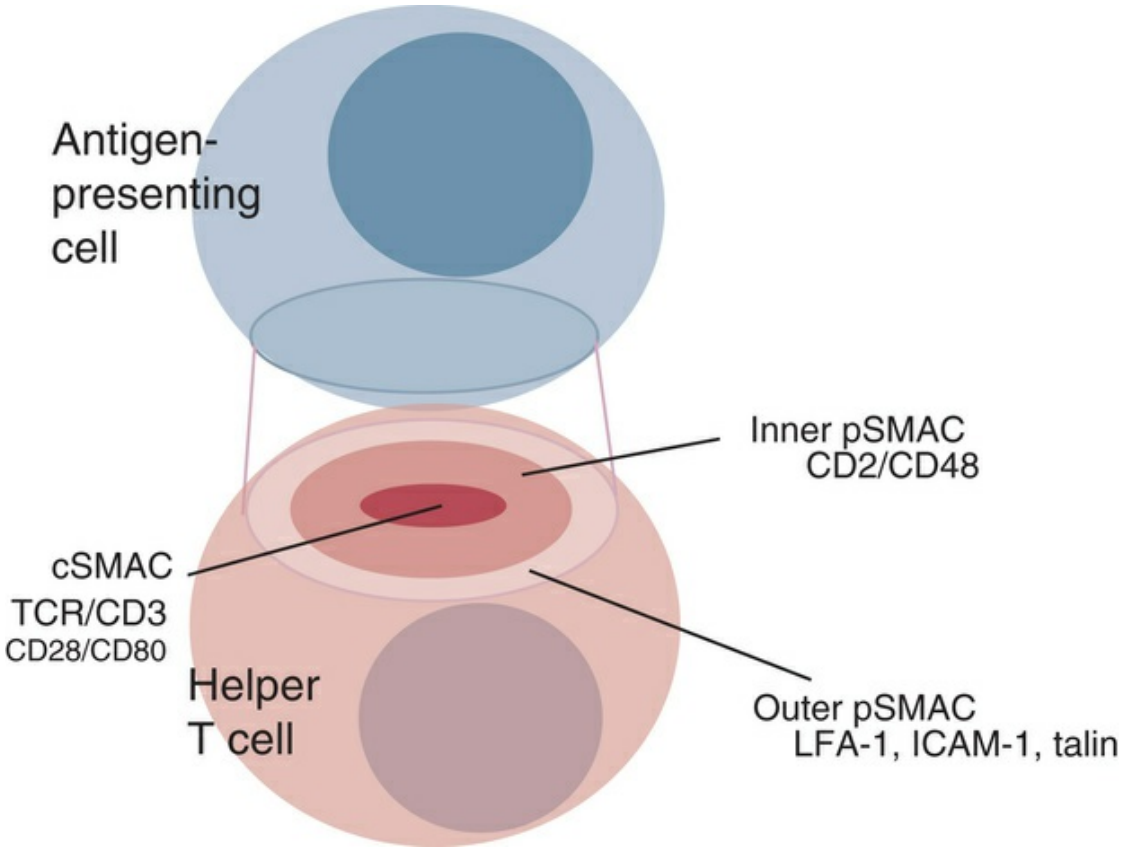


FIG. 14.9 Interaction between a T cell and an antigen-presenting cell generates the supramolecular structure called an immunological synapse. Thus a series of concentric rings form around the interacting TCR-MHC complex. The center contains the antigen-binding TCR. The middle ring contains different co-stimulating molecules. The outer ring contains the cell-binding proteins.

Th2 cells, in contrast, do not form a “bull's eye” synapse with APCs. They form multifocal immunological synapses at high antigen concentrations. Whereas the immunological synapse is on the cell surface, mitochondria in the cytosol migrate beneath the synapse and reduce the local concentration of calcium ions. This inactivates Ca channels in the plasma membrane, resulting in a sustained Ca influx and activation of transcription factors such as NF-AT.

It is important to note that T cells may initially form synapses with multiple antigen-presenting cells but then polarize toward the cell providing the strongest stimulus. Thus, in effect, the T cell seeks the antigen that binds most strongly to its TCR. Once signaling is complete, the synapse is endocytosed and degraded, terminating cell interactions.

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Signal Transduction

Once a TCR binds to antigen on a presenting cell and an immunological synapse forms, the receptor signals to the T cell. Several TCRs cluster so that the immunoreceptor tyrosine-based activation motifs (ITAMs) on the CD3 chains can activate their tyrosine kinases (Chapter 8). These form a signaling complex that acts through calcineurin to activate NF-AT. It also activates the ras-mitogen-activated protein kinase (MAPK) pathway that triggers AP-1 production and a protein kinase C-dependent pathway that activates NF-κB. These three transcription factors activate multiple cytokine genes (see Fig. 8.11). As a result, the T cells enter the cell cycle and synthesize and secrete a mixture of cytokines (Fig. 14.10). These newly produced cytokines trigger the next stages of the immune responses.

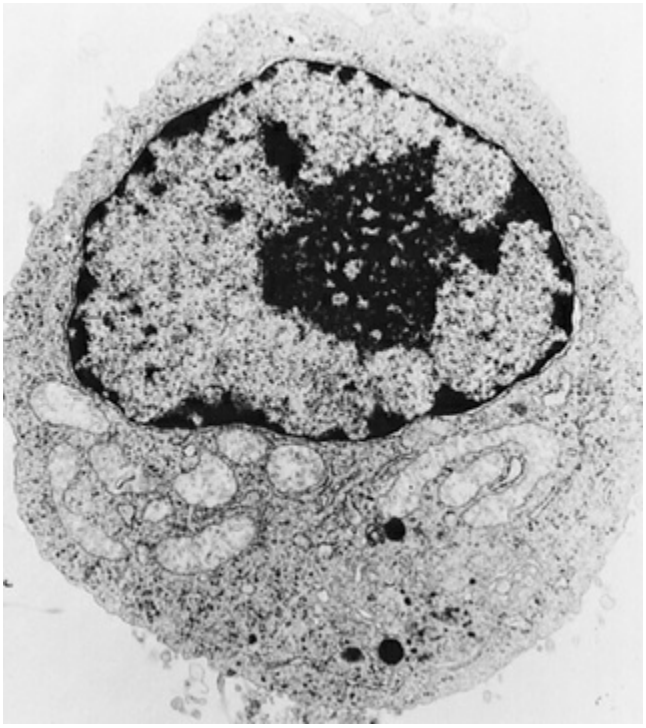


FIG. 14.10 Transmission electron micrograph of a lymphoblast. Compare this with an unstimulated lymphocyte in Fig. 13.2. Note the extensive cytoplasm, ribosomes, and large mitochondria.
(Courtesy Dr. S. Linthicum.)

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Overall Considerations

T cells are highly mobile cells. As described in Chapter 12, they migrate rapidly through lymph nodes while continuously scanning the surfaces of dendritic cells for antigens. When it recognizes a foreign antigen, a T cell changes its behavior. It slows down, stops, and eventually binds strongly to the antigen-presenting cell and forms a synapse. Whether or not a T cell stops depends on how strongly it binds to the target antigen. It will not stop for weakly binding antigens.

Once the synapse forms, TCRs and co-stimulatory molecules signal to the T cell. However, the TCR does not function simply as an on/off switch. Instead, differences in the strength of binding, in the amount of co-stimulation, and in the duration of the cell interaction affect T cell responses.

Because MHC molecules can bind many different antigenic peptides, any individual peptide will only be displayed in small amounts. T cells must be able to recognize these few specific peptide-MHC complexes among a vast excess of MHC molecules carrying irrelevant peptides. The number of MHC-peptide complexes signaling to the T cell is also important since the stimulus needed to trigger a T cell response varies. For example, only one MHC-peptide complex is needed to trigger a $CD8^+$ T cell response. At least 8000 TCRs must bind antigen for a $CD4^+$ T cell to become activated in the absence of CD28, but only about 1000 TCRs need be engaged if CD28 is present (Fig. 14.11). The duration of signaling also determines a T cell's response. In the presence of appropriate antigens, T cells need to bind DCs for less than 15 seconds before pulling away. T cells can make 30 to 40 APC contacts within a minute. Sustained signaling is, however, required for maximal T cell activation and is maintained by serial triggering of its TCRs. Thus during the prolonged cell interaction process, each MHC-peptide complex may trigger up to 200 TCRs. This serial triggering depends on the kinetics of TCR-ligand interaction. CD28, for example, reduces the time needed to trigger a T cell and lowers the threshold for TCR triggering. Adhesion molecules stabilize the binding of T cells to antigen-processing cells and allow the signal to

be sustained for hours.

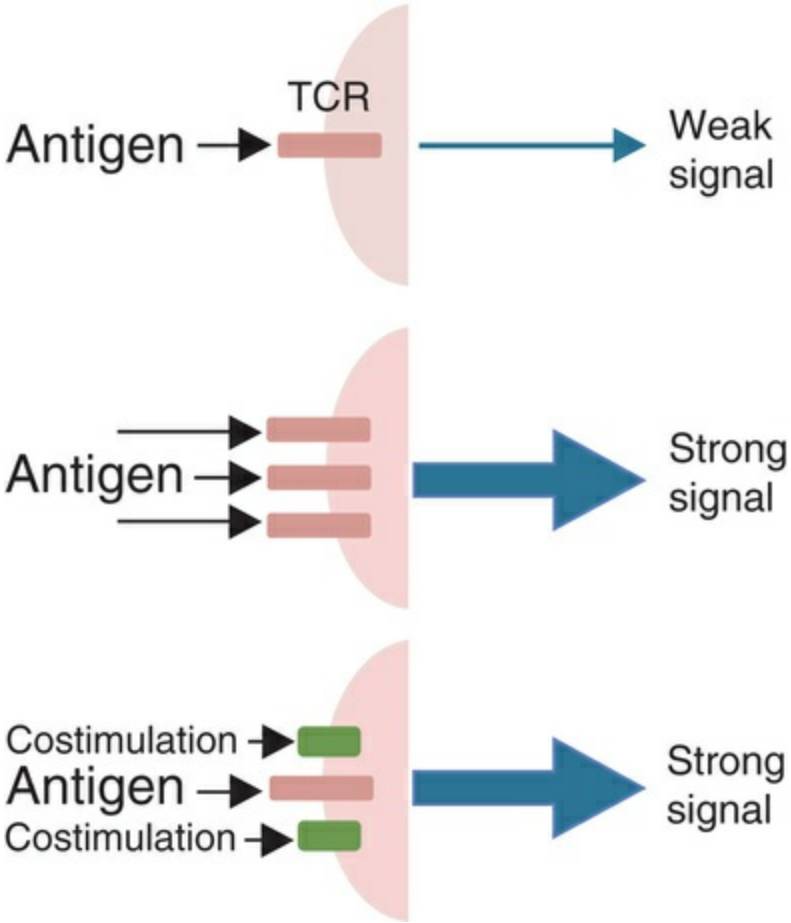


FIG. 14.11 Successful stimulation of a T cell requires the additive effects of multiple signals. Depending on the antigen, the T cell may be activated by signals from multiple TCRs or by appropriate costimulation.

The properties of a helper T cell are determined by the type of antigen-presenting cell used and by the nature of the signals received from it. Thus naïve T cells have strict requirements for activation. They must receive a sustained signal for at least 10 hours in the presence of co-stimulation or for up to 30 hours in its absence. This level of co-stimulation can only be provided by dendritic cells, which supply high levels of co-stimulatory and adhesion molecules. In contrast, other antigen-presenting cells act only transiently. Although macrophages and B cells can briefly trigger a TCR, they are unable to complete the process and thus fail to activate naïve T cells. Once primed, however, T cells require about an hour to reach commitment. Only then can they be

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activated by macrophages and B cells. In the absence of effective co-stimulation, T cells undergo abortive activation. They do not divide or produce cytokines but either become unresponsive to antigen (anergic) or undergo apoptosis.

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Superantigens

Fewer than 1 in 10,000 T cells can bind and respond to any specific foreign antigen. However, some microbial molecules called superantigens are unique in that they may stimulate as many as one in five T cells. These molecules are not simply nonspecific mitogens. Superantigens only activate T cells with specific TCR Vβ domains. Unlike conventional antigens that must bind within the grooves of both an MHC molecule and a TCR, superantigens directly link a TCR Vβ domain to an MHC class II molecule on the antigen-presenting cell. All superantigens come from microbial sources such as streptococci, staphylococci, and mycoplasma, and from viruses such as rabies. The responses to superantigens are not MHC restricted (i.e., they do not depend on specific MHC haplotypes), but the presence of MHC antigens is required for an effective response since superantigens do not bind to the antigen-binding groove of the MHC class II molecule but attach elsewhere on its surface (Fig. 14.12). As a result, they bind the T cell and the antigen-presenting cell together. Because of this strong binding, superantigens trigger a powerful T cell response. Some superantigens may stimulate the secretion of so many cytokines that they trigger a toxic shock syndrome (Chapter 7).

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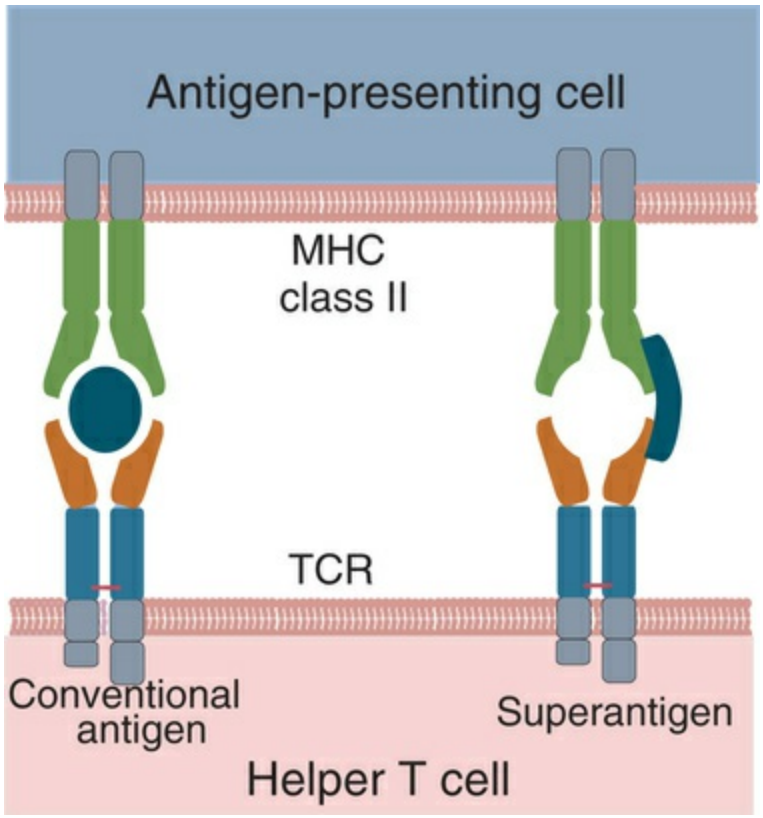


FIG. 14.12 Differences in binding to a TCR between a conventional antigenic peptide that fills the groove between the α and β chains as opposed to a superantigen that binds only to the β chain.

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Helper T Cell Subpopulations

$CD4^+$ T cells are very diverse. As naïve T cells develop and differentiate, four major subpopulations emerge. They are called helper 1 (Th1), helper 2 (Th2), helper 17 (Th17), and regulatory (Treg) cells, and each is distinguished by the mixture of cytokines that they secrete (Fig. 14.13). As always, many of the details of their function have been investigated in mice and humans, and it must not be assumed that they function in a completely identical manner in other mammals.

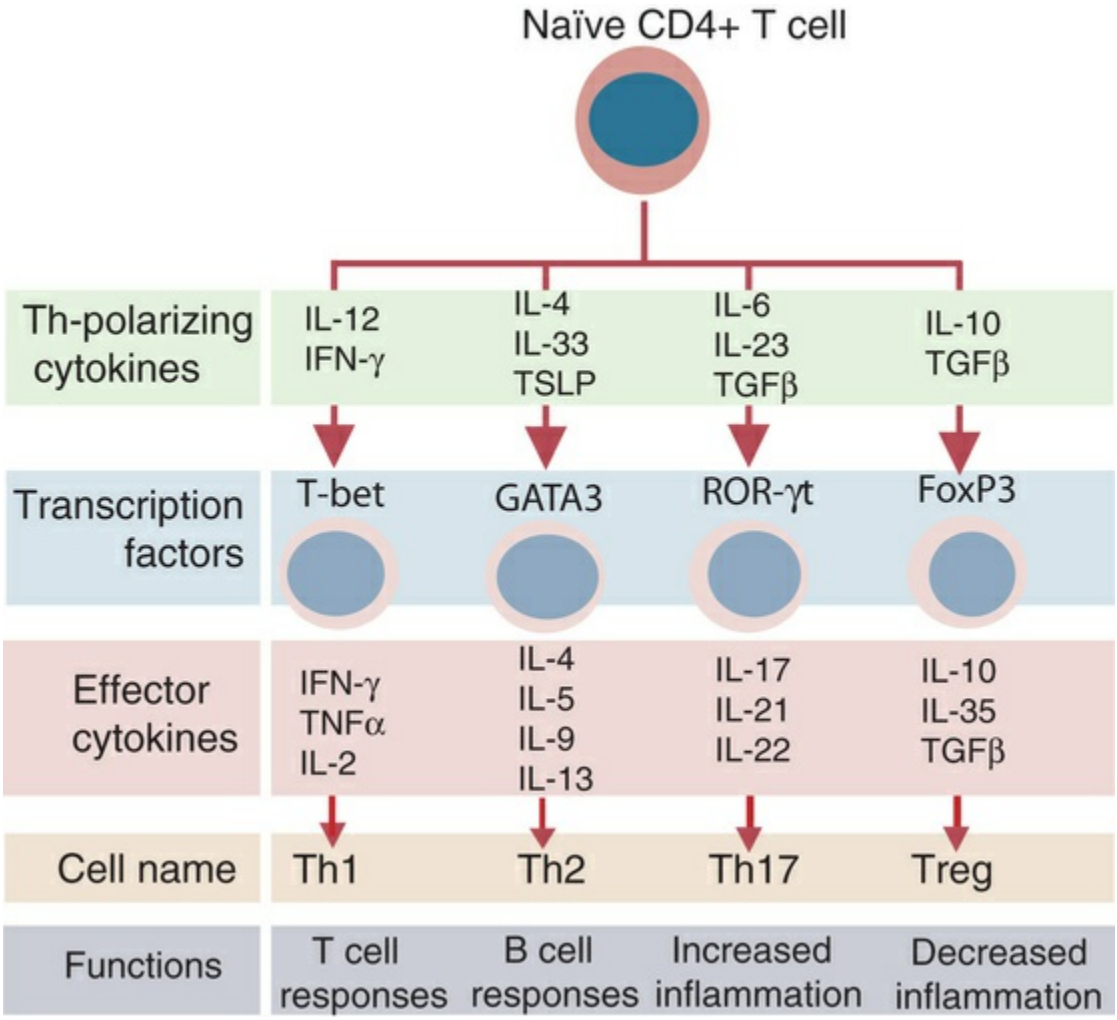
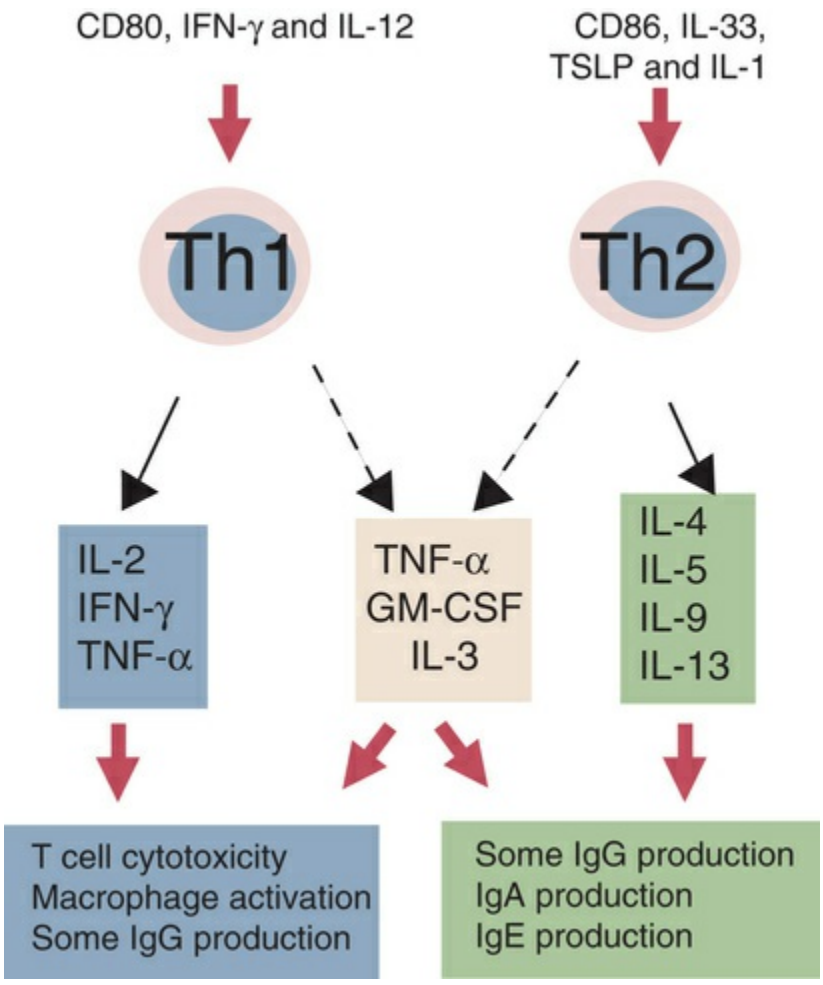


FIG. 14.13 The major populations of helper T cells. Note that their differentiation is induced by different mixtures of polarizing cytokines. These induce specific transcription factors in each population. Once polarized, the T cells synthesize and secrete different mixtures of effector cytokines.

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Th1 Cells

The production of Th1 cells is driven by IL-12 from antigen-presenting dendritic cells (cDC1), macrophages (M1), and B cells, plus co-stimulation by CD80. Complete Th1 cell activation, proliferation, and IFN-γ production is achieved by additional stimulation by IFN-γ. This signaling activates the transcription factor T-bet. T-bet is the master regulator of Th1 cell differentiation. Once activated, Th1 cells produce IL-2, IFN-γ, and TNF-α (Fig. 14.14). Th1 cells promote cell-mediated immune responses such as the delayed hypersensitivity reaction and macrophage activation. They thus generate immunity to intracellular organisms such as mycobacteria and viruses (Fig. 14.15). These "type-1"–associated cytokines also inhibit type-2 immune responses.



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FIG. 14.14 Major differences between Th1 and Th2 populations. Note that the polarizing cytokines that trigger them are different, as are the set of effector cytokines they secrete.

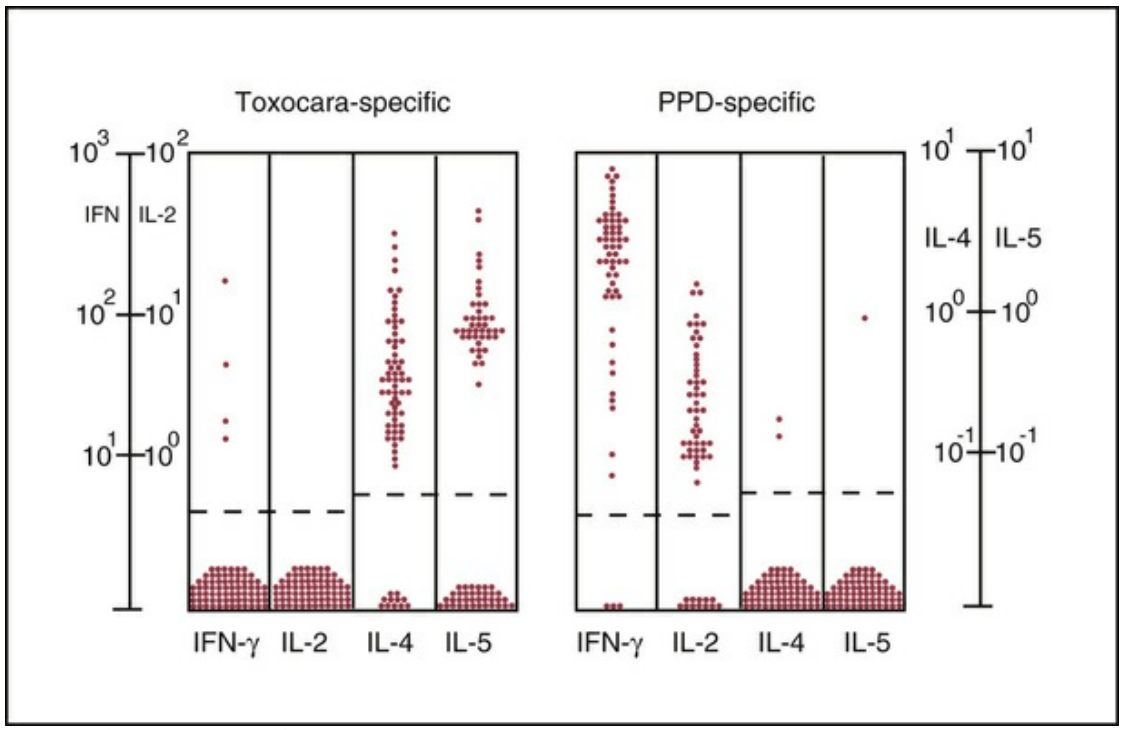


FIG. 14.15 Different antigens can trigger distinctly different Th cell subpopulations. For example, T cells exposed to a parasite antigen from the roundworm *Toxocara canis* mount a type 2 response and secrete IL-4 and IL-5. In contrast, T cells exposed to PPD, an antigen from *Mycobacterium tuberculosis,* mount a type 1 response characterized by secretion of IFN-γ and IL-2. (From Del Prete G, De Carli M, Mastromauro C, et al: Purified protein derivative of *Mycobacterium tuberculosis* and excretory-secretory antigen(s) of *Toxocara canis* expand in vitro human T cells with stable and opposite (type 1 T helper or type 2 T helper) profile of cytokine production, *J Clin Invest* 88:346-350, 1991.)

Interferon-γ

Interferon-γ has some antiviral activity, but its major function is the regulation of Th1 cell responses (Fig. 14.16). IFN-γ is mainly produced by Th1 cells, $CD8^+$ cytotoxic T cells, and natural killer (NK) cells, with lesser amounts from antigen-presenting cells, B cells, and natural killer T (NKT) cells (Chapter 19). It activates cells through the JAK-STAT pathway. It promotes macrophage activation, suppresses Th2 cells, and enhances NK cell activity (Box

14.1).

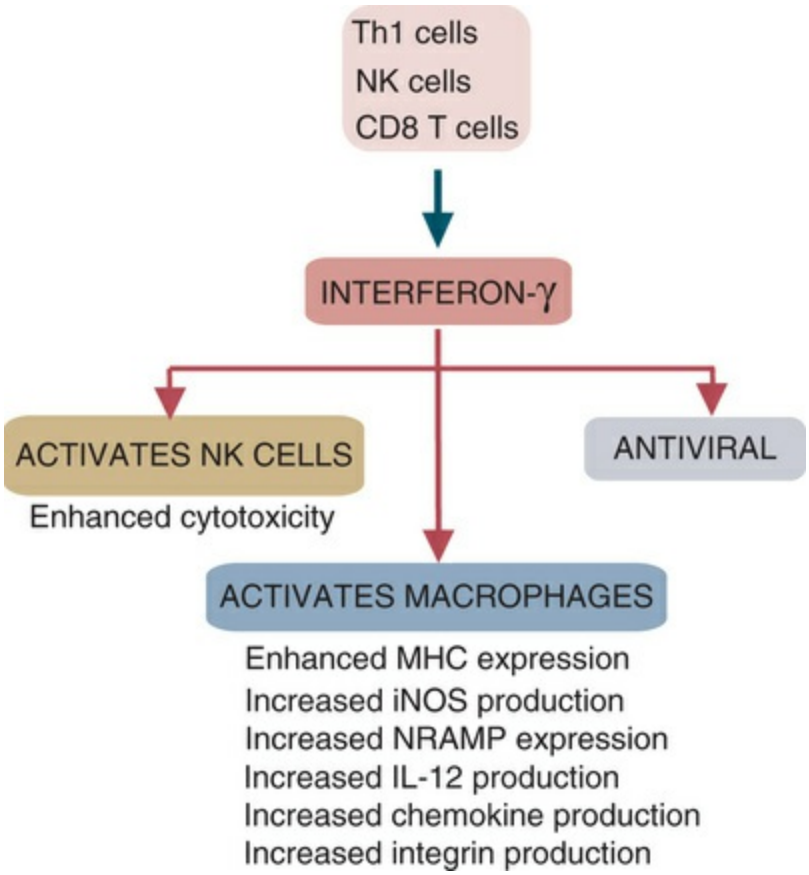


FIG. 14.16 The origins and some properties of IFN-γ.

Box 14.1

Type 1 and Type 2 Immunity

There is a growing tendency among immunologists to classify protective immune responses into two types. Type 1 immunity encompasses the use of Th1 cells. However, it also involves the use of Th17 cells, cytotoxic T cells, group 1 and 3 innate lymphoid cells, and M1 macrophages, as well as immunoglobulins G, M and A. Type 1 responses are responsible for immunity to bacteria, viruses, protozoa and fungi. It employs the cytokines IFN-γ, IL-12, -17, and -18. Type 2 immunity, in contrast, encompasses the use of Th2 cells. In addition, it includes group 2 innate lymphoid cells, basophils, mast cells, eosinophils, M2 macrophages, and immunoglobulin E. It also employs the cytokines IL-4, -5, -9, -13, -25, -33, and TSLP.

Type 2 responses are responsible for immunity to parasitic helminths and arthropods as well as for allergic responses (Fig. 14.17).

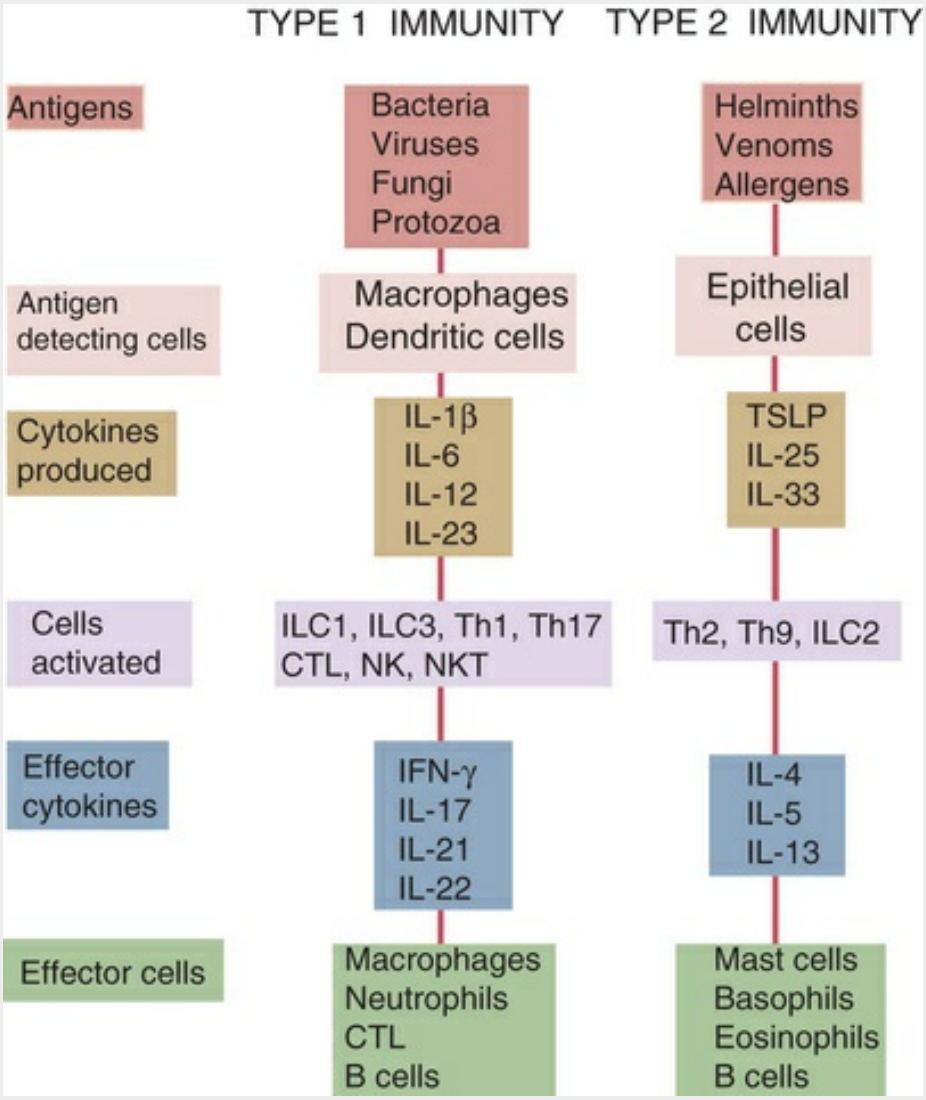


FIG. 14.17 A comparison of the key features of type 1 and type 2 immune responses.

Interleukin-2

IL-2 is produced by activated $CD4^+$ Th1 cells. Some is also produced by $CD8^+$ cells, NKT cells (Chapter 19), dendritic cells, and mast cells. Its targets are T, B, and NK cells and macrophages. IL-2 is a potent stimulator of T cell proliferation, IFN-γ production, and antibody production by B cells. It enhances the cytotoxicity of $CD8^+$

and NK cells (Fig. 14.18). It promotes T cell differentiation into Th1 and Th2 subsets while also inhibiting Th17 differentiation. IL-2 is essential for the survival of regulatory T cells as well as activation-induced cell death. Thus it has a broad range of essential functions.

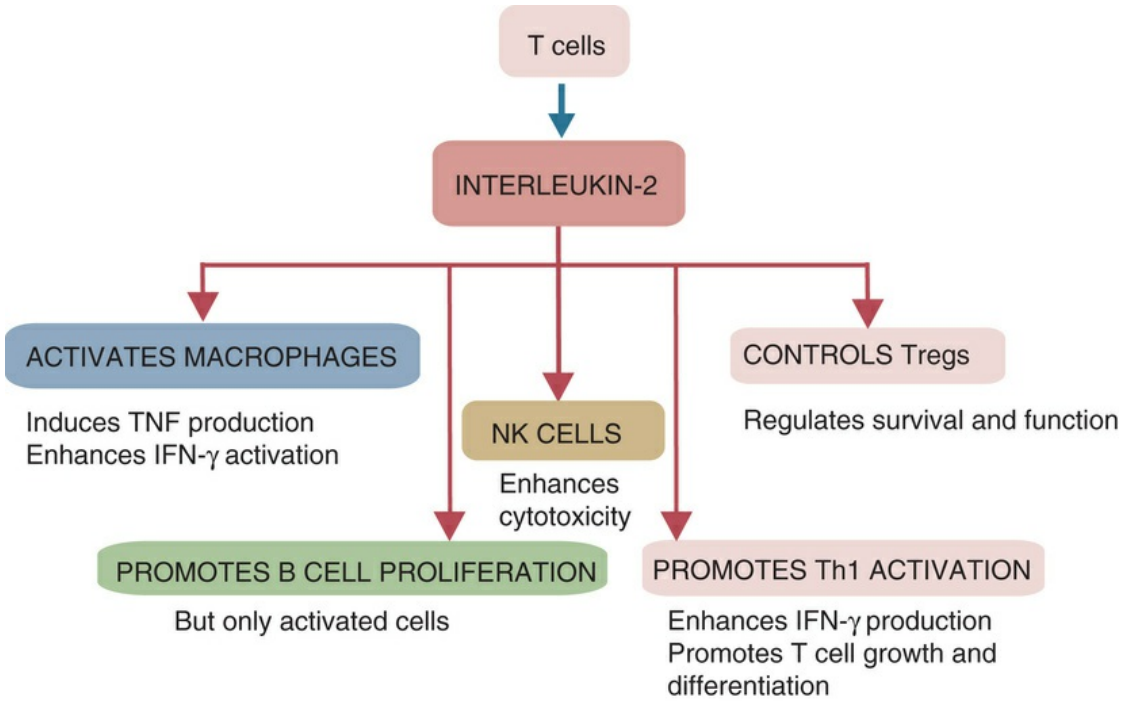


FIG. 14.18 The origins and some properties of interleukin-2.

Th2 Cells

cDC2 cells preferentially promote Th2 cell differentiation. The major Th2 stimulators are IL-4, IL-33, and TSLP. These Th2 cells respond optimally to antigen presented by cDC2 and macrophages, and less well to antigen presented by B cells. The cDC2 cells provide additional co-stimulation through CD86. Th2 cells may require co-stimulation by IL-1 from macrophages or dendritic cells.

Activated Th2 cells secrete IL-4, IL-5, IL-9, and IL-13 and so mediate "type 2" immune responses (Fig. 14.19). These cytokines stimulate B cell proliferation and immunoglobulin secretion but tend to suppress cell-mediated reactions. The cytokines from Th2 cells enhance B cell production of immunoglobulin G (IgG) and IgA up to 20-fold and production of IgE up to 1000-fold. Type 2 responses are associated with enhanced immunity to parasitic

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worms but decreased resistance to mycobacteria and other intracellular organisms. They suppress some autoimmune diseases, and they neutralize toxins and regulate wound and tissue repair following infection and injury. When not carefully regulated, type 2 responses may trigger damaging allergic responses.

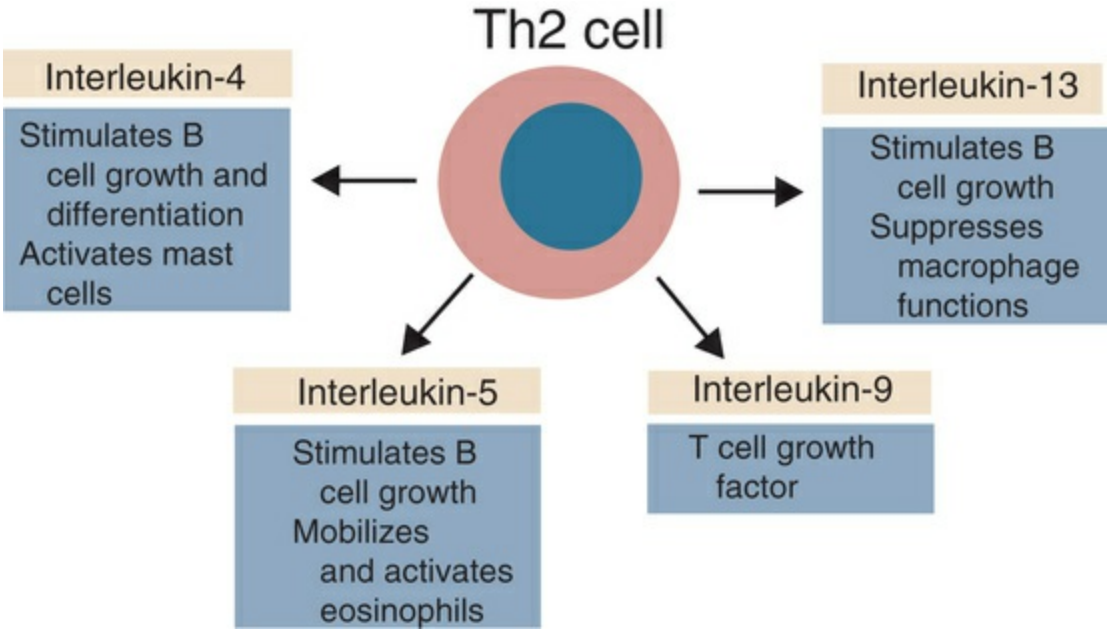


FIG. 14.19 The cytokines produced by Th2 cells and their major properties.

Interleukin-4

IL-4 is a glycoprotein produced by Th2 cells and mast cells. Its targets are T cells, B cells, and macrophages. Signaling through STAT6, IL-4 activates the Th2-specific transcription factor GATA3. GATA3 is the master regulator of Th2 differentiation. IL-4 promotes IgG and IgE production and inhibits IFN-γ expression and Th17 cell production. In humans and rodents, IL-4 is essential for antibody production because it stimulates B cell activity (Fig. 14.20). In pigs, however, IL-4 blocks antibody and IL-6 production and suppresses antigen-induced B cell proliferation. Thus IL-4 may play a very different role in pigs than it does in mice or humans. IL-4 shares overlapping intracellular signaling pathways and biological functions with IL-13.

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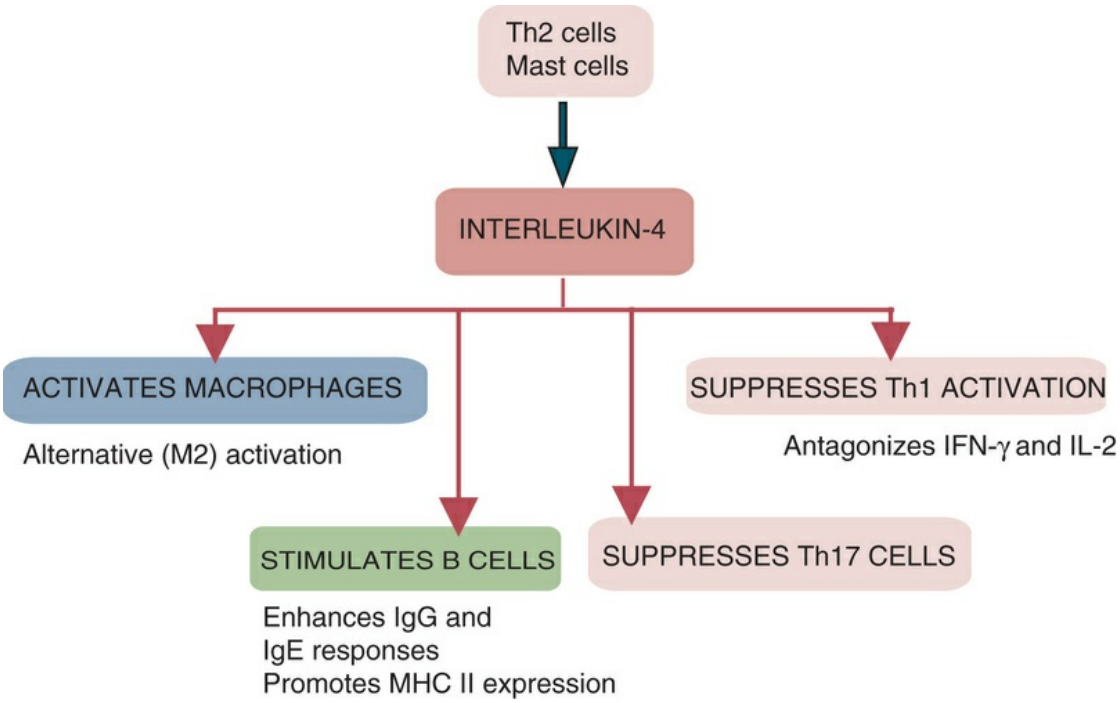


FIG. 14.20 The origins and some properties of interleukin-4.

Some helper T cells secrete a mixture of Th1 and Th2 cytokines. These cells may be precursors of Th1 and Th2 or cells that are in transition between the two populations. Some IL-2-secreting T cells may switch to become IL-4–secreting cells after exposure to antigen, implying a change in phenotype from Th1 to Th2. The principal molecules that control this switch are IL-4 and IL-12. When cultured in the presence of IL-4, undifferentiated helper cells become Th2 cells. When cultured in the presence of IL-12, they become Th1 cells. Mixed cell populations are most obvious early after initiation of an immune response, whereas Th1 and Th2 subsets are more obvious in chronic diseases where the antigens are persistent and cannot be easily removed.

Th17 Cells

The second major population of $CD4^+$ T cells produce IL-17 and hence are called Th17 cells (see Fig. 14.13) (Box 14.2). The development of Th17 cells is initiated by IL-23. IL-23 triggers the production of a unique transcription factor called ROR-γt. Th17 growth is then promoted by TGF-β, IL-6, and IL-21. These molecules induce the Th17 cells to produce a mixture of cytokines, namely IL-17A, IL-17F, IL-21, and IL-22. Th17 cells have two major

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functions: they mediate inflammation and they are potent B-cell helpers (see Fig. 20.14). Cytokines of the IL-17 family play a key role in protective type 1 responses to extracellular bacteria and assist in the clearance of fungi. Under some circumstances, Th17 cells may convert into IFN-γ–producing Th1 cells. Likewise, they can differentiate into regulatory T cells (Tregs) when inflammation is resolved. The balance between Th17 and Treg cells is critical to maintaining homeostasis during immune responses and inflammation. Excessive Th17 activity can lead to the development of chronic inflammatory diseases.

Box 14.2

Interleukin-17

The interleukin-17 family contains multiple members (IL-17A through F). IL-17F is also called IL-25. They have no sequence similarity to any other cytokines. The most important is IL-17A since it induces inflammation. IL-17 is produced by Th17 cells under the influence of IL-23. IL-17 binds to a family of cell surface receptors (IL-17RA through RE) that signal through NF-κB.

IL-17 plays a key role in immunity to extracellular bacteria and fungi since it recruits granulocytes through its actions on stem cells. It stimulates the production GM-CSF leading to a neutrophilia (Chapter 5). It promotes the recruitment and survival of macrophages, and it stimulates the production of proinflammatory cytokines and antibacterial peptides from many cell types. It attracts neutrophils and macrophages (but not eosinophils) to inflammatory sites. It triggers production of many other cytokines such as IL-1, IL-6, GM-CSF, and TNF-α, as well as chemokines and prostaglandins. IL-17 contributes significantly to immunologic diseases such as asthma, lupus, and rheumatoid arthritis.

Regulatory T Cells (Tregs)

Regulatory T cells are typical lymphocytes that express CD4 and CD25 (the α chain of the IL-2 receptor, which is discussed in detail in Chapter 20). Their most characteristic feature, however, is their

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use of the transcription factor, FoxP3. Treg cells act through several pathways. They contact other cells to deliver suppressive molecules such as TGF-β, or cytotoxic granzymes and perforins. They also produce suppressive cytokines such as IL-10 and IL-35. As a result, Treg cells suppress the responses of both Th1 and Th2 cells and prevent inappropriate T cell activation in the absence of antigens. The balance between Treg cells and Th2 cells regulates many inflammatory and allergic diseases.

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Species Differences

The details of helper T cell subpopulations described above have largely been derived from studies in laboratory mice and humans. Cattle most certainly possess Th1 and Th2 cells and can mount polarized immune responses. Bovine IgG1 expression is positively regulated by IL-4 and IgG2 expression by IFN-γ. Many bovine $CD4^+$ cells produce IL-2, IL-4, IL-10, and IFN-γ.

γ/δ T Cells

The proportion of T cells with γ/δ TCRs differ greatly between mammals. In some species, such as humans and mice, fewer than 5% have γ/δ TCRs (γ/δ-low). In others, such as pigs and ruminants, up to 66% of T cells in young calves and 85% in piglets express γ/δ TCRs (γ/δ-high). This decreases with age but still remains relatively high in adults (8%-18% in adult cattle). These cells have different functions in these two groups of mammals (Fig. 14.21).

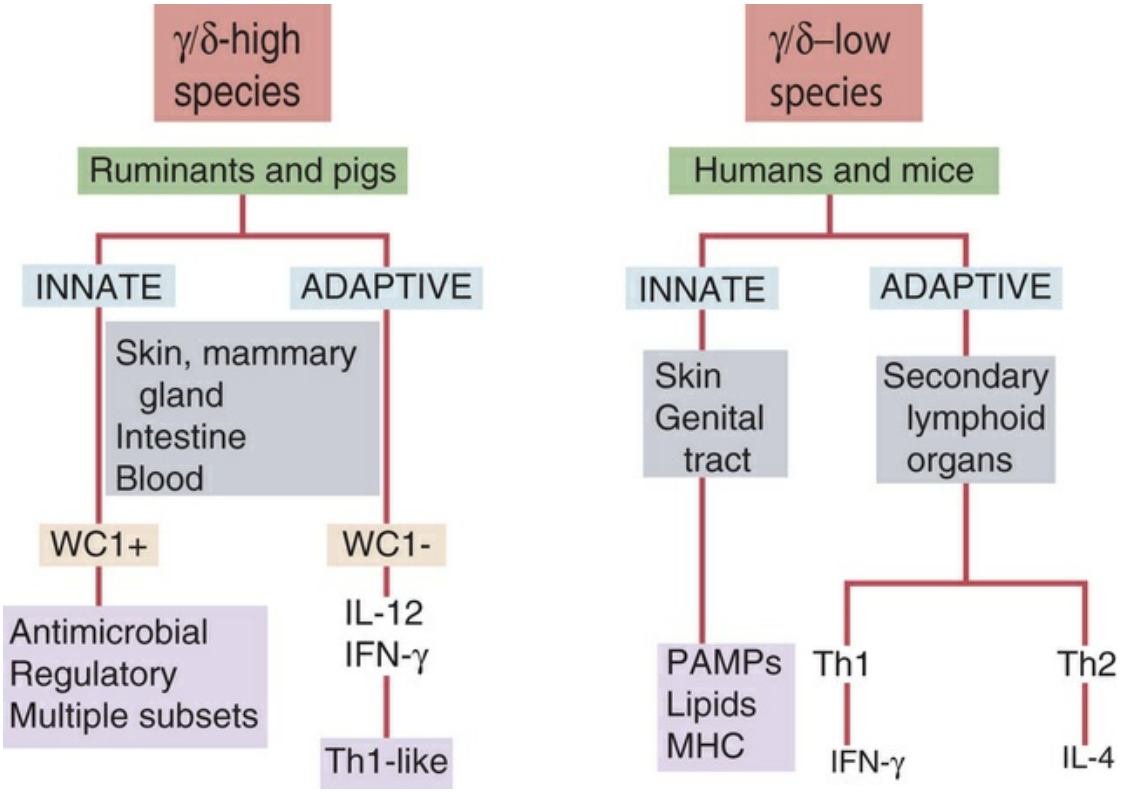


FIG. 14.21 γ/δ T cells may, depending on the species, act as innate immune cells with an invariant antigen receptor. Others may act as classic helper T cells with diverse TCRs of polyclonal origin.

In γ/δ-low species, there are two subsets of γ/δ cells. One subset is engaged in innate immunity, has limited γ/δ receptor diversity, and is mainly found in the skin and genital tract. The other subset is engaged in adaptive immunity, has extensive receptor diversity, and is found in secondary lymphoid organs and the digestive tract. The innate γ/δ cells preferentially bind microbial PAMPs, especially heat-shock proteins and phospholigands (carbohydrates or nucleotides with a phosphate group). They also respond to the MHC class Ib molecules, MIC-A and MIC-B, produced by stressed cells, cancer cells, and virus-infected cells (Chapter 19). These innate γ/δ T cells also respond to lipid antigens presented by CD1 molecules (see Chapter 19). When stimulated, they secrete IL-17 and IFN-γ. Like Th17 cells, innate γ/δ T cells are activated by IL-23.

In contrast, the adaptive subset of γ/δ T cells can be further subdivided into helper and effector cells. These effector cells can destroy cells infected with mycobacteria and some leukemic cells. In γ/δ-high species, these T cells colonize the skin, mammary gland, reproductive organs, tonsils, and the intestinal wall where they form the major T cell population. They are polyclonal at birth, but their diversity decreases with age. Between 50% and 99% of ruminant blood γ/δ T cells express WC1 and are engaged in innate immunity, whereas WC1-negative cells are regulatory cells. $WC1^+$ and $WC1^-$ cells have a different tissue distribution. $WC1^-$ predominate in the spleen and uterus. $WC1^+$ γ/δ T cells are found in granulomas surrounding schistosomes and mycobacteria. In these cases the initial T cell infiltration is dominated by γ/δ T cells and this is followed by α/β T cells. A second wave of γ/δ T cells may terminate the response. These $WC1^+$ cells secrete IL-12 and IFN-γ and may promote a Th1 bias in the immune response. IFN-γ–producing bovine γ/δ $WC1^+$ cells are the major population involved in recall responses to *Leptospira.*

Bovine γ/δ T cells respond to microbial PAMPs by increasing the expression of lymphotactin (XCL1), MIP-1β, TNF-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF). They express TLR3, TLR9, mannose-binding lectin, and CD36. These γ/δ

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T cells may well be major contributors to innate immunity, especially since many of them have non-polymorphic TCRs. They recognize microbial glycolipids presented by CD1-positive antigen-presenting cells and release cytokines and lyse target cells just like conventional α/β NKT cells (Chapter 19). The role of γ/δ T cells on mucosal surfaces is discussed further in Chapter 22. Some bovine γ/δ T cells serve a regulatory function. They spontaneously secrete IL-10 and can inhibit antigen-specific and nonspecific $CD4^+$ and $CD8^+$ T cell proliferation *in vitro.*

Pigs also have multiple phenotypically distinct subpopulations of γ/δ T cells. Some may produce IFN-γ alone, TNF-α alone, or both IFN-γ and TNF-α. Other subpopulations can produce IL-17. Some have differing levels of TCR γ/δ expression.

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Memory T Cells

As described, when naïve T cells encounter antigens with appropriate co-stimulation, they differentiate into multiple effector T cell populations. These effector cells are usually short lived because they are eliminated by apoptosis. However, some resist apoptosis, and develop into long-lived memory cells. These can be thought of as "antigen-experienced" cells. Memory T cells can be the most abundant T cell population in the body, especially in older animals since they accumulate throughout life. Compared to naïve T cells, memory cells are easier to activate, live longer, and have enhanced effector activity. As a result, they mount a strong rapid cytokine response the next time they encounter the antigen and can provide life-long protection against pathogens. The differences in behavior between naïve and memory T cells likely results from epigenetic modifications that alter gene transcription (mainly histone methylation [Chapter 20]) and hence cell functions.

This development of effector and memory T cell populations results from asymmetrical T cell division (Fig. 14.22). As described earlier, naïve T cells interact with antigen-presenting cells for several hours through an immunological synapse. Once it receives sufficient stimulation, a T cell begins to divide even before it separates from the antigen-presenting cell. The dividing T cell is polarized since one pole of the cell contains the immunological synapse and associated structures. The other pole contains molecules excluded from the synapse. Thus when the cell divides, it forms two distinctly different daughter cells. The daughter cell adjacent to the synapse is the precursor of the effector T cells. The daughter cell formed at the opposite pole is the precursor of the memory T cells.

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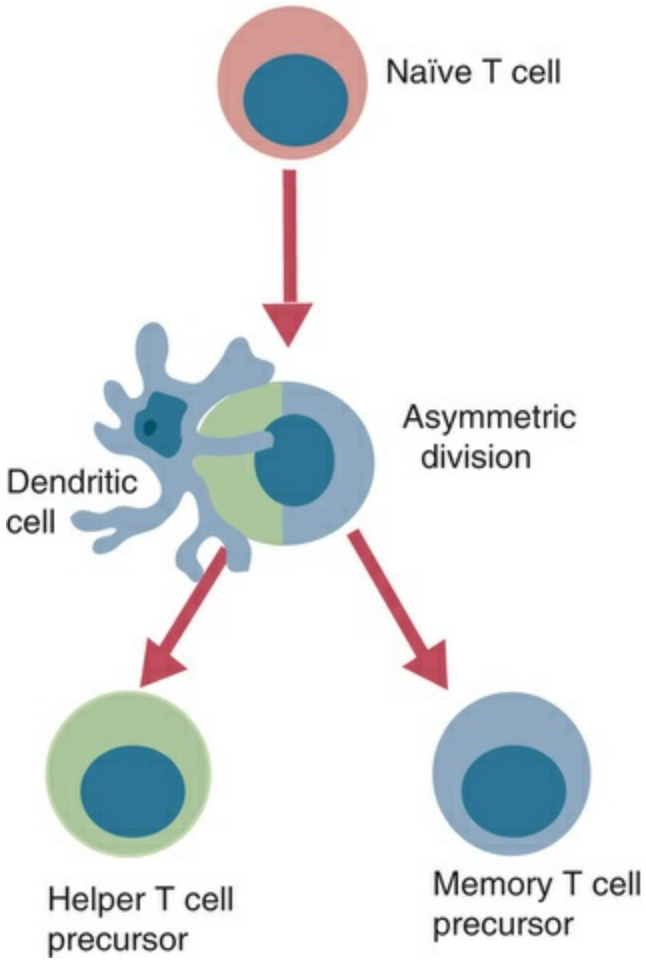


FIG. 14.22 Following T cell-DC interactions, the T cell divides asymmetrically. The cell at the pole in contact with the antigen-presenting dendritic cell synapse becomes a helper T cell. The cell at the opposite pole receives different signals and so becomes a memory T cell.

Three types of memory T cells have been characterized. These are central memory cells, tissue-resident memory T cells, and effector memory T cells. Central memory T cells circulate through secondary lymphoid tissues, such as lymph nodes, awaiting the arrival of invaders. They lack immediate effector function but have very rapid recall responses. Effector memory T cells, in contrast, have receptors enabling them to hone to inflamed tissues, where they immediately attack invaders without the need to differentiate further. Tissue-resident memory T cells occupy tissues and provide a first response to pathogens invading through body surfaces. They rapidly produce cytokines after infection. They do not circulate in peripheral blood. All memory T cell populations express either CD4 or CD8 and persist in the absence of antigen. $CD8^+$ memory cells

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tend to accumulate under epithelial surfaces, while $CD4^+$ memory cells are scattered through the tissues in memory lymphocyte clusters. These cells slowly divide and replenish their numbers. In fact, they can be thought of as adult stem cells. IL-7 and IL-15 are required for the survival of memory $CD8^+$ T cells, whereas only IL-7 is needed for the survival of $CD4^+$ T cells. These maintain the cells in a state of slow proliferation. In humans, memory $CD4^+$ T cells have a half-life of 8 to 12 years, whereas memory $CD8^+$ T cells have a half-life of 8 to 15 years. The size of the immune system is fixed somewhat, but the effector memory $CD8^+$ T cell pool can double without loss of preexisting memory cells.

Human memory T cells express TLR2. If exposed to its ligand lipopeptide in the presence of either IL-2 or IL-15, they will proliferate. Thus it is possible that bacterial PAMPs such as the lipopeptides may promote the long-term survival of memory T cells even in the absence of persistent antigen.

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B Cells and Their Response to Antigens

CHAPTER OUTLINE

B Cell Antigen Receptors, 148
Antigen-Binding Component, 148
Light Chains, 148
Heavy Chains, 148
Variable Regions, 148
Constant Regions, 149
Hinge Region, 149
Signal Transducing Component, 149
Antigen Presentation by B Cells, 150
Co-Stimulation of B Cells, 150
T Cell Help, 150
Cytokine Secretion, 151
Cell-Cell Signaling, 151
Complement Help, 151
Toll-Like Receptors and PAMP Help, 151
B Cell Response, 151
Differential Signaling, 152

Cellular Responses, 154
Plasma Cells, 154
Memory B Cells, 154
Germinal Centers, 156
B Cell Subpopulations, 157
Myelomas, 157
Polyclonal Gammopathies, 159
Hybridomas, 159

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Briefly describe the origins and life history of B cells.
- Describe the basic structure of a B cell antigen receptor (BCR).
- Explain how BCRs are shed into body fluids to form immunoglobulins or antibodies.
- Understand that an optimal B cell response normally requires additional stimulation by helper T cells.
- Describe how helper T cells stimulate B cells through an immunologic synapse.
- Explain how B cells also require co-stimulation by cytokines.
- Understand how responding B cells may become either memory cells or antibody-secreting plasma cells.
- Identify plasma cells by their characteristic morphology.
- Explain how somatic mutation within germinal centers results in a progressive increase in antibody affinity.
- Explain how B cells may also act as antigen-presenting cells.
- Describe how cancerous plasma cells (myeloma cells), produce large quantities of very pure immunoglobulin.
- List the signals required for B cell activation.
- Define light chain, heavy chain, hypervariable region, germinal center, myeloma, somatic mutation, affinity selection, hybridomas, and monoclonal antibody.
- Describe how hybridomas and monoclonal antibodies may be produced.

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The division of the adaptive immune system into two major components is based on the need to recognize two distinctly different forms of foreign invaders. Some invaders enter the body and grow in extracellular fluids. These “exogenous” invaders are destroyed by antibodies. Other invaders grow inside cells, where antibodies cannot reach. They are destroyed by T cell-mediated responses. Antibodies are produced by the lymphocytes called B cells. This chapter describes B cells and their response to antigens.

B cells are found in the cortex of lymph nodes, in the marginal zone in the spleen, in the bone marrow, throughout the intestine, and in Peyer's patches. Few B cells circulate in the blood. Like T cells, B cells have a large number of identical antigen-binding receptors on their surface. Each B cell, therefore, can only bind and respond to a single antigen. Antigen receptors are generated at random during B cell development in a process described in Chapter 17. If a B cell encounters an antigen that binds its receptors, it will, with appropriate co-stimulation, respond by secreting its receptors into body fluids, where they are called antibodies.

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B Cell Antigen Receptors

Each B cell is covered with about 200,000 to 500,000 identical antigen receptors (BCRs), many more than the 30,000 antigen receptors (TCRs) expressed on each T cell. Each BCR is constructed from multiple peptide chains and, like the TCR, can be divided into antigen-binding and signaling components. Unlike the TCR, however, the BCR can also bind antigens when in solution. Antibodies are simply soluble BCRs released into body fluids; they all belong to the family of proteins called immunoglobulins (Chapter 14).

Antigen-Binding Component

The antigen-binding component of the BCR (or immunoglobulin) is a glycoprotein of 160 to 180 kDa consisting of four linked peptide chains. These chains consist of two identical pairs: two heavy chains, each 60 kDa in size; and two light chains, about 25 kDa each (Fig. 15.1). The light chains are linked by disulfide bonds to the heavy chains so that the complete molecule forms the letter Y. The tail of the Y (called the Fc region) is formed from paired heavy chains and attaches to the B cell surface. The arms of the Y (called the Fab regions) are formed by paired light and heavy chains, and they bind antigens (Fig. 15.2). The antigen-binding sites are formed by the grooves between the light and heavy chains. Thus each BCR has two identical antigen-binding sites.

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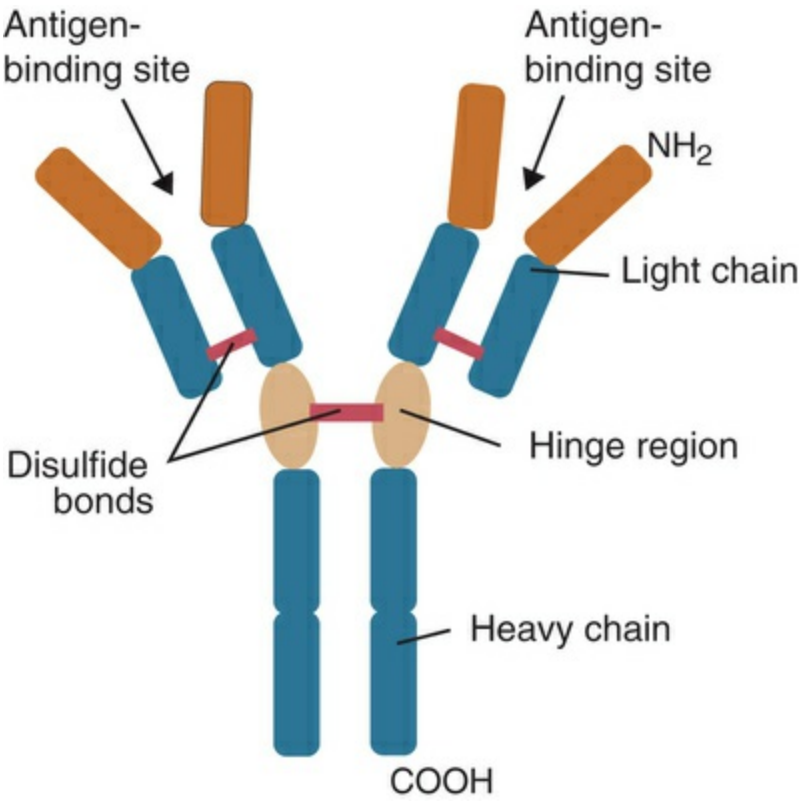


FIG. 15.1 The overall structure of an immunoglobulin molecule. When expressed on a B cell surface, this molecule acts as an antigen receptor (BCR). When released by the B cell and free in the circulation, it acts as an antibody. Note that unlike a TCR, it has two antigen-binding sites.

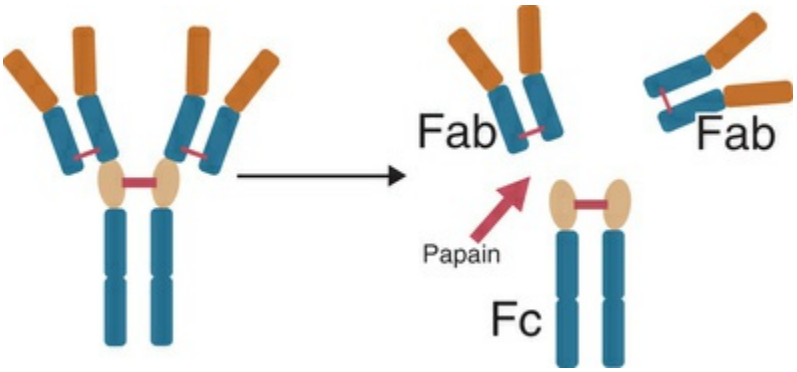


FIG. 15.2 The effect of treating an immunoglobulin molecule with the proteolytic enzyme papain. The names of these fragments denote the nomenclature of different regions of an immunoglobulin molecule. *Fab,* Fragment-antibody binding; *Fc,* Fragment crystallizable.

Light Chains

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Light chains are constructed from two domains each containing about 110 amino acids. The amino acid sequences in the C-terminal domains in the BCRs from different B cells are identical and are called constant domains (C_L). In contrast, the sequences in the N-terminal domains differ in each B cell and so form variable domains (V_L). Mammals also make two types of light chains, called κ (kappa) and λ (lambda). Although their amino acid sequences are different, they are functionally identical. The ratio of κ to λ chains in BCRs varies among mammals, ranging from mice and rats, which have more than 95% κ chains, to cattle and horses, which have 95% λ chains. Primates such as the rhesus monkey and the baboon have 50% of each, whereas humans have 70% κ chains. Carnivores such as cats and dogs have 90% λ chains.

Heavy Chains

Immunoglobulin heavy chains are constructed from four or five domains each of about 110 amino acids. The N-terminal domain is a variable (V_H) domain. The remaining three or four domains show few sequence differences and thus are constant (C_H) domains.

Mammalian B cells make five different classes of heavy chain that differ in their sequence and domain structure. As a result, each class has a different biological activity. The five different immunoglobulin heavy chains are called α, γ, δ, ε, and μ. These heavy chains determine the immunoglobulin class (or isotype). Thus immunoglobulin molecules that use α heavy chains are called immunoglobulin A (IgA), and those that use γ chains are called IgG; μ chains are used in IgM, δ chains in IgD, and ε chains in IgE.

Variable Regions

When the sequences of V domains from light and heavy chains are examined in detail, two features become apparent. First, their sequence variation is largely confined to three regions each containing 6 to 10 amino acids, within the variable domain (Fig. 15.3). These regions are said to be hypervariable. Between these three hypervariable regions are relatively constant sequences called framework regions. The hypervariable regions on paired light and heavy chains determine the shape of the antigen-binding site and

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thus the specificity of antigen binding. Since the shape of the antibody-binding site is complementary to the conformation of the antigenic determinant, the hypervariable sequences are also called complementarity determining regions (CDRs). Each V-domain is folded in such a way that its three CDRs come into close contact with the antigen (Fig. 15.4).

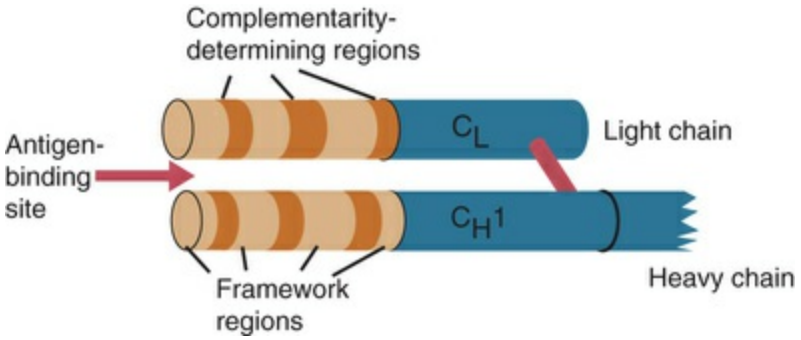


FIG. 15.3 The variable regions of the light and heavy chains of an immunoglobulin molecule are divided into three highly variable complementarity-determining regions separated by relatively constant framework regions.

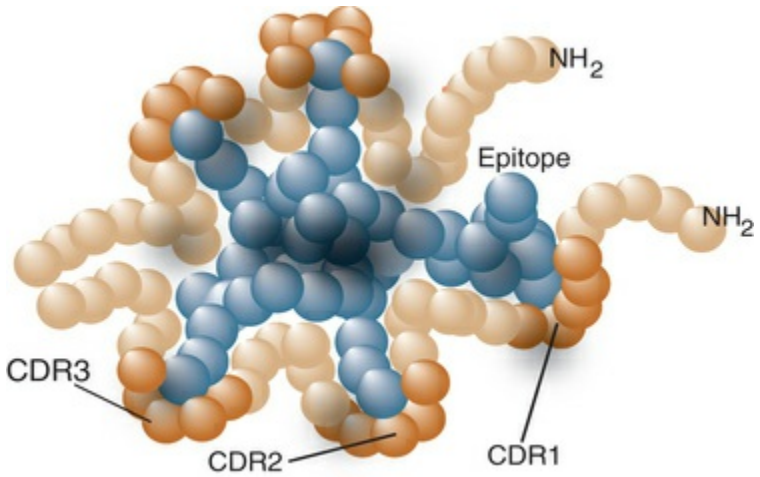


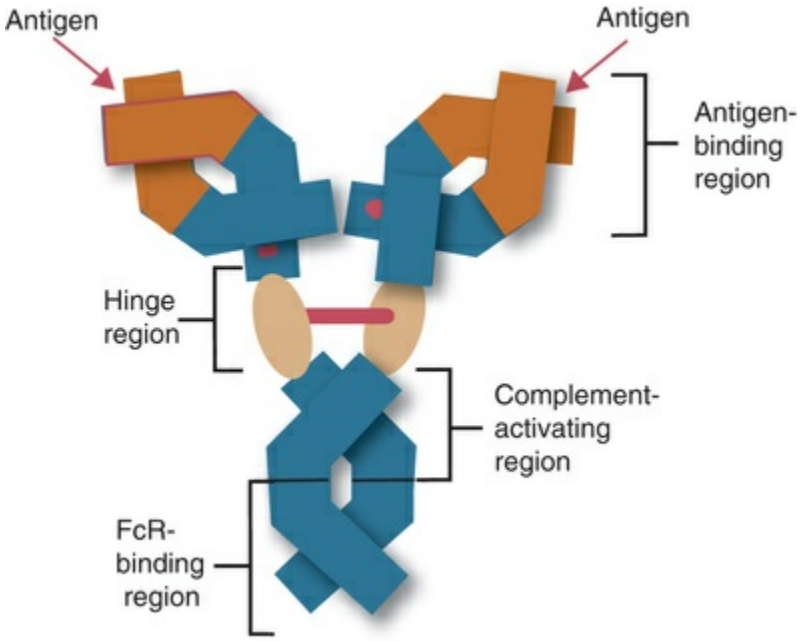
FIG. 15.4 The way in which the complementarity-determining regions are folded to form the antigen binding site on an immunoglobulin molecule. A similar folding occurs in the peptide chains of the TCR.

Constant Regions

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The number of constant domains differs between immunoglobulin heavy chain classes. There are three constant domains in a γ heavy chain; they are labeled, from the N-terminal end, C_H1, C_H2, and C_H3. Three constant domains are also found in α and most δ chains, whereas μ and ε chains have an additional constant domain called C_H4.

Since heavy chains are paired, the domains in each chain come together to form structures by which antibody molecules can exert their biological functions. Thus V_H and V_L together form an antigen-binding site, and C_H1 and C_L together stabilize the antigen-binding site. The paired C_H2 domains of IgG contain a site that activates the classical complement pathway (Chapter 4) and a site that binds to Fc receptors on phagocytic cells (Fig. 15.5). The heavy chain also regulates the transfer of IgG into colostrum (Chapter 23) and antibody-mediated cellular cytotoxicity (Chapter 18). When immunoglobulin molecules act as BCRs, their Fc region is embedded in the B cell surface membrane. These cell-bound immunoglobulins differ from the secreted form in that they have a small transmembrane domain located at their C-terminus. This contains hydrophobic amino acids that associate with the cell-membrane lipids.



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FIG. 15.5 The structure of an IgG molecule, showing how the light and heavy chains intertwine to form clearly defined regions of the molecule. Each region has defined biological functions.

Hinge Region

One important feature of the immunoglobulins is that their Fab regions that contain the antigen-binding sites can swing freely around the center of the molecule as if hinged. This hinge consists of a short domain of about 12 amino acids located between the C_H1 and C_H2 domains. The hinge region contains many hydrophilic and proline residues that cause the peptide chain to unfold and make this region readily accessible to proteases (see Fig. 16.10). This region also contains the interchain disulfide bonds that bind the four peptide chains together. Proline, because of its configuration, produces a 90-degree bend when inserted in a polypeptide chain. Because amino acids can rotate around peptide bonds, the effect of closely spaced proline residues is to produce a universal joint around which the immunoglobulin chains can swing freely. The μ chains of IgM do not possess a hinge region.

Signal Transducing Component

BCR immunoglobulins cannot signal directly to their B cell since their cytoplasmic domains contain only three amino acids. However, their C_H4 and transmembrane domains associate with two glycoprotein heterodimers formed by pairing CD79a (Ig-α) with CD79b (Ig-β). These CD79 heterodimers are the signal transducers of the BCR (Fig. 15.6). The CD79b chains are identical in all BCRs. The CD79a chains differ depending on their associated heavy chains and employ different signaling pathways.

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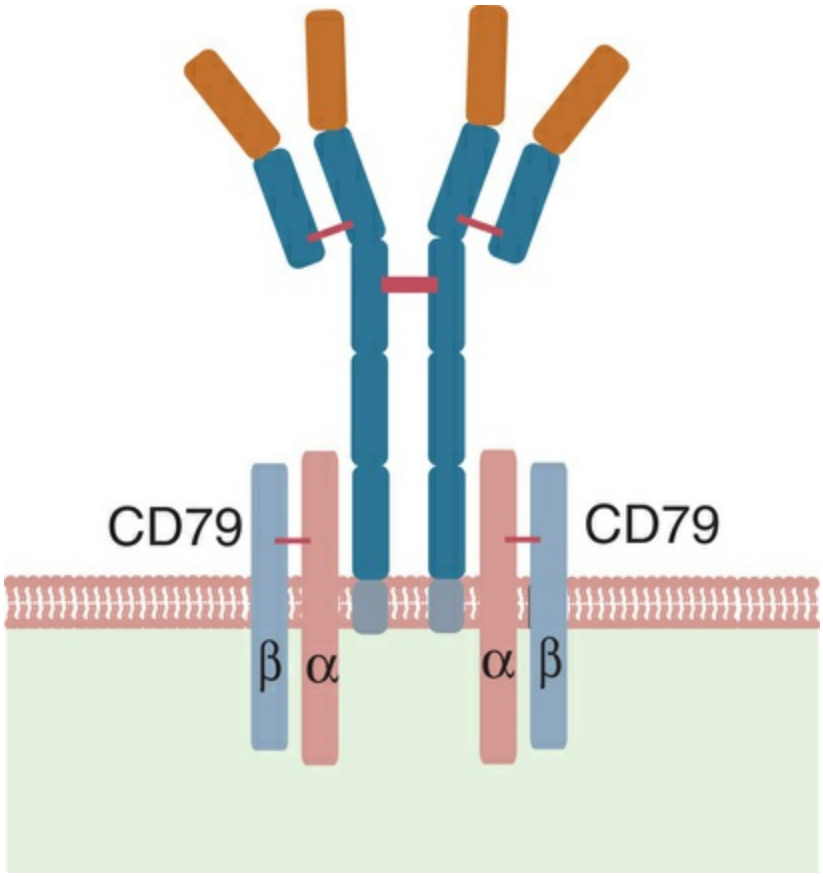


FIG. 15.6 The structure of a complete BCR, showing both the antigen-binding component (immunoglobulin) and the signal transducing components (CD79). Note the small transmembrane domain at the end of each heavy chain.

Antigen–BCR binding and cross-linking of two receptors exposes ITAMs on CD79a and CD79b. Phosphorylation of these ITAMs by src kinases leads to phosphorylation of a phospholipase C and a G-protein (see Fig. 8.12). Subsequent hydrolysis of phosphatidylinositol and calcium mobilization generates a protein kinase C and calcineurin and activates the transcription factors NF-κB and NF-AT. This eventually results in cell division and immunoglobulin production—providing the B cell also receives appropriate costimulatory signals from other sources.

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Antigen Presentation by B Cells

B cells are effective antigen-presenting cells. Following antigen binding, the BCR is internalized and either degraded or transported to an intracellular compartment, where major histocompatibility (MHC) class II molecules and antigen fragments combine. These antigen-MHC class II complexes are then carried to the B cell surface and presented to helper T cells (Fig. 15.7). If antigen presented by an APC binds to a BCR, the antigen may be extracted from the presenting cell and endocytosed by the B cell. This antigen is delivered to endosomes where it also complexes with MHC class II molecules. Since all the antigen receptors on a single B cell are identical, each B cell can bind only one antigen. This makes them much more efficient antigen-presenting cells than macrophages that must present any foreign material that comes their way. This is especially true in primed animals, in which large numbers of B cells can bind and present a specific antigen. As a result, B cells can activate Th cells with 1/1000 of the antigen required to activate macrophages.

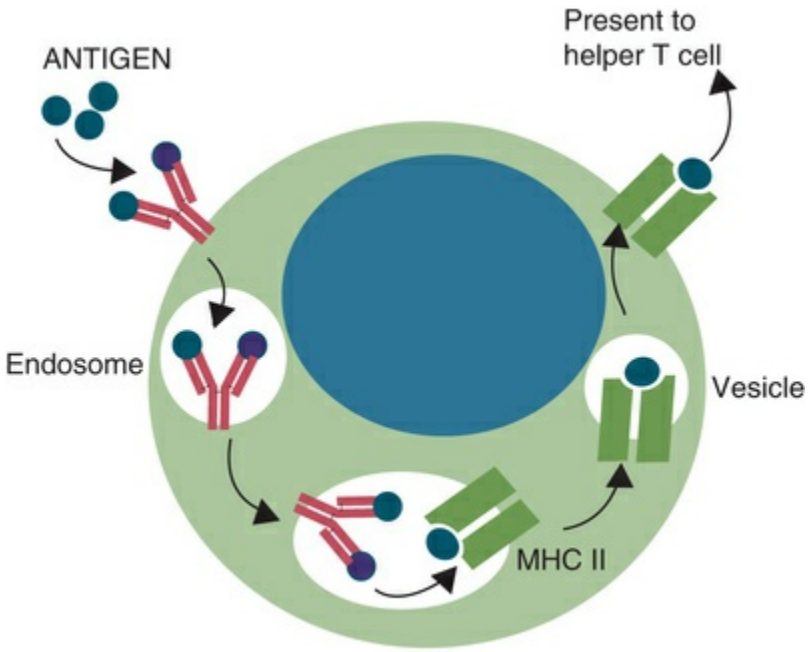


FIG. 15.7 The processing of antigens by B cells. This is a very efficient process since the BCRs capture large amounts of identical

antigen molecules.

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Co-stimulation of B Cells

Although the binding of antigen to a BCR is an essential first step, it alone is usually insufficient to activate B cells. Complete activation of a B cell requires multiple signals from other sources. Thus it requires co-stimulation by helper T cells and cytokines, by complement, and by pathogen-associated molecular patterns (PAMPs; Fig. 15.8).

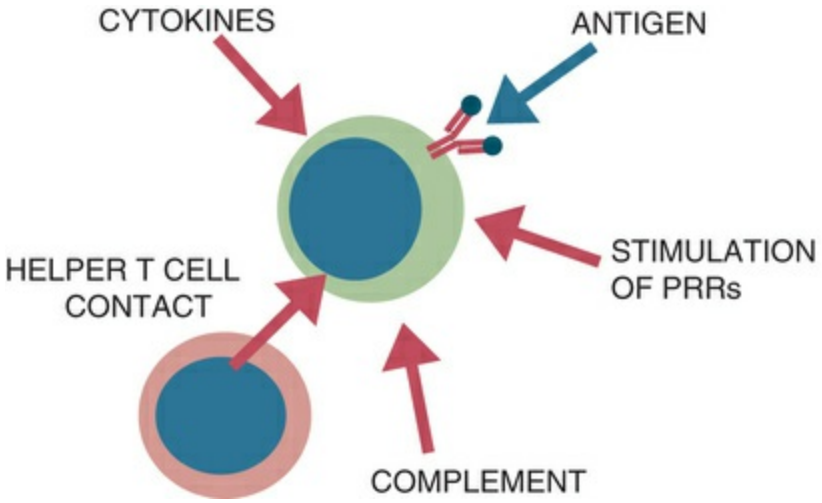


FIG. 15.8 Not only must B cells be stimulated by antigen, but they must also receive costimulation from helper T cells and their cytokines, as well as complement and PRRs, if they are to respond optimally.

T Cell Help

When helper T cells "help" B cells they promote several different B cell activities. These signals result in increased expression of IgM BCR and MHC class II, as well as receptors for IL-4, IL-5, IL-6, tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β), and start the process that leads to B cell division and differentiation into antibody-secreting cells. They stimulate B cell proliferation and survival through CD40L, IL-21, and IL-4. The "help" also triggers somatic mutation within germinal centers and thus changes antibody binding affinity. Helper T cells also trigger

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immunoglobulin class switching.

In order to do all this, however, the helper T cells must themselves encounter the antigen. This antigen can be presented by one of the professional antigen-presenting cells, such as dendritic cells, macrophages, or even B cells. Thus a B cell can capture and process antigen, present it to a T cell, and then receive co-stimulation from the same T cell. B cells thus play two roles. They respond to antigen by making antibodies while at the same acting as antigen-processing cells. The helper T cells provide B cells with co-stimulatory signals from cytokines as well as through interacting receptor pairs.

Cytokine Secretion

Th2 cells produce multiple cytokines that activate B cells. The most important of these are interleukin-4 (IL-4), IL-5, IL-6, IL-13, and IL-21.

IL-4 stimulates the growth and differentiation of B cells and enhances their expression of MHC class II and Fc receptors. It also induces immunoglobulin class switching and thus stimulates IgA and IgE production (Table 15.1). The actions of IL-4 are neutralized by IFN-γ, which inhibits both IgA and IgE synthesis as well as B cell proliferation.

TABLE 15.1

Immunoglobulins Produced by B Cells in the Presence of Th1 and Th2 Antigen-Specific Helper T Cell Clones in Mice

Class	Th1 Cells (ng/mL)	Th2 Cells (ng/mL)
IgG1	<8	21,600
IgG2a	14	39
IgG2b	<8	189
IgG3	<8	354
IgM	248	98,000
IgA	<1	484
IgE	<1	187

Adapted from Coffmann RL, Seymour BW, Lebman DA, et al: The role of helper T cell products in mouse B cell differentiation and isotype regulation, *Immunol Rev* 102:5, 1988.

IL-5 promotes the differentiation of activated B cells into plasma cells. It stimulates IgG and IgM production and enhances IL-4-induced IgE production. IL-5 selectively stimulates IgA production

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in mucosal B cells.

IL-6 is needed for the final differentiation of activated B cells into plasma cells. It acts together with IL-5 to promote IgA production and with IL-1 to promote IgM production.

IL-13 has biological activities similar to those of IL-4 because it acts through a receptor (IL-13R) that shares a common α chain with the IL-4R. This stimulates B cell proliferation and increases immunoglobulin secretion. IL-13 is required for optimal induction of IgE, especially if IL-4 is low or absent.

IL-21 is produced by several Th populations including both Tfh cells and Th17 cells. It induces the differentiation of B cells into plasma cells and memory B cells. It stimulates IgG production in conjunction with IL-4. IL-21 promotes the IgM to IgG class switch, while IL-4 induces the switch to IgE.

Cell-Cell Signaling

Cytokines alone cannot fully activate B cells. Complete activation also requires signaling between Th cells and B cells through receptor pairs such as CD40 and CD154. CD154 is expressed on activated helper T cells while its receptor CD40 is expressed on resting B cells. CD40 must receive a signal from CD154 in order for the B cell to begin its cell cycle and upregulate its IL-4 and IL-5 receptors (Figs. 15.9 and 15.10). The signals from CD154 synergize with those from IL-4 and IL-5 receptors to drive B cell activation, memory cell development, and immunoglobulin class switching. CD28, also found on helper T cells must also provide co-stimulation by binding CD86 on activated B cells.

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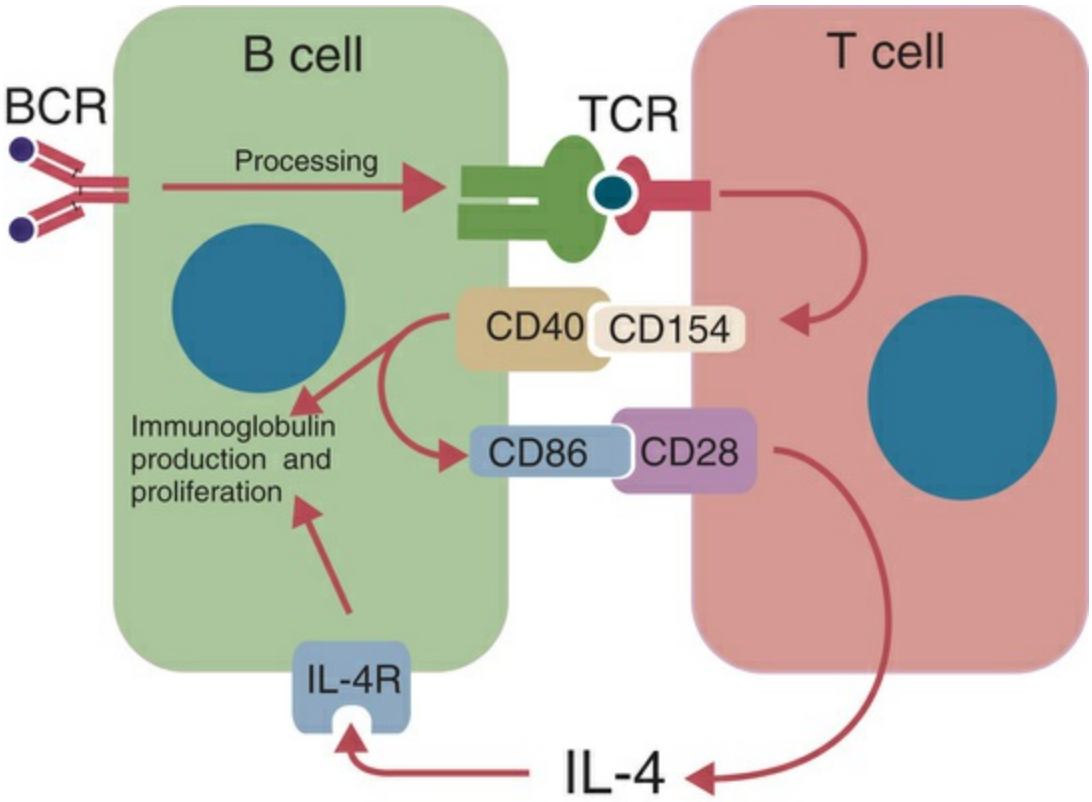


FIG. 15.9 The sequence of events that occurs when an antigen-processing B cell interacts with a helper T cell. During a primary immune response, antigen is processed by a dendritic cell and presented to the helper T cell. During a secondary immune response, the B cell itself can act as an antigen-presenting cell. Co-stimulators, such as CD154 and CD28, engage serially to trigger IL-4 secretion by the T cell and IL-4R production by the B cell.

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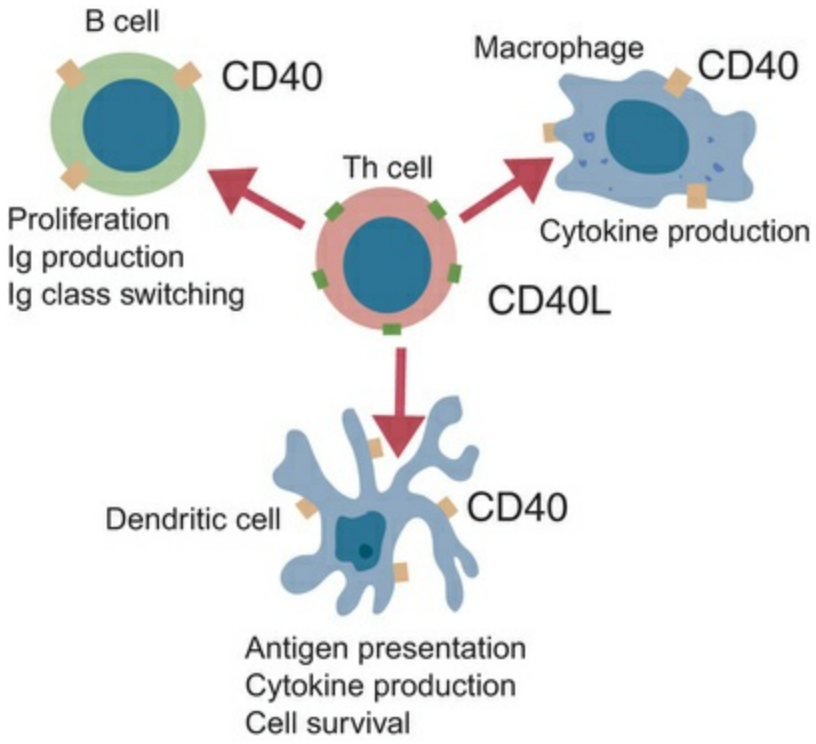


FIG. 15.10 CD40 and CD154 participate in a dialog between T cells and the three populations of antigen-presenting cells. In each case, both cell types are stimulated. In the case of B cells, T cell stimulation permits B cell proliferation and immunoglobulin production.

Complement Help

Effective co-stimulation of B cells also requires signals from complement transmitted through CD21/CD19 on the B cell surface. CD21 is a receptor (CR2) whose ligand is C3d. CD19 is its accompanying signaling component. If an antigen with C3d attached binds to CD21, a signal is transmitted through CD19 to the B cell (Fig. 15.11). Stimulation of a BCR plus CD19/CD21 lowers the threshold for B cell activation 100-fold. The importance of complement in stimulating B cells is emphasized by the observation that mice deficient in the complement components C3, C4, or CR2 cannot mount effective antibody responses.

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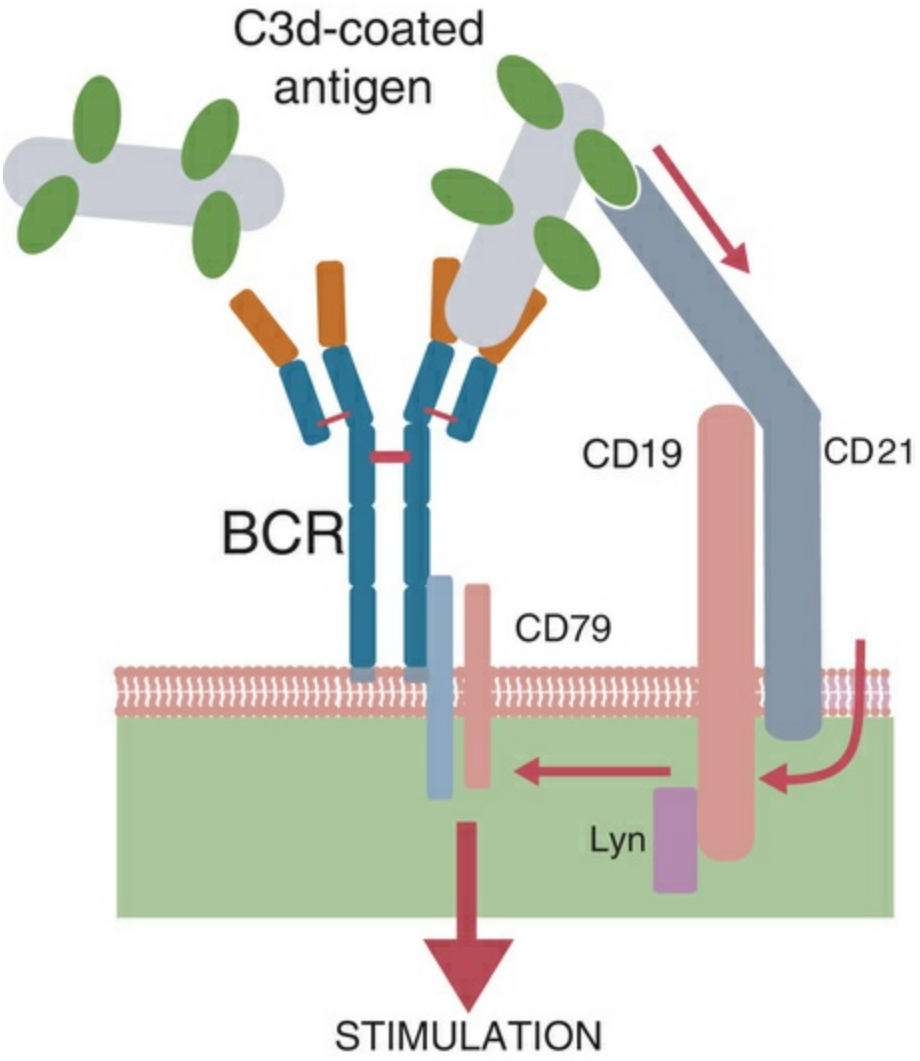


FIG. 15.11 The stimulation of B cells through the CD21/CD19 complex. CD21 binds to C3d on the antigen. Signaling through CD19, it generates a potent costimulatory signal to enhance B cell responses.

The B cell Fc receptor, FcγRIIb, is a negative regulator of B cell function. When an IgG molecule binds and links this receptor to a BCR through antigen, it inhibits antibody formation. This has important practical consequences when vaccinating young animals (Chapter 25).

Toll-Like Receptors and PAMP Help

Although BCR-antigen-binding plus T cell co-stimulation trigger initial B cell division, they cannot induce a prolonged, self-sustaining B cell response. Complete activation of B cells also requires coordinated signals from their toll-like receptors (TLRs).

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The B cell–stimulating ligands include flagellins, lipopolysaccharides, and CpG DNA. Signaling by TLR4 enhances B cell antigen presentation, promotes germinal center formation, and is required for optimal antibody production against T-dependent antigens. TLR signaling to memory B cells increases antibody production, but it does not appear to be required for IgA and IgE production. Thus TLR signaling can substitute in part for T cell help and explains why antibody production still occurs in AIDS patients despite their lack of T cells.

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B Cell Response

Once it receives help from the multiple sources described above, the B cell is ready to respond.

Differential Signaling

Like the TCR, the BCR probably produces a tunable signal. That is, it generates signals that depend on the properties of the antigen and the amount of co-stimulation received. The affinity of a BCR for its antigen influences B cell proliferation and antibody secretion. On the other hand, receptor occupancy influences MHC class II expression and signal transduction. The direction of the immunoglobulin class switch also depends on signals received from Th1 or Th2 cytokines.

Certain antigens can provoke antibody formation in the absence of helper T cells. These so-called T-independent antigens are usually simple repeating polymers (and PAMPs), such as *Escherichia coli* lipopolysaccharide, polymerized salmonella flagellin, and pneumococcal polysaccharide. T-independent antigens bind directly to B cell TLRs and cross-link several BCRs, providing a sufficient signal for B cell proliferation. Characteristically, T-independent antigens only trigger IgM responses and fail to generate memory cells (Fig. 15.12).

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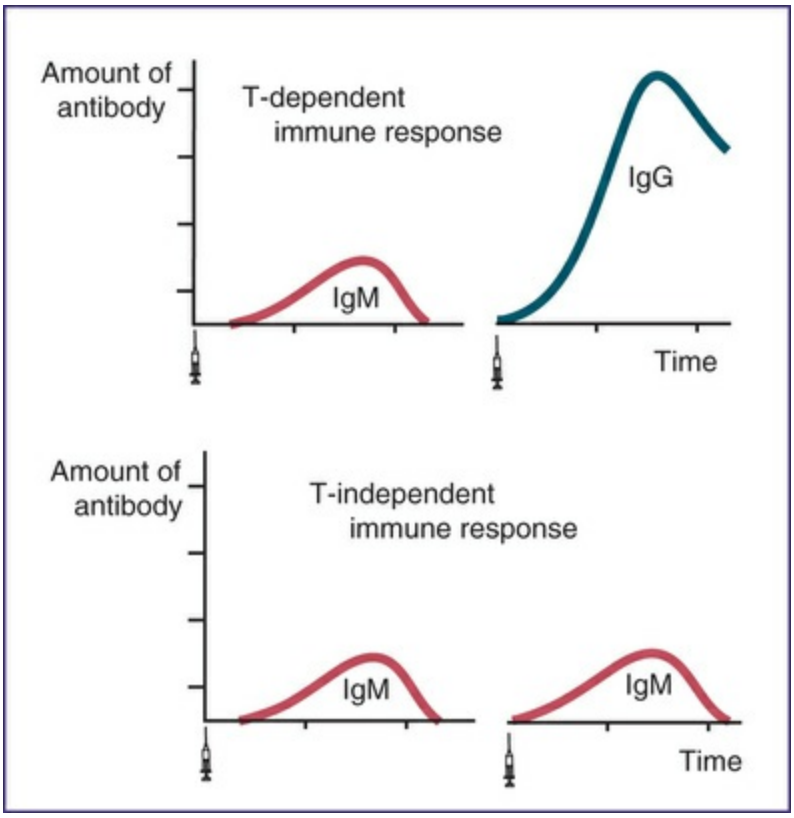


FIG. 15.12 The differences in the time course of a T-dependent and T-independent antibody response. T-independent antigens cannot induce an immunoglobulin switch or immunological memory, as demonstrated by a secondary antibody response.

It is appropriate to emphasize at this stage that the BCR has the same antigen-binding ability as antibody molecules. Thus, antibodies can bind free intact antigens in solution. This is very different from the α/β TCR that can only bind processed antigen fragments bound to an MHC molecule (Fig. 15.13). This difference in the antigen-binding ability of B and T cells is significant in that B cells can respond to a greater variety of antigens than T cells. Likewise, antibodies are directed, not against breakdown products of antigens, but against intact antigen molecules. As a result, antigen-antibody interactions usually depend on maintenance of the conformation of an antigen. A good example of this is seen with tetanus toxoid. Antibodies raised against the intact molecule will bind only to the intact molecule and may be unable to bind proteolytic fragments such as those produced by macrophage processing.

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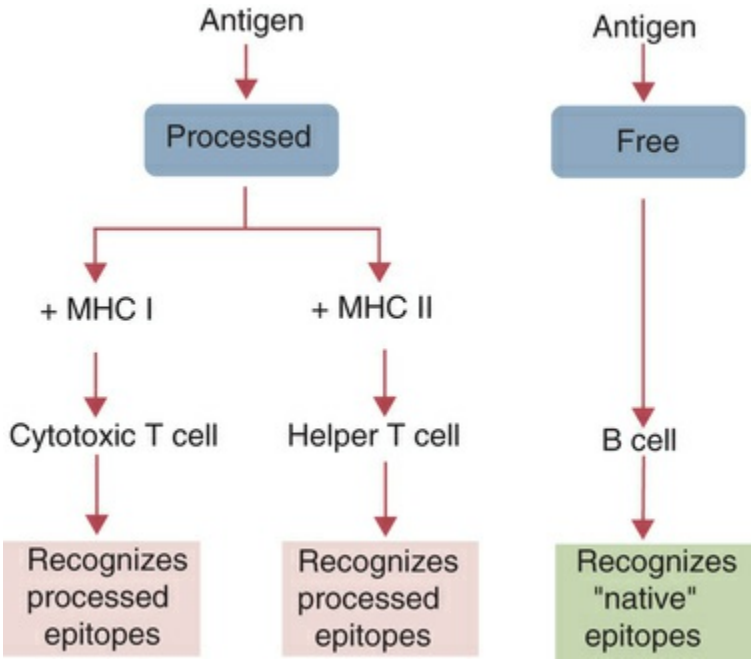


FIG. 15.13 TCRs and BCRs recognize antigen in a very different fashion. Thus a BCR can bind to free, soluble antigen. A TCR, in contrast, can recognize only antigen processed and presented on a MHC molecule.

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Cellular Responses

The term *clonotype* is used to describe a clone of B cells expressing a BCR capable of responding to a single epitope. A newborn animal with few B cells has available only a limited variety of clonotypes, but their diversity increases with age as a result of increased use of alternative sets of V genes and somatic mutation in these V genes (Chapter 17). This diversity is also increased by the effects of the intestinal microbiota (Chapter 21). In an adult animal the number of B cells within a given clonotype depends on their exposure to different antigens over the animal's lifetime. Thus the most used clonotypes will increase greatly in number and there may be as many as 10^4 responsive B cells per clonotype (Box 15.1). Conversely, the numbers of unused clonotypes will remain very small, and an individual may have as few as 10 responsive cells in their spleen or bone marrow.

Box 15.1

Cell Membrane Exchange

It has generally been assumed that individual cells conserve their major structural components and do not share them with other cells. In recent years, however, it has become abundantly clear that different cells may exchange cell surface membranes and their associated receptors. Thus macrophages may accept fragments of neutrophil membranes. Activated B cells can also donate their antigen receptors to nearby bystander cells. This process is mediated by membrane transfer between adjacent B cells and is amplified by the interaction of the BCR with specific antigen. The net effect is to permit a dramatic expansion of the number of antigen-binding B cells in vivo. The B cells with their newly acquired receptors can act as antigen-presenting cells for $CD4^+$ T cells. This is yet another example of the remarkable efficiency of the adaptive immune system in responding to a specific antigen once an animal is primed.

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In most newborn mammals, each B cell initially expresses both IgM and IgD BCRs on its surface, with about 10 times as many IgD molecules as IgM. These unstimulated B cells may secrete small amounts of monomeric IgM.

When appropriately stimulated and co-stimulated, B cells undergo repeated division. The B cell division that results from this is asymmetric so that one daughter cell gets lots of antigen while the other daughter cell gets very little or none. The cell that gets lots of antigen then differentiates into a plasma cell. The cell that gets none continues the cycle of dividing and mutating and eventually becomes a memory cell. The cells destined to become plasma cells develop a rough endoplasmic reticulum, increase their rate of synthesis, and secrete large quantities of immunoglobulins. Within a few days, these responding cells switch from making IgM to making another immunoglobulin class. This switch occurs within the germinal center and leads to production of IgG, IgA, or IgE. The class switch results from deletion of unwanted heavy chain genes and the joining of variable-region genes to the next available heavy chain genes (Chapter 16). The specificity of the antibody produced remains unchanged.

Class switching is controlled by IL-4, IFN-γ, and TGF-β. Thus IL-4 from Th2 cells directs mouse B cells to produce IgG1 and IgE, whereas it directs human B cells to produce IgG4 and IgE (see Table 15.1). IL-4 alone is insufficient for class switching, and additional signals are required from CD40 and CD154. IFN-γ from Th1 cells stimulates a switch to IgG2a and IgG3 in mouse B cells and effectively suppresses the effects of IL-4. IFN-γ acts by promoting the production of the B cell-stimulating cytokines BAFF and APRIL (Box 15.2). TGF-β promotes the switch to IgA production on body surfaces. As described previously, signals from IL-5, IL-6, IL-13, and IL-21 also contribute to class switching.

Box 15.2

BAFF/APRIL System

B cell activating factor (BAFF; CD257) and "a proliferation-inducing ligand" (APRIL; CD256) are two related cytokines. BAFF is produced by monocytes, dendritic cells, T cells, and neutrophils.

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APRIL is produced by monocytes, dendritic cells, T cells, and intestinal epithelial cells. They bind to the same or related receptors on B cells. BAFF is expressed on the cell membranes of antibody-producing cells but can be cleaved off as a soluble cytokine. It functions in both situations. APRIL only functions as a soluble cytokine. They both promote B cell division and inhibit their apoptosis. Both BAFF and APRIL are crucial survival factors for B cells and essential for their production and differentiation. Overexpression of BAFF results in severe autoimmune disease.

Ng LG, Mackay CR, Mackay F: The BAFF/APRIL system: life beyond B lymphocytes, *Mol Immunol* 42:763-772, 2005.

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Plasma Cells

Plasma cells develop from antigen-stimulated B cells (Fig. 15.14). Early plasma cells (plasmablasts) can be identified in the lymph node cortex and paracortex and in the marginal zone in the spleen. Fully developed plasma cells emigrate from these areas to the spleen, the medulla of lymph nodes, and the bone marrow.

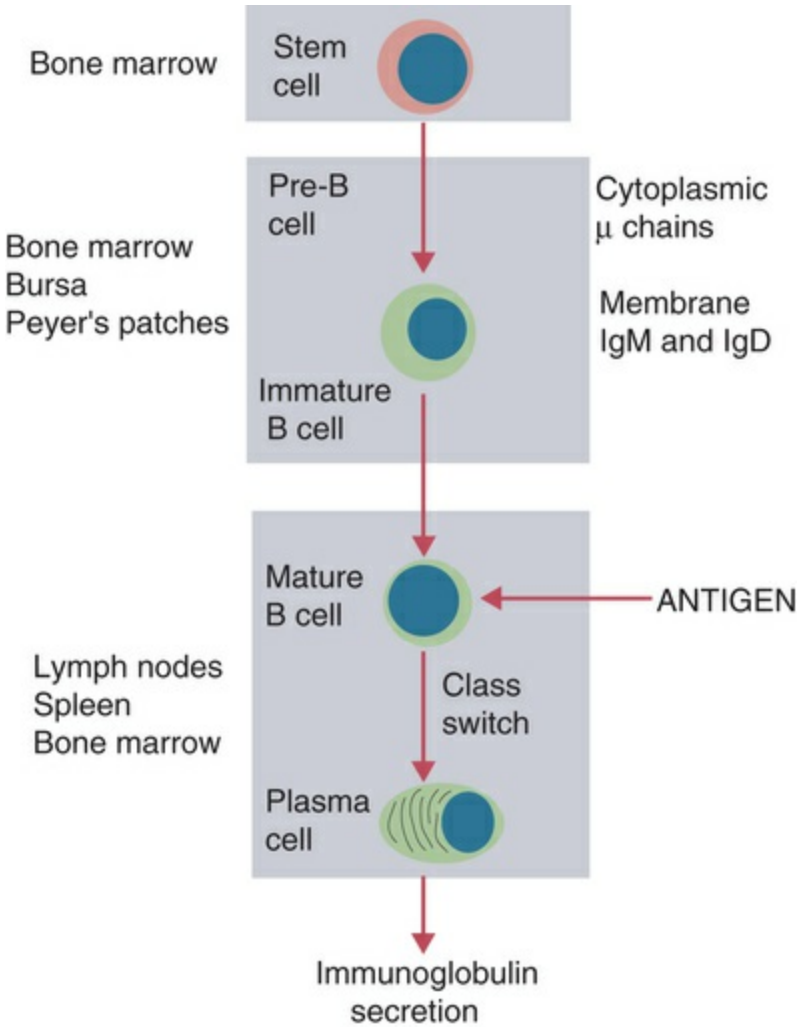


FIG. 15.14 B cells originate in the bone marrow and proceed through a series of differentiation stages before becoming able to respond to antigen. When B cells respond to antigen, they respond by division and differentiation of their progeny into plasma cells.

Plasma cells are ovoid cells, 8 to 9 μm in diameter (Fig. 15.15). They have a round, eccentrically placed nucleus with unevenly

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distributed chromatin. As a result, the nucleus may resemble a clock face or cartwheel. Plasma cells have an extensive cytoplasm that is rich in rough endoplasmic reticulum and stains strongly with basic dyes and pyronin. They have a large, pale-staining Golgi apparatus (Figs. 15.16 and 15.17). Plasma cells can secrete up to 10,000 molecules of immunoglobulin per second. The immunoglobulin produced by a plasma cell is of identical specificity to the BCRs on its parent B cell.

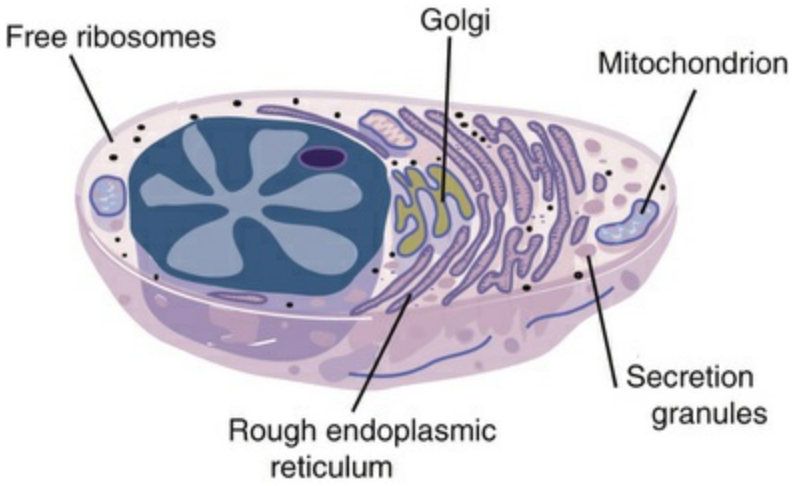


FIG. 15.15 The structure of a typical plasma cell. The possession of an extensive rough endoplasmic reticulum is typical of a cell dedicated to the rapid production of large amounts of immunoglobulin.

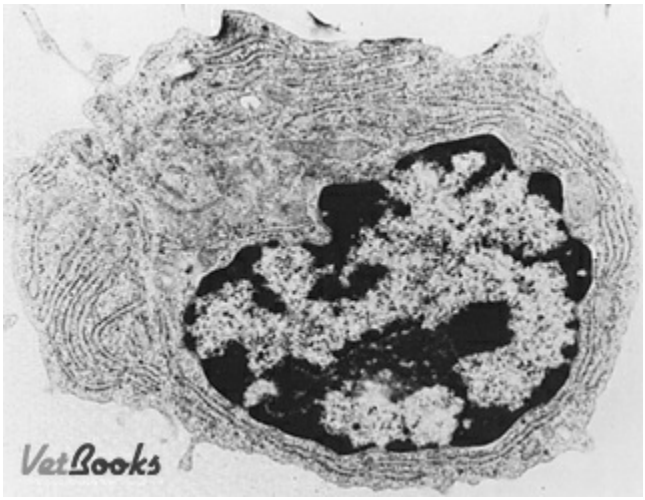


FIG. 15.16 A transmission electron micrograph of a plasma cell from a rabbit. Note how its cytoplasm is densly packed with rough

endoplasmic reticulum, a sign of massive protein synthesis. (Courtesy Dr. S. Linthicum.)

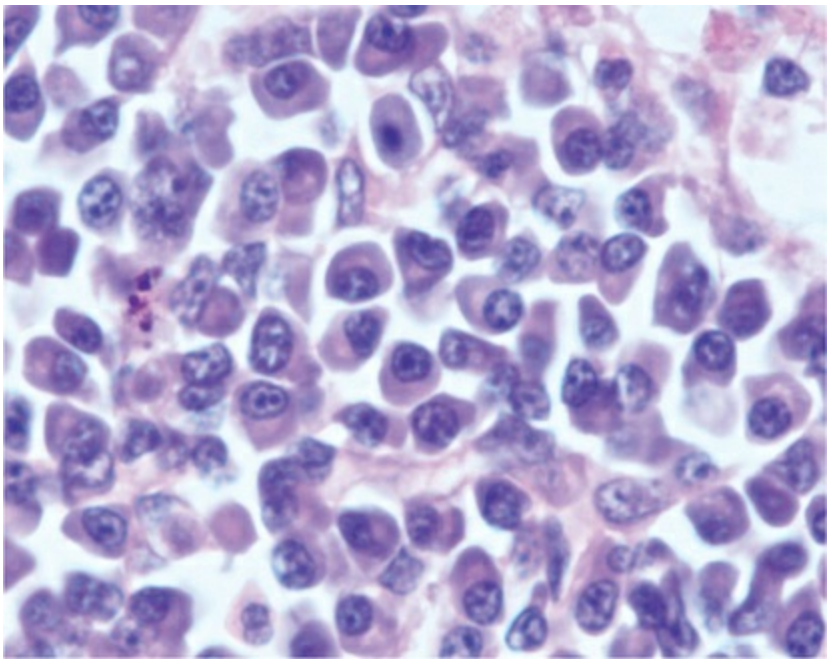


FIG. 15.17 Plasma cells in the medulla of a dog lymph node. Their cytoplasm is rich in ribosomes and thus stains intensely with pyronin, giving a dark red appearance. Original magnification ×450. (From a specimen provided by Drs. N. McArthur and L.C. Abbott.)

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Memory B Cells

One reason that the primary immune response ends is that the responding B cells and plasma cells are simply removed by apoptosis. If all these cells died, however, immunological memory could not develop. Clearly some B cells must survive as memory cells. B cells are activated by antigen and helper T cells in the paracortex of lymph nodes. Most of these B cells differentiate into plasma cells and migrate to the bone marrow, spleen, and other organs, but some memory precursors remain in the cortex, proliferate, and form germinal centers. (Asymmetric division, as described above, likely accounts for these two different fates.) These cells persist under the influence of programming and rescue signals. Thus, memory cells are first screened for their ability to bind antigen. This induces CD154 on nearby T cells, which in turn promotes expression of bcl-2. Bcl-2 protects them against apoptosis and allows them to differentiate into memory cells.

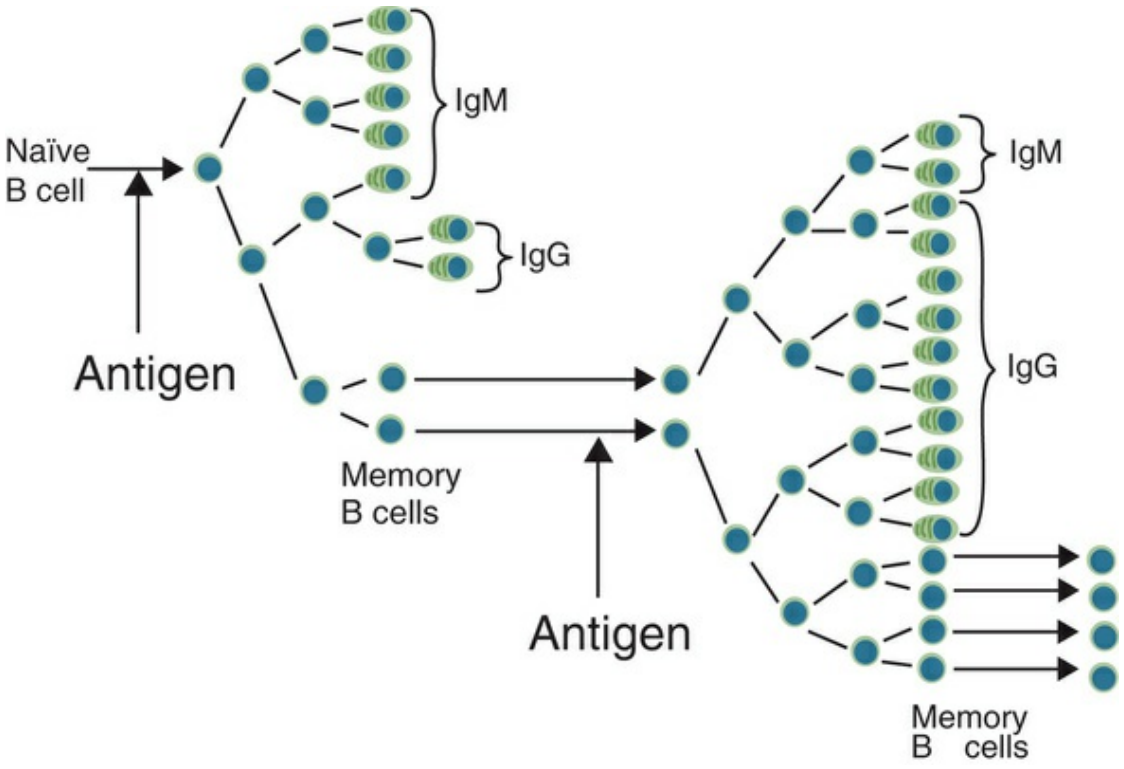
Memory cells form a reserve of long-lived antigen-sensitive cells to be called on following subsequent exposure to an antigen. There are several classes of memory B cells distinguishable by their immunoglobulin class, their location, and their passage through germinal centers. For example, one population consists of small, long-lived resting cells with IgG BCR. These cells, unlike plasma cells, look like generic lymphocytes. Their survival does not depend on antigen contact. On exposure to antigen, they proliferate and differentiate into plasma cells without undergoing further mutation. It has been calculated that in a secondary immune response, the clonal expansion of memory B cells results in 8- to 10-fold more plasma cells than does a primary immune response.

A second memory B cell population consists of large, dividing cells with IgM BCR. These cells persist in germinal centers, where their continued survival depends on exposure to antigen on follicular dendritic cells. There are two distinct populations of plasma cells: a short-lived population that lives for 1 to 2 weeks and produces large amounts of antibodies shortly after antigen exposure, and a long-lived population that can survive for months or years. (In humans these plasma cells have a half-life of 8 to 15

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years.) These antibodies provide immediate immunity to microbial pathogens. The short-lived cells are found in the spleen and lymph nodes soon after immunization. The long-lived plasma cells, in contrast, accumulate in the bone marrow. These long-lived plasma cells probably develop from a population of self-renewing, slowly dividing memory B cells. These memory B cells require a functional BCR to survive, suggesting that constant low-affinity antigen binding keeps them alive. Thus cats immunized with killed panleukopenia virus will continue to produce antibodies at low levels for many years. The source of these antibodies is believed to be the long-lived plasma cells stimulated to secrete antibodies by exposure to PAMPs and T cell help.

If a second dose of antigen is given to a primed animal, it will encounter large numbers of memory B cells, which respond in the manner described previously for antigen-sensitive B cells (Fig. 15.18). As a result, a secondary immune response is much greater than a primary immune response. The lag period is shorter since more antibodies are produced, and they can be detected earlier. IgG is also produced in preference to the IgM characteristic of the primary response.



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FIG. 15.18 The time course of a B cell response and the cellular events that accompany it. Note that some IgG is made in the primary immune response, whereas a small amount of IgM is also made in a secondary immune response.

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Germinal Centers

As mentioned above, a key feature of the humoral immune response is the progressive increase in antibody binding affinity over time. This process takes place within germinal centers (Fig. 15.19). Thus germinal centers are sites where antigen-driven B cell proliferation, somatic mutation, and positive and negative selection of B cell populations occur. Germinal centers are divided into two zones based on staining patterns, a light zone containing dendritic cells, some B cells and Tfh cells, and a dark zone that mainly consists of dividing B cells (see Fig. 12.10). In the early stages of the reaction, B cells stimulated by antigen and Tfh cells migrate to the dark zone. There they proliferate and mutate their antibody V genes. B cells divide every 6 to 8 hours so that within just a few days a single B cell develops into a clone of several thousand cells. During this phase of rapid B cell division, the BCR V region genes mutate randomly, on average once per division. This repeated mutation generates large numbers of B cells whose BCRs differ from the parent cell. Once these cells have been clonally expanded, a process that takes 10 to 20 days, they migrate to the light zone where they are presented with antigen by the dendritic cells. Because of their V gene mutations, some of these B cells bind the antigen with greater affinity, and others bind it less strongly. A process of selection thus occurs. If a mutation has resulted in greater affinity for the antigen, this stimulates more B cell proliferation. Thus cycles of rapid somatic mutation and selection lead to a rapid improvement in antigen binding—a process called affinity maturation. These antigen-selected B cells eventually leave the germinal center to form either plasma cells or memory B cells. In contrast, those B cells that have reduced antigen binding undergo apoptosis and are removed by macrophages. Thus the B cell population that emerges from a germinal center is very different from the population of cells that entered it. In addition to somatic mutation of BCR V genes, BCRs also undergo class switching within germinal centers. The germinal center eventually dissipates after the B cell response has peaked (Chapter 12).

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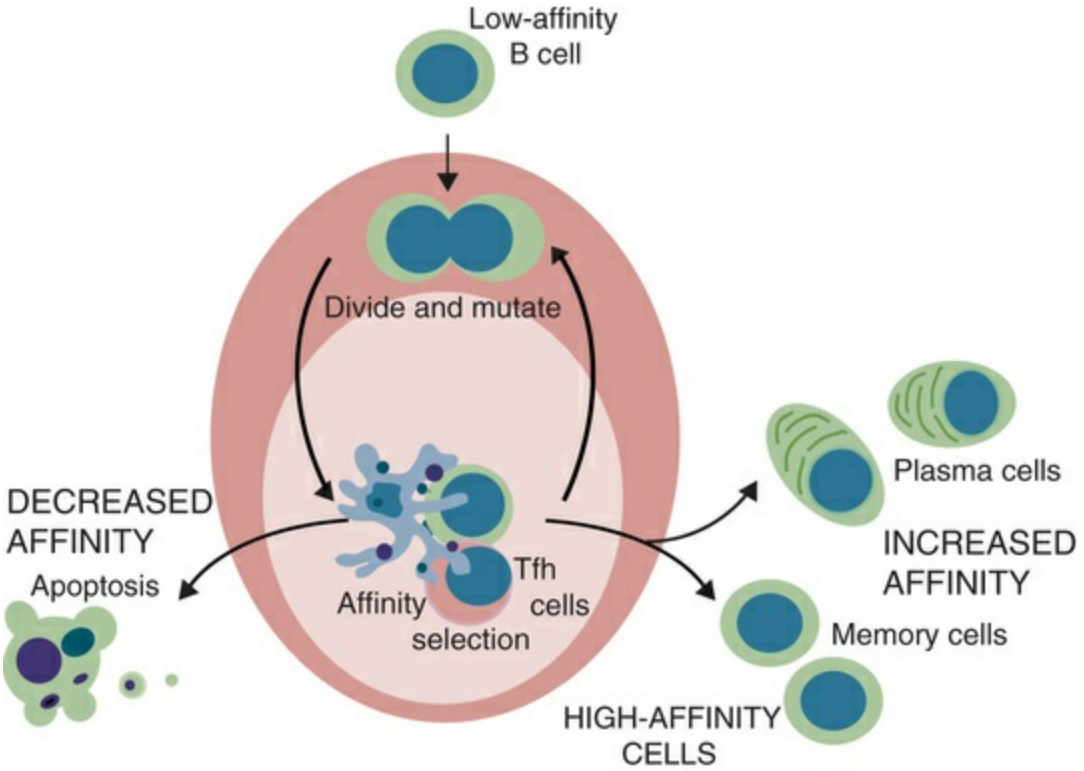


FIG. 15.19 B cells in the germinal center undergo somatic mutation as they respond to antigen presented by dendritic cells. If the mutation enables them to bind antigen with increased affinity, they will be stimulated to continue dividing. If, on the other hand, the mutation reduces their antigen-binding affinity, they will undergo apoptosis.

B Cell Subpopulations

In laboratory mice, there are two subpopulations of B cells that develop from different precursor stem cells. These are called B1 and B2 cells. B2 cells are conventional B cells that are central to the adaptive antibody responses and are discussed in this chapter and throughout the book. B2 cells appear late in neonatal life and are the predominant population in adult bone marrow and produce most of the body's IgG.

Mouse B1 cells originate from stem cells in the fetal liver or omentum rather than the bone marrow. They are innate-like cells that share some features with macrophages. They are, for example, phagocytic and microbicidal (they produce reactive oxygen species) and can present antigen to $CD4^+$ T cells. There are two subpopulations of B1 cells termed B1a and B1b. B1a cells develop exclusively in the neonate, are self-replenishing, and are responsible

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for most “natural” IgM in serum. They thus participate in innate immunity. B1a cells express CD5, an adhesion and receptor molecule. (CD5 is the receptor for CD72.) They recognize common bacterial molecules such as phosphocholine as well molecules such as immunoglobulins and DNA. They produce antibodies in a T-independent manner. B1a cells also differ from conventional B2 cells in that they are found in the peritoneal and pleural cavities and can renew themselves. B1b cells are distinguished from B1a cells by lacking CD5. They are, however, required for protection against several parasites and bacteria. B1b cells are produced throughout adult life. Many of the IgA-producing cells in the intestine originate from B1 cells. B1 cells have been identified in humans, mice, rabbits, guinea pigs, pigs, sheep, and cattle. It is unclear, however, whether the B1-B2 classification applies to all these species.

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Myelomas

If a B cell turns cancerous it may generate a clone of immunoglobulin-producing tumor cells. These cells are usually recognizable as plasma cells (Fig. 15.20). Plasma cell tumors are called myelomas or plasmacytomas. Because myelomas arise from a single precursor cell or clone, they secrete a homogeneous immunoglobulin called a myeloma protein. On serum electrophoresis, this homogeneous myeloma protein will appear as a sharp, well-defined peak. This is called a monoclonal gammopathy (Fig. 15.21).

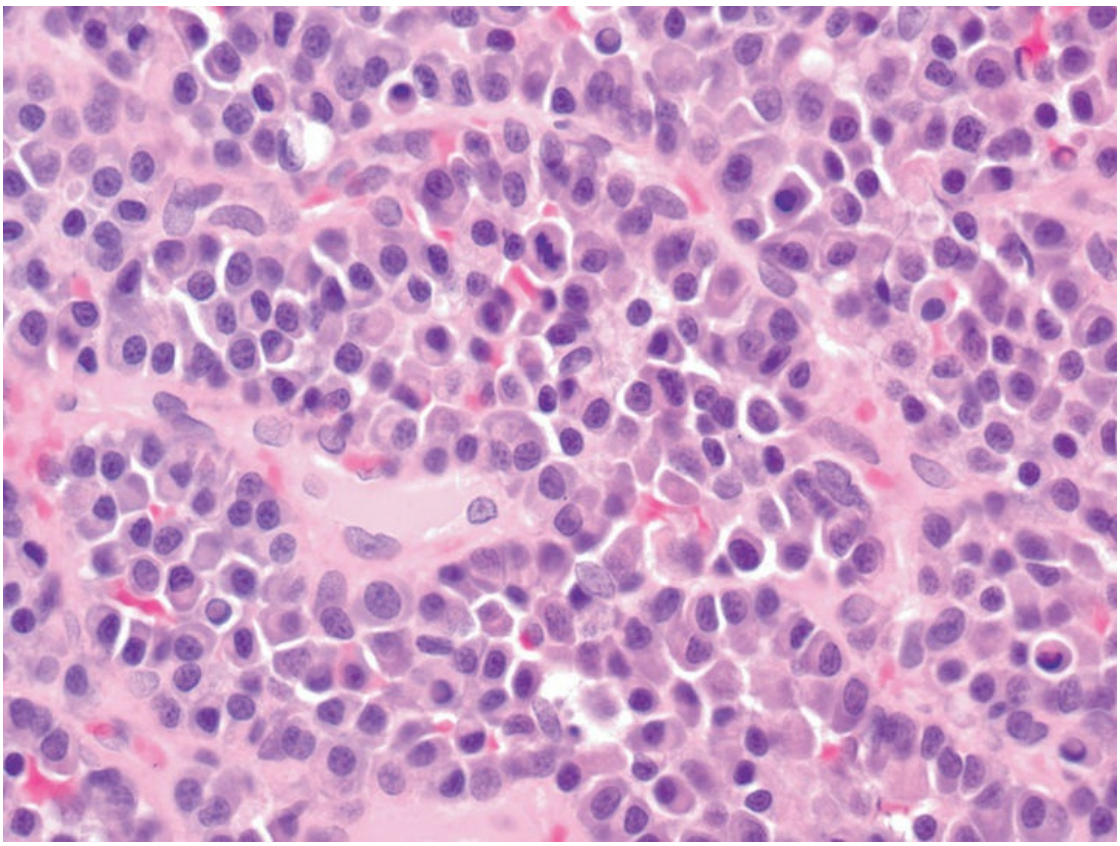


FIG. 15.20 A section of a myeloma tumor mass in a dog. Original magnification ×600. These cells are clearly plasma cells. (Courtesy Dr. B. Porter.)

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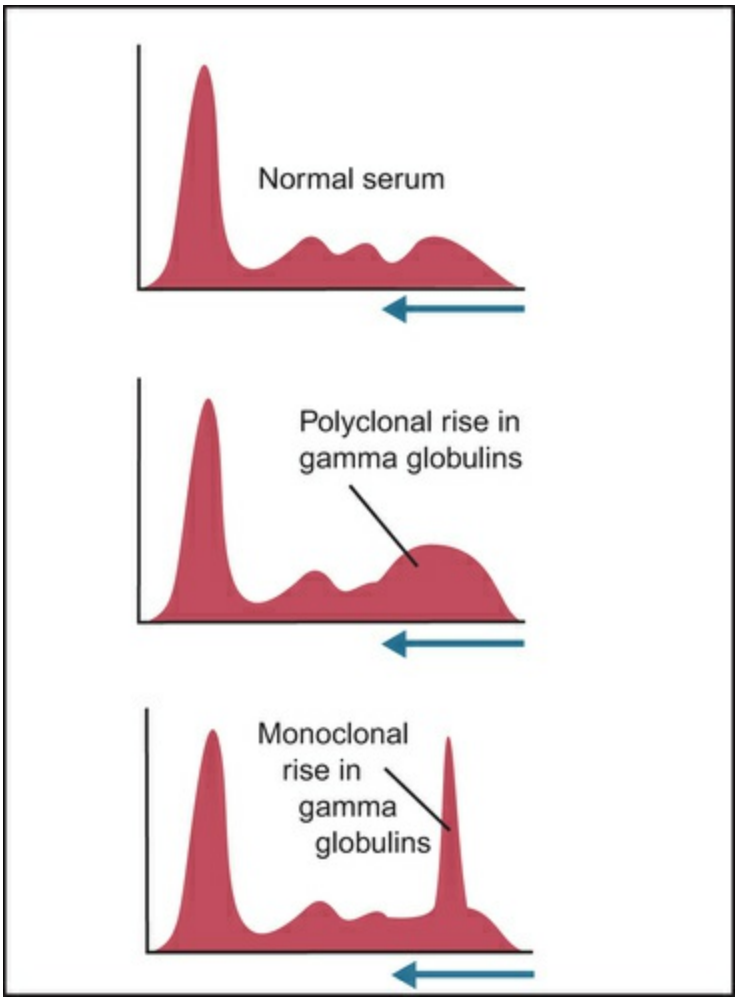


FIG. 15.21 Serum electrophoretic patterns showing the normal pattern and the characteristic features of monoclonal and polyclonal gammopathies. The monoclonal antibody spike reflects the production of large amounts of homogenous immunoglobulins. Monoclonal gammopathies commonly result from the presence of a myeloma. The *arrow* denotes the direction of migration.

Myeloma proteins may belong to any immunoglobulin class. For example, IgG, IgA, and IgM myelomas have been reported in dogs. In humans, in addition to myelomas of the major immunoglobulin classes, rare cases of IgD and IgE myelomas have also been described. The prevalence of myelomas expressing the various immunoglobulin classes in myeloma proteins correlates with their quantities in normal serum. Light chain disease is caused by a myeloma in which light chains alone are produced or the production of light chains is greatly in excess of the production of heavy chains. Similarly, there is a very rare form of myeloma in which Fc fragments alone are produced. This condition is erroneously termed heavy chain disease.

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Myelomas have been described in humans, mice, dogs, cats, horses, cows, pigs, ferrets, and rabbits. They account for less than 1% of all canine tumors, and they are considerably rarer in the other domestic species. The clinical presentations of myelomas include bleeding disorders, hyperviscosity, renal failure, and hypercalcemia. Other signs include lethargy, recurrent infections, anemia, lameness, bone fracture, and neurological signs, including dementia and peripheral neuropathy. The most common clinical manifestation in dogs is excessive bleeding as a result of a thrombocytopenia and a loss of clotting components as they bind to myeloma proteins. The presence in serum of abnormally large quantities of immunoglobulins results in a hyperviscosity syndrome, which is especially severe in animals with IgM myelomas (macroglobulinemia). As a result of the increase in blood viscosity, the heart must work harder, and congestive heart failure, retinopathy, and neurological signs may result. Because myeloma cells stimulate osteoclast activity, the presence of tumors in bone marrow may lead to severe bone destruction. Multiple radiolucent osteolytic lesions and diffuse osteoporosis develop and are readily seen by radiography (Fig. 15.22). These lesions result in pathological fractures. Light chains, being relatively small, are excreted in the urine. Unfortunately, they are toxic for renal tubular cells and, as a result, may cause renal failure. The light chains may be detected by electrophoresis of concentrated urine or, in some cases, by heating the urine. Light chains precipitate when heated to 60°C but redissolve as the temperature is raised to 80°C. Proteins possessing this curious property are called Bence-Jones proteins, and their presence in urine suggests a myeloma. They occur in about 40% of canine cases. Nonsecretory myelomas are occasionally diagnosed in dogs (Fig. 15.23).

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FIG. 15.22 A radiograph of a dog showing the round radiolucent areas where bone has been eroded by the presence of a myeloma. (Courtesy Dr. Claudia Barton.)

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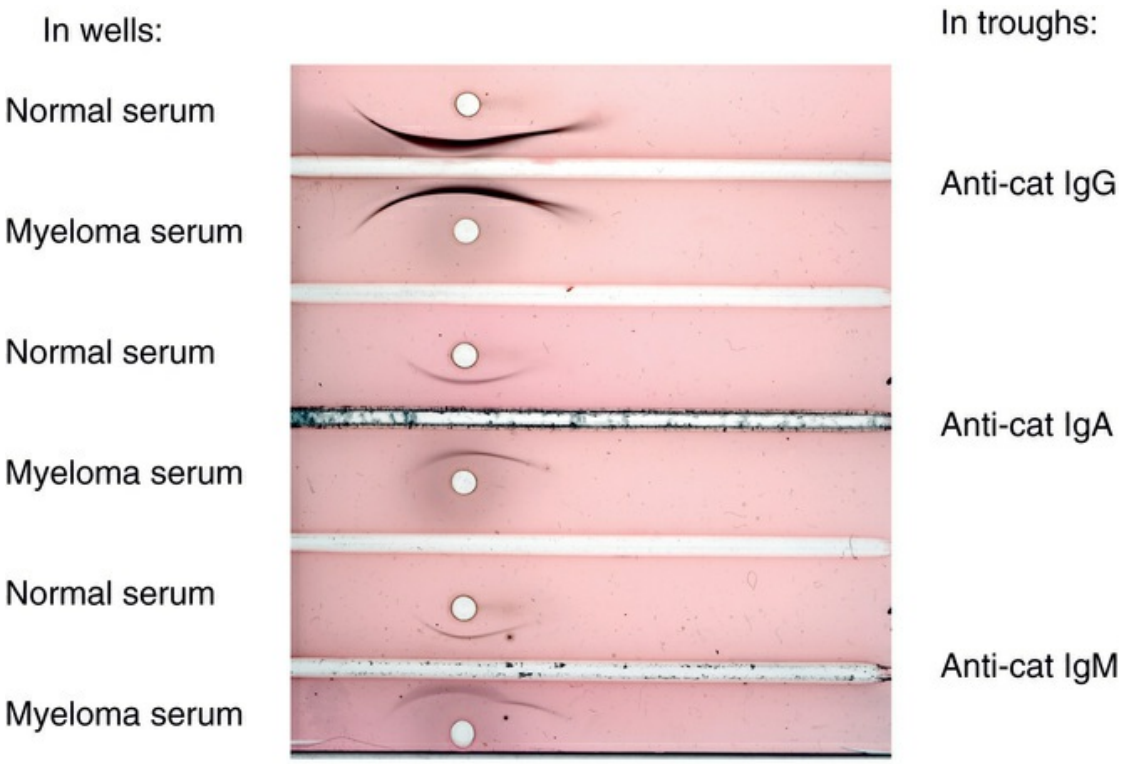


FIG. 15.23 Immunoelectrophoresis of serum from a cat with an IgM-secreting myeloma. Note that the line of precipitate formed by the reaction between anti-cat IgM and the myeloma serum is distorted *(bottom well).* The line is much thicker than the control, and it forms two distinct joined arcs as a result of the presence of large quantities of monoclonal IgM myeloma protein. (Details of this technique can be found in Chapter 42.) (Courtesy Dr. G. Elissalde.)

Because of the overwhelming commitment of the body's immune resources to the production of neoplastic plasma cells, as well as the replacement of normal marrow tissue by tumor cells and the negative feedback induced by elevated serum immunoglobulins, animals with myelomas are often immunosuppressed and anemic. In humans, renal failure and overwhelming infection are the most common causes of death in myeloma patients.

Affected animals should receive supportive therapy. Antibiotics can be used to control secondary infections, and fluid therapy should be administered to combat dehydration resulting from renal failure. Steroids and diuretics may assist in promoting calcium excretion. The serum hyperviscosity may be reduced by plasmapheresis to remove the myeloma protein. The tumor itself can be treated with specific chemotherapy. The drug of choice is melphalan, an alkylating agent. Prednisone may be used in association with melphalan. In unresponsive cases,

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cyclophosphamide or thalidomide may be employed. In humans, major improvements in survival have resulted from the use of monoclonal antibodies against B cell antigens.

Sometimes, in clinically normal humans, dogs, and horses, a monoclonal gammopathy may develop that is not due to a myeloma. These monoclonal antibodies are usually an accidental finding on serum electrophoresis, and their origin is unclear. They may disappear spontaneously within a short period, or they may persist for many years. Affected animals may show abnormally large numbers of plasma cells in their internal organs on necropsy.

Polyclonal Gammopathies

In contrast to monoclonal gammopathies, which are usually produced by a myeloma, polyclonal gammopathies are observed in many different diseases. Polyclonal gammopathies are characterized by an increase in all immunoglobulins as a result of excessive activity of many different clones of plasma cells. The condition that most resembles a myeloma is Aleutian disease in mink (Chapter 27). Animals infected by the Aleutian disease parvovirus show marked plasmacytosis and lymphocyte infiltration of many organs and tissues, as well as polyclonal (occasionally monoclonal) gammopathy. As a result of the elevated immunoglobulin levels, affected mink experience a hyperviscosity syndrome and are severely immunosuppressed.

Other causes of polyclonal gammopathy include autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, and myasthenia gravis (Chapter 38), as well as infections such as tropical pancytopenia of dogs due to *Ehrlichia canis,* African trypanosomiasis, and chronic bacterial infections such as pyometra and pyoderma. In horses heavily parasitized with *Strongylus vulgaris,* polyclonal IgG3 levels rise significantly. Polyclonal gammopathy also occurs in virus diseases such as feline infectious peritonitis and African swine fever, and in diseases in which there is extensive liver damage.

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Hybridomas

The plasma cells in myelomas become neoplastic in an entirely random manner, so the immunoglobulins that they secrete are not usually directed against any antigen of practical importance. Nevertheless, myeloma cells can be grown in tissue culture, where they survive indefinitely. It is desirable to obtain large quantities of absolutely pure, specific immunoglobulins directed against an antigen of interest. This can be done by fusing normal plasma cells making the antibody of interest with myeloma cells that can grow in tissue culture. The resulting mixed cell is called a hybridoma.

The first stage in making a hybridoma is to generate antibody-producing plasma cells (Fig. 15.24). This is done by immunizing a mouse against the antigen of interest and repeating the process several times to ensure that a good antibody response is mounted. Two to four days after the antigen is administered, the spleen is removed and broken up to form a cell suspension. These spleen cells are suspended in culture medium, together with cultured mouse myeloma cells. Generally, myeloma cells that do not secrete immunoglobulins are used since this simplifies purification later on. Polyethylene glycol is added to the mixture. This compound induces many of the cells to fuse (although it takes about 200,000 spleen cells on average to form a viable hybrid with one myeloma cell). If the fused cell mixture is cultured for several days, any unfused spleen cells will die. The myeloma cells would normally survive, but they are eliminated by blocking their nucleic acid synthesis.

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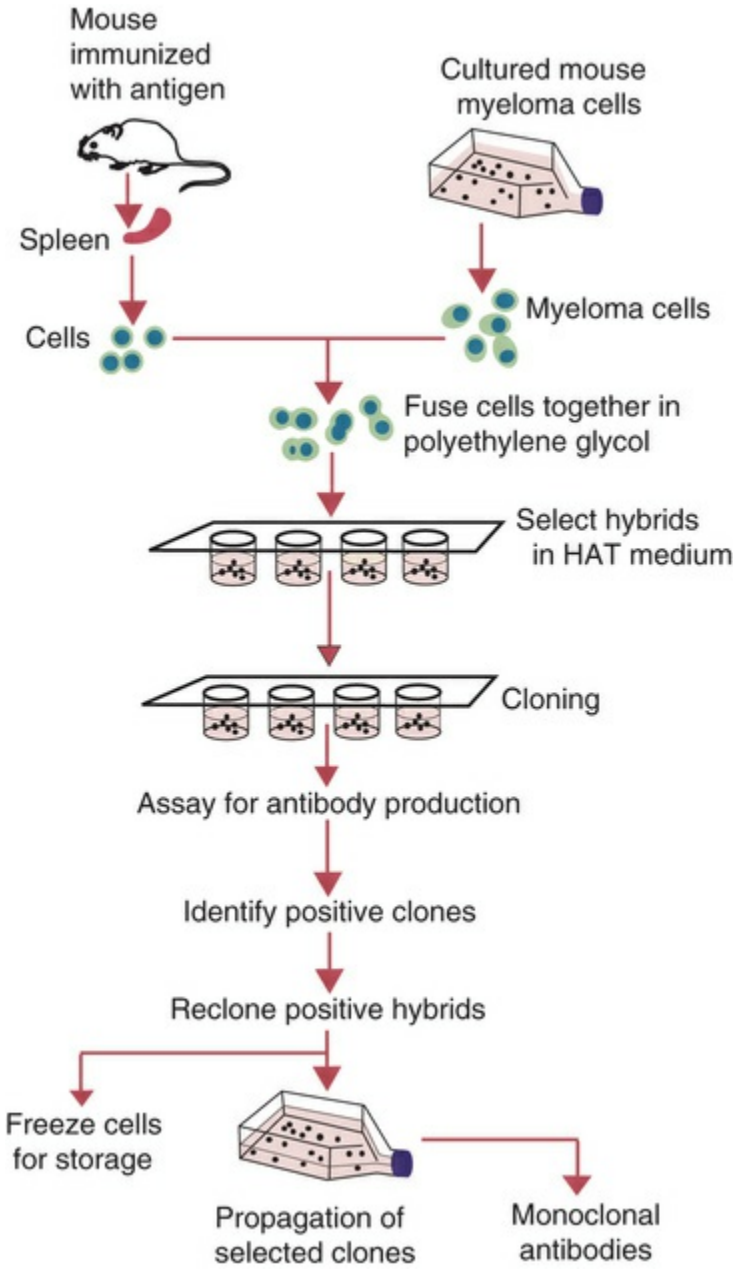


FIG. 15.24 A schematic diagram showing the method of production of monoclonal antibodies. Antibody-producing plasma cells are fused with myeloma cells. The resulting hybridoma cells are cultured, cloned, and selected so that they produce antibodies against the antigen of interest.

There are three pathways by which cells can synthesize nucleotides and therefore nucleic acids. The myeloma cells are selected so that they lack two enzymes: hypoxanthine phosphoribosyl transferase and thymidine kinase. As a result, they cannot use either thymidine or hypoxanthine and are obliged to use an alternative biosynthetic pathway to convert uridine to nucleotides. The fused cell mixture is therefore grown in a culture

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containing three compounds: hypoxanthine, aminopterin, and thymidine (known as HAT medium). Aminopterin is a drug that prevents cells from making their own nucleotides from uridine. Since the myeloma cells cannot use hypoxanthine or thymidine and the aminopterin stops them from using the alternative synthetic pathway, they cannot make nucleic acids and soon die. Hybrid cells made from a myeloma and a normal cell are able to survive and grow since they possess the critical enzymes. The hybridomas divide rapidly in the HAT medium, doubling their numbers every 24 to 48 hours. On average, about 300 to 500 different hybrids can be isolated from a mouse spleen, although not all will make antibodies of interest.

If a mixture of cells from a fusion experiment is cultured in wells on a plate with about 50,000 myeloma cells per well, it is usual to obtain about one hybrid in every three wells. After culturing for 2 to 4 weeks, the growing cells can be seen, and the supernatant fluid can be screened for the presence of antibodies. It is essential to use a sensitive assay at this time. Radioimmunoassays or enzyme-linked immunosorbent assays are preferred (Chapter 42). Clones that produce the desired antibody are grown in mass culture and recloned to eliminate non–antibody-producing hybrids.

Unfortunately, antibody-producing clones tend to lose this ability after being cultured for several months. Thus it is usual to make large stocks of hybridoma cells and store them frozen in small aliquots. These can then be thawed as required and grown up in bulk culture. Alternatively, the hybridoma cells can be injected intraperitoneally into mice. Since they are tumor cells, the hybridomas grow rapidly and provoke the effusion of a large volume of fluid into the mouse peritoneal cavity. This fluid is rich in monoclonal antibody and can be readily harvested.

The classical methods of making hybridomas produce only mouse immunoglobulins, and these are of limited usefulness in mammals of other species. Mouse antibodies are regarded as foreign in these species and are removed by the recipient's antibody response. Two strategies have been used to prevent or minimize this. One involves the genetic manipulation of hybridomas so that they produce antibodies of reduced antigenicity. Thus we can use only purified Fab'2 fragments—this eliminates the immunogenic Fc

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region but reduces their biological activities. It is possible, however, to join this fragment to the Fc region of the target species to produce a chimeric molecule. For example, mouse myeloma variable regions can be attached to dog constant regions to make a canine monoclonal antibody for use in that species. By subsequently modifying the sequence in the V region framework regions, the monoclonal antibody may be fully "caninized." For example, a caninized monoclonal antibody directed against interleukin-31 is used to prevent itch in dogs with atopic dermatitis (Chapter 30). Alternatively, it is possible to produce monoclonal antibodies of the desired species using "display technologies" such as phage displays. In these cases, huge libraries of antibody fragments are generated from the species of interest and then screened for their antigen-binding abilities.

Monoclonal antibodies are the preferred source of antibodies for much immunological research. They are absolutely specific for single epitopes and are available in large amounts. Because of their purity, they are used in clinical diagnostic tests in which large quantities of antibodies of consistent quality are required. Although mouse cells have been the preferred source, studies have shown that cattle and goats can be genetically engineered to produce monoclonal antibodies in their milk. It has even proved possible to incorporate antibody genes into plants such as soy, corn, and tobacco. These "plantibodies" are produced in very large quantities and appear to be functional. "Humanized" monoclonal antibodies are being employed to treat cancers and suppress inflammatory and autoimmune diseases. Similar species-modified monoclonal antibodies are being increasingly employed in veterinary medicine (Chapter 41).

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16

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Antibodies

Soluble Antigen Receptors

CHAPTER OUTLINE

Immunoglobulins, 162
Immunoglobulin Classes, 163
Immunoglobulin G, 163
Immunoglobulin M, 164
Immunoglobulin A, 165
Immunoglobulin E, 165
Immunoglobulin D, 165
Three-Dimensional Structure of Immunoglobulins, 167
Immunoglobulin Variants, 168
Subclasses, 168
Allotypes, 168
Idiotypes, 168
Production of Immunoglobulin Heavy Chains, 168
Class Switch Recombination, 169
B Cell Antigen Receptors and Soluble Immunoglobulins, 169
Immunoglobulins of Domestic Mammals, 170

Horses, 170
Cattle, 170
Sheep, 171
Pigs, 172
Dogs and Cats, 172
Primates, 172
Other Mammals, 172

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Explain the relationship between BCRs and circulating antibodies.
- Recognize that mammals make five classes of immunoglobulins: immunoglobulin G (IgG), IgM, IgA, IgE, and IgD.
- Describe how IgG is the predominant immunoglobulin in serum and is mainly responsible for systemic defense.
- Explain why IgM is mainly produced during a primary immune response.
- Briefly describe the role of IgA on body surfaces. What is its function?
- Explain that IgE functions in immunity to parasitic worms and as a cause of allergies.
- Recognize that IgD is found on the surface of immature lymphocytes and its function is unclear.
- Describe the basic structure of each of the five immunoglobulin classes.
- Explain the significance of immunoglobulin subclasses (or isotypes).
- Explain how immunoglobulin heavy chain genes can undergo class switching.
- Explain how immunoglobulins can exist in both cell-bound and soluble forms.
- Understand the unique structure of bovine and camel immunoglobulins.

The properties of the B cell antigen receptors (BCRs) were discussed

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in the previous chapter. These receptors are, however, not restricted to the B cell surface. Once a B cell response is triggered, it becomes a plasma cell and its antigen receptors are produced in huge amounts and shed into the surrounding fluid where they act as antibodies. These antibodies bind to foreign antigens and mark them for destruction or elimination. Antibodies are found in many body fluids but are present in highest concentrations and are most easily obtained from blood serum. Antibodies have to defend an animal against many different types of microbes, including bacteria, viruses, helminths, and protozoa. They must also act in several different environments, for example, in blood or milk, or on body surfaces. It is not surprising, therefore, that multiple immunoglobulin classes exist. Each class is optimized for action in a specific environment; for instance, IgA protects body surfaces. Immunoglobulins may also be optimized for activity against a specific group of pathogens. For example, IgE is important in the defense against parasitic worms.

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Immunoglobulins

Antibody molecules are glycoproteins called immunoglobulins (abbreviated to Ig). There are five structural classes (or isotypes) of immunoglobulins. The class found in highest concentrations in serum is called immunoglobulin G (abbreviated IgG). The class with the second highest serum concentration (in most mammals) is immunoglobulin M (IgM). The third highest concentration in most mammals is immunoglobulin A (IgA). IgA is, however, the predominant immunoglobulin in secretions such as saliva, milk, and intestinal fluid. Immunoglobulin D (IgD) is primarily a BCR and is rarely encountered in body fluids. Immunoglobulin E (IgE) is found in very low concentrations in serum and mediates allergic reactions. The characteristics of each of these classes are shown in Table 16.1.

TABLE 16.1

Major Immunoglobulin Classes in the Domestic Mammals

Property	IMMUNOGLOBULIN CLASS				
	IgM	IgG	IgA	IgE	IgD
Molecular weight	900,000	180,000	360,000	200,000	180,000
Subunits	5	1	2	1	1
Heavy chain	μ	γ	α	ε	δ
Largely synthesized in:	Spleen and lymph nodes	Spleen and lymph nodes	Intestinal and respiratory tracts	Intestinal and respiratory tracts	Spleen and lymph nodes

When serum is electrophoresed, its proteins separate into four major fractions (Fig. 16.1). The most negatively charged fraction consists of a single homogeneous protein called serum albumin. The other three fractions contain proteins classified as α, β, and γ globulins, according to their electrophoretic mobility (Fig. 16.2). Most immunoglobulins are found in the γ globulins. Immunoglobulin molecules consist of four linked peptide chains. Together they form a bilaterally symmetrical Y-shaped molecule with two identical Fab regions linked to a stem consisting of an Fc region (Chapter 15). The Fab regions bind antigens, and the Fc region binds to cells and activates complement.

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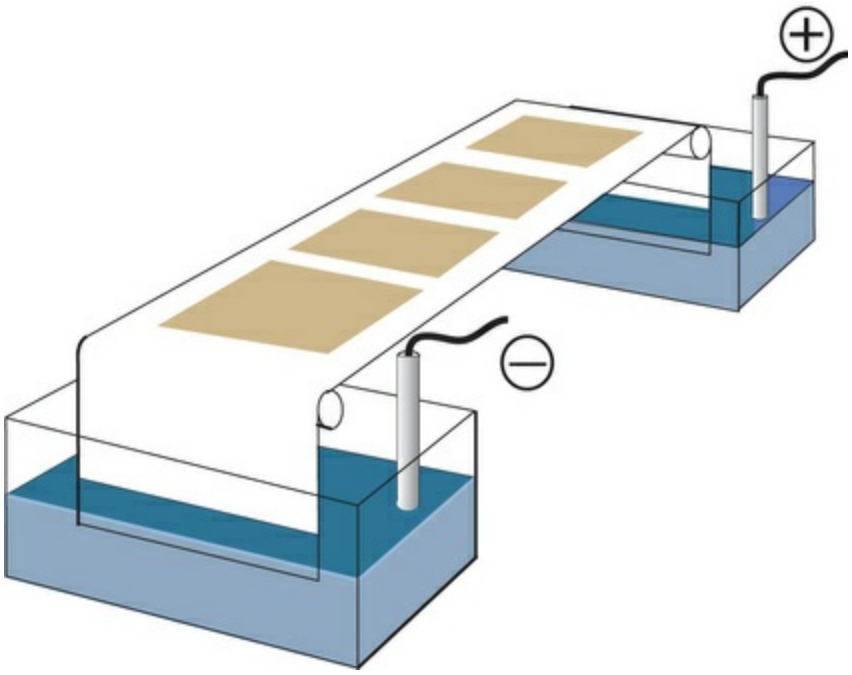


FIG. 16.1 The electrophoresis of a protein mixture on a strip of paper or other support. The support bridges two buffer baths, and an electrical potential is applied across them. It provides a convenient way to analyze serum proteins.

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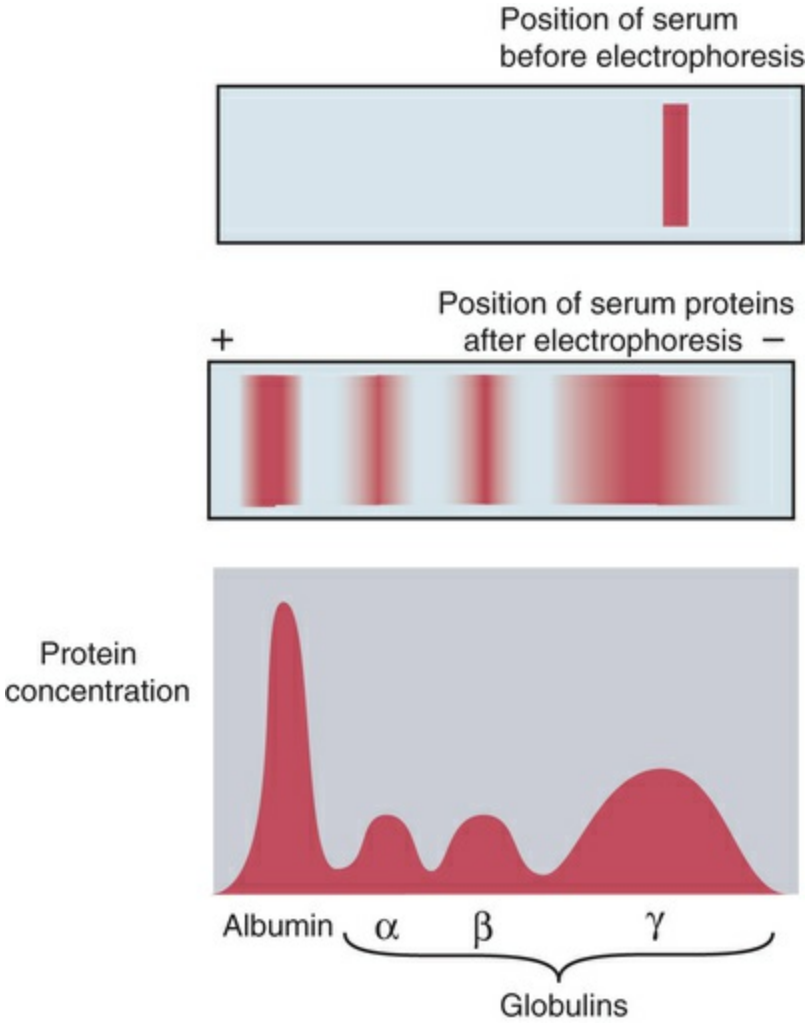


FIG. 16.2 Schematic diagram showing the results of electrophoresis of whole serum. Four major peaks develop consistently: the albumin and three globulin peaks. The globulins are classified as α, β, and γ globulins.

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Immunoglobulin Classes

Immunoglobulin G

IgG is produced by plasma cells in the spleen, lymph nodes, and bone marrow. It is the immunoglobulin found in highest concentration in the blood (Table 16.2) and plays the major role in antibody-mediated defenses. It has a molecular weight of about 180 kDa and a typical BCR structure with two identical light chains and two identical γ heavy chains (Fig. 16.3). Its light chains may be of the κ or λ type. Because it is the smallest of the immunoglobulin molecules, IgG can escape from blood vessels more easily than the others. This is especially important in inflammation, where increased vascular permeability allows IgG to participate in the defense of tissues and body surfaces. IgG binds to specific antigens such as those found on bacteria. Binding of these antibody molecules to bacteria can cause clumping (agglutination) and opsonization. IgG antibodies activate the classical complement pathway only when sufficient molecules have clustered on the antigenic surface (Chapter 4).

TABLE 16.2

Serum Immunoglobulin Levels in the Domestic Animals and Humans

Species	IMMUNOGLOBULIN LEVELS (mg/dL)			
	IgG	IgM	IgA	IgE
Horses	1000-1500	100-200	60-350	4-106
Cattle*	1700-2700	250-400	10-50	
Sheep	1700-2000	150-250	10-50	
Pigs	1700-2900	100-500	50-500	
Dogs	1000-2000	70-270	20-150	2.3-4.2
Cats†	400-2000	30-150	30-150	
Chickens	300-700	120-250	30-60	
Humans	800-1600	50-200	150-400	0.002-0.05

*Cattle show significant seasonal differences in serum immunoglobulin levels.

†Immunoglobulin levels in pathogen-free cats are about half those in pet cats.

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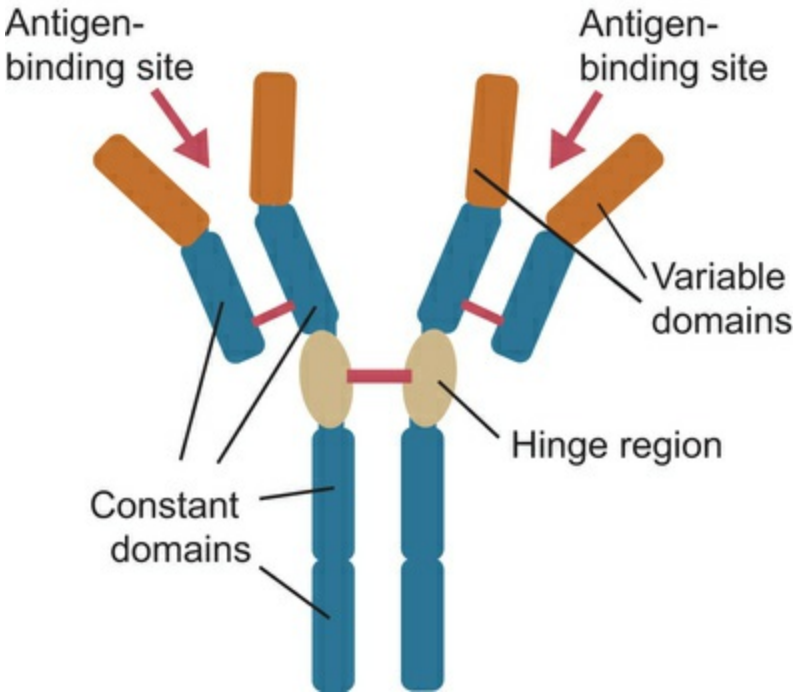


FIG. 16.3 The structure of IgG, the typical immunoglobulin molecule. Compare this with Fig. 15.1, a typical BCR.

Immunoglobulin M

IgM is produced by plasma cells in the secondary lymphoid organs. It has the second highest concentration after IgG in most mammalian sera. When attached to the B cell surface and acting as a BCR, IgM is a 180-kDa monomer. However, the secreted form of IgM consists of five (occasionally six) 180-kDa units linked by disulfide bonds in a circular fashion. Its total molecular weight is then 900 kDa (Fig. 16.4). A small polypeptide called the J chain (15 kDa) joins two of the units to complete the circle. Each IgM monomer is of conventional immunoglobulin structure and consists of two κ or λ light chains and two μ heavy chains; μ chains differ from γ chains in that they have an additional fourth constant domain (C_H4), as well as an additional 20-amino acid segment on their C-terminus but have no hinge region. The complement activation site on IgM is located on the C_H4 domain.

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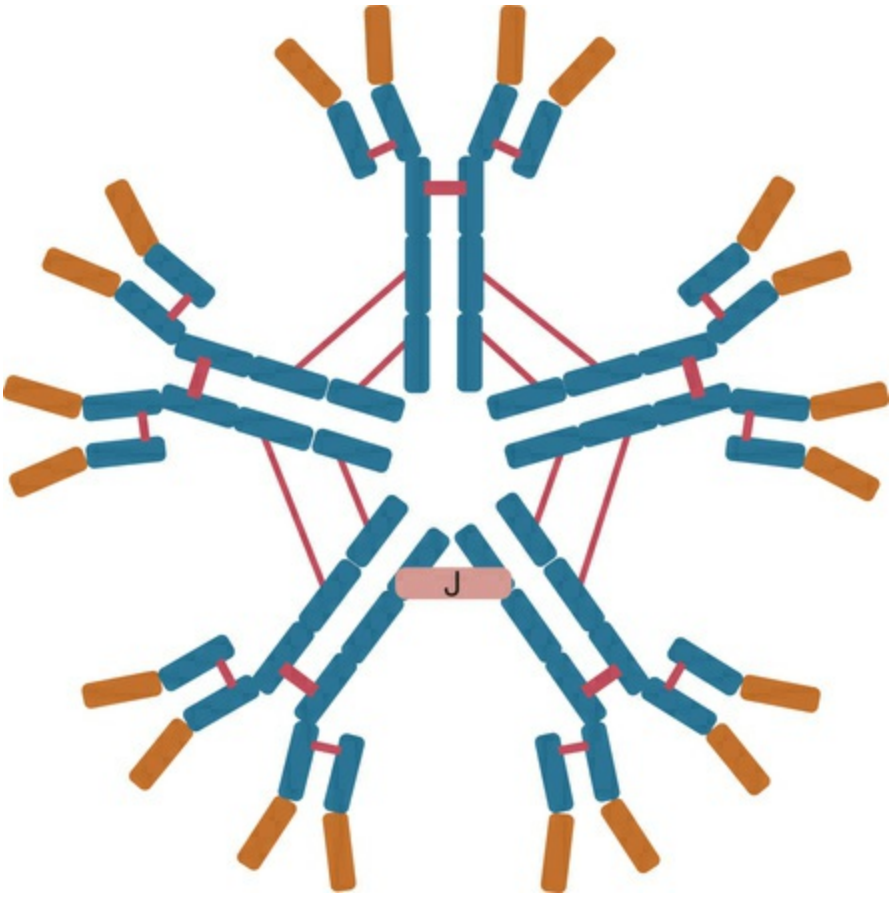


FIG. 16.4 The structure of IgM.

IgM is the major immunoglobulin produced during primary immune responses (Fig. 16.5). Smaller quantities are also produced in secondary responses. Although produced in small amounts, IgM is more efficient (on a molar basis) than IgG at complement activation, opsonization, neutralization of viruses, and agglutination. Because of their very large size, IgM molecules rarely enter tissue fluids, even in acute inflammation.

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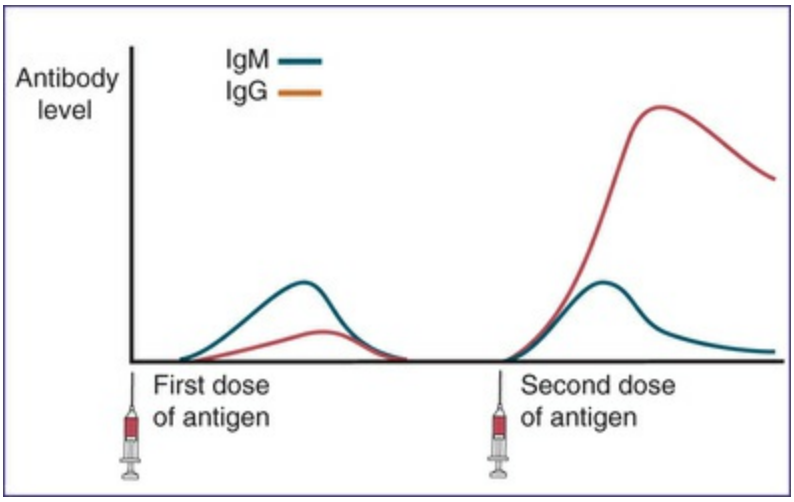


FIG. 16.5 The relative amounts of each immunoglobulin class produced during the primary and secondary immune responses. Note that IgM predominates in a primary immune response, whereas IgG predominates in later responses.

Immunoglobulin A

IgA is produced by plasma cells located under body surfaces. Thus it is made in the walls of the intestine, respiratory tract, urinary system, skin, and mammary gland. Although produced in large amounts, most goes into the intestine, bronchi, or milk. As a result, its serum concentration in most mammals is usually lower than that of IgM. IgA monomers have a molecular weight of 150 kDa, but they are normally secreted as dimers. Each IgA monomer consists of two light chains and two α heavy chains containing three constant domains. In dimeric IgA, the two monomers are joined by a J chain (Fig. 16.6). Higher polymers of IgA are occasionally found in serum.

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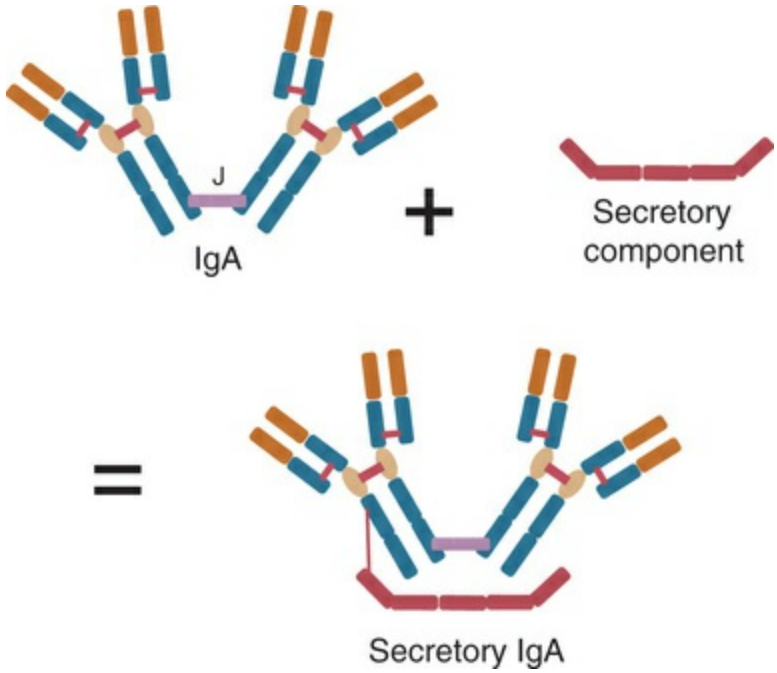


FIG. 16.6 The structure of IgA and secretory IgA. Secretory component is found on the surface of certain epithelial cells, where it acts as a receptor for polymeric immunoglobulins (pIgR). It can also bind to IgM.

IgA produced in body surfaces is transported through epithelial cells into external secretions bound to the polymeric immunoglobulin receptor (pIgR) or secretory component (see Fig. 22.13). Secretory component is a peptide that binds to IgA dimers to form secretory IgA (SIgA). It protects the IgA from digestion by intestinal proteases.

SIgA is the major immunoglobulin in the external secretions of nonruminants. As such, it is of critical importance in protecting the intestinal, respiratory, and urogenital tracts, the mammary gland, and the eyes against microbial invasion. IgA does not activate the classical complement pathway, nor can it act as an opsonin. It can, however, agglutinate particulate antigens and neutralize viruses. IgA prevents the adherence of invading microbes to body surfaces. Because of its importance, IgA is examined in more detail in Chapter 22.

Immunoglobulin E

IgE, like IgA, is mainly made by plasma cells located beneath body surfaces. It is a typical Y-shaped, four-chain immunoglobulin with four constant domains in its ε heavy chains and a molecular weight

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of 190 kDa (Fig. 16.7). Most IgE is bound to tissue mast cells and occurs in extremely low concentrations in serum. As a result, IgE cannot act simply by binding and coating antigens, as the other immunoglobulins do. IgE triggers acute inflammation by acting as a signal-transducing molecule. Thus IgE molecules bind tightly to FcεRI receptors on mast cells and basophils. When this IgE binds antigen, it triggers the release of inflammatory molecules from the mast cells. The resulting acute inflammation enhances local defenses and helps eliminate invaders such as parasitic worms. IgE has the shortest half-life of all immunoglobulins (2-3 days) and is readily destroyed by mild heat treatment. IgE is described in more detail in Chapter 29.

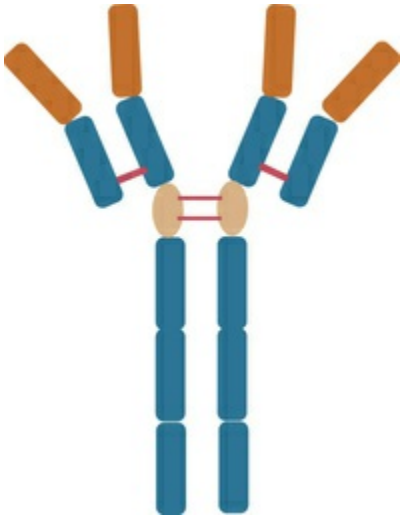


FIG. 16.7 The structure of IgE. Note the presence of four constant domains in addition to a hinge in the heavy chain.

Immunoglobulin D

IgD is present in horses, cattle, sheep, pigs, dogs, rodents, and primates but has not yet been detected in rabbits or cats. It is present in many bony fish and many birds but not in chickens. IgD remains attached to B cells, and very little is released into the blood. IgD molecules consist of two δ heavy chains and two light chains. In contrast to the other immunoglobulin classes, IgD is evolutionarily labile and shows many structural variations. For example, mouse IgD lacks a Cδ2 domain and thus has only two

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constant domains in its heavy chains. It has a molecular weight of about 170 kDa (Fig. 16.8). Horse, cow, sheep, dog, monkey, and human IgD, in contrast, has three heavy chain constant domains and a very long hinge domain coded for by two exons (Fig. 16.9). Pig IgD has a short hinge coded for by a single exon. In cattle, sheep, and pigs, but not horses or dogs, the Cδ1 domain is almost identical to the Cμ1 domain of IgM, whereas the other constant domains are distinctly different. In mice, the two constant region domains (Cδ1 and Cδ3) are separated by a very long exposed hinge region. Because of this long hinge region and the fact that it has no interchain disulfide bonds, mouse IgD is unusually susceptible to destruction by proteases and cannot be detected in serum, although it may be detected in plasma (Box 16.1). Like IgE, IgD is destroyed by mild heat treatment.

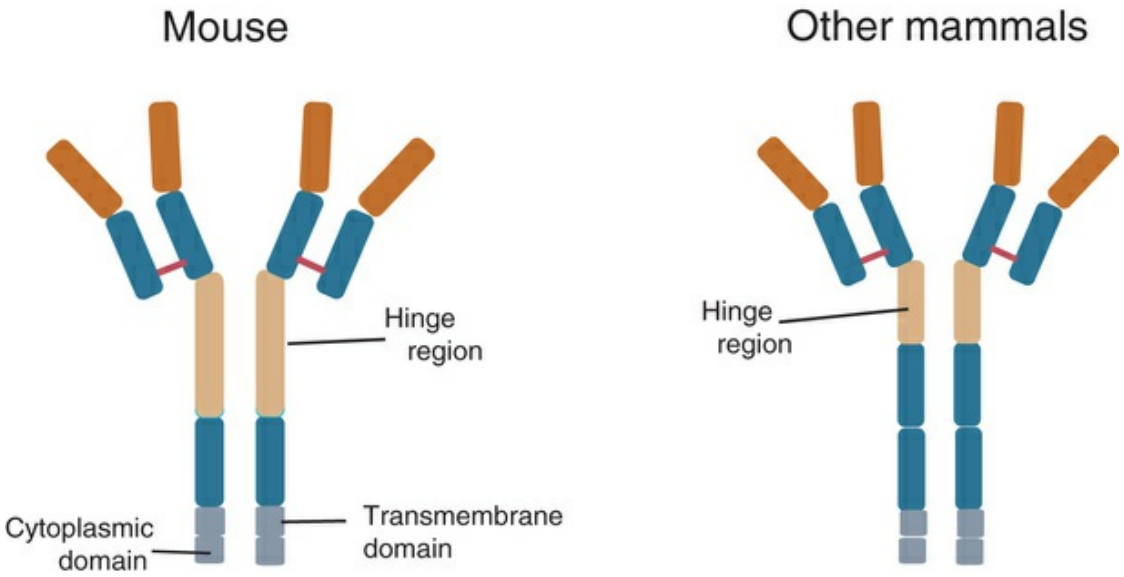


FIG. 16.8 The structure of IgD in mice and other mammals. Note the long exposed hinge region in mouse IgD that makes this molecule very unstable.

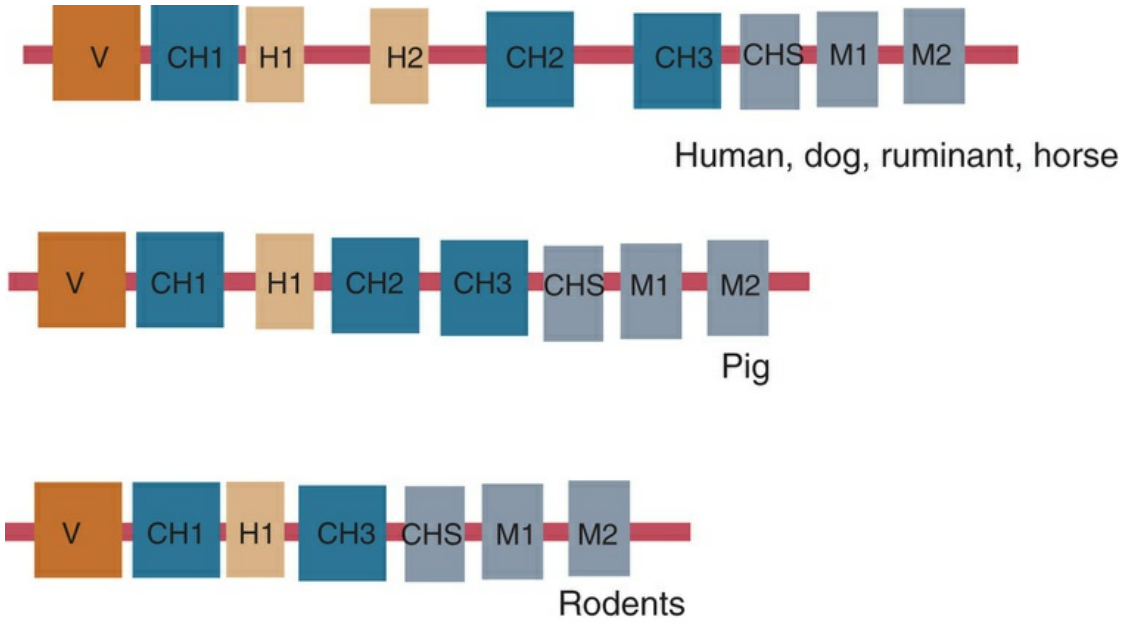


FIG. 16.9 The gene structure of IgD differs greatly among mammals. This diagram shows the exon structure of IgD heavy chains in different species. No other immunoglobulin class shows such variation, and its significance is unknown.

Box 16.1

IgD and the Microbiota

Recent studies on the role of IgD have determined that B cell class switching from IgM to IgD in mice is initiated by the intestinal microbiota! In mice, much IgD is produced by the B cells of the intestinal mucosa-associated lymphoid tissues. However, it is not produced in germ-free mice, nor is it produced in MyD88-deficient mice, indicating that production is triggered through toll-like receptors. The IgD produced in the intestine is active against the intestinal bacteria. Microbiota-associated IgD class switching can also be detected in the nasal mucosa. Thus IgD may play a role in regulating the normal microbiota.

Choi JH, Wang KW, Zhang D, et al: IgD class switching is initiated by microbiota and limited to mucosa-associated lymphoid tissue in mice, *Proc Natl Acad Sci USA* 114: E1196-E1204, 2002.

The role of IgD has so far defied explanation, but it probably regulates B cell responses. IgM to IgD class switching has been described in the upper respiratory mucosa of humans. This

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generates IgD-producing plasma cells whose products bind respiratory bacteria. The circulating IgD also binds to basophils and induces them to produce cathelicidins IL-1, IL-4, and B cell–activating factor (BAFF; see Box 15.2). Thus in humans, IgD orchestrates a defense system at the interface between innate and adaptive immunity.

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Three-Dimensional Structure of Immunoglobulins

Immunoglobulin peptide chains fold in such a way that an IgG molecule consists of three globular regions (two Fab regions and one Fc region) linked by flexible hinges (Fig. 16.10). Each globular region is made up of paired domains. Thus the Fab regions each consist of two pairs of interacting domains (V_H-V_L and C_H1-C_L), whereas the Fc region contains either two or three paired domains, depending on the immunoglobulin class (i.e., C_H2-C_H2, C_H3-C_H3, and in IgE or IgM, C_H4-C_H4). The peptide chains within each domain are closely intertwined. In the Fab regions, a groove is formed between the two variable domains, V_H and V_L. The amino acids of the complementarity determining regions (CDRs) line this groove, and as a result, the surface of the groove has a highly variable shape. This groove forms the antigen-binding site. The CDRs from both light and heavy chains contribute to the binding of an antigen, although the heavy chain contributes most to the process. Because immunoglobulins are bilaterally identical, the CDRs on each of the Fab regions are also identical. Thus the molecule has two identical antigen-binding sites and binds two identical epitopes. The presence of a hinge region in the middle of their heavy chains makes immunoglobulins such as IgG very flexible. Since the antigen-binding sites on each Fab region are identical, immunoglobulins are able to cross-link two antigens at the same time.

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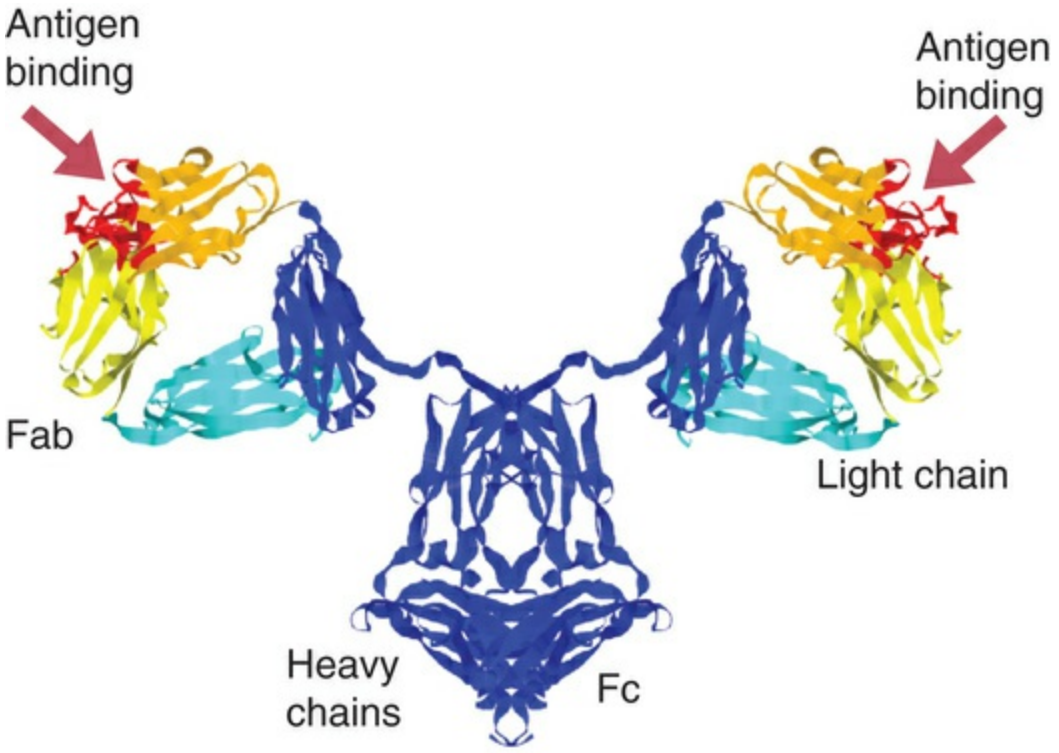


FIG. 16.10 A ribbon diagram showing the folding of the peptide chains in bovine IgG. Compare this with the very schematic diagrams of IgG structure seen elsewhere in the text. The globular domain structure is very obvious. It is also clear that the peptide chains in the hinge region are very exposed to breakage by proteases. Blue denotes constant domains, orange/yellow denotes variable domains, and red denotes the antigen-binding site. (Courtesy Dr. B. Breaux.)

It has generally been assumed that once an antibody molecule has formed, its structure remains unchanged until it is destroyed by catabolic processes. That assumption is incorrect. The IgG4 subclass in humans can exchange regions with other antibody molecules to generate a hybrid antibody with two different Fab arms. As a result, this antibody can cross-link two different antigens. This exchange of Fab arms between IgG4 molecules is dynamic. Thus a homogeneous IgG4 antibody, when administered to a human, will rapidly begin to swap arms. It is not known whether this occurs in domestic mammals.

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Immunoglobulin Variants

Subclasses

All immunoglobulin molecules are made of two heavy and two light chains. Several different heavy chains are employed in making these molecules. Thus when γ chains are used, the resulting immunoglobulin is IgG. IgM contains μ chains; IgA contains α chains, and so on. However, closer examination shows that even these immunoglobulin classes consist of mixtures of molecules using structurally different heavy chains known as subclasses.

Immunoglobulin subclasses have arisen as a result of gene duplication. Thus during the course of evolution, heavy chain *(IGH)* genes have been duplicated, and each new gene then is gradually changed through mutation. The amino acid sequences coded by these new genes may differ from the original in only minor respects. For example, bovine IgG is a mixture of three subclasses, IgG1, IgG2, and IgG3, coded for by the heavy chain genes *IGHG1*, *IGHG2*, and *IGHG3*, respectively. They differ in amino acid sequence and in physical properties such as electrophoretic mobility. These immunoglobulin subclasses may also have very different biological activities; for example, bovine IgG2 agglutinates antigenic particles, whereas IgG1 does not. Canine IgG subclasses differ in their ability to bind Fc receptors and thus have different functional abilities. All animals of a species possess each of these subclasses.

The number and properties of immunoglobulin subclasses vary among species. For example, most mammals have only one or two IgA subclasses, but rabbits have 13. These species differences are probably not of major biological significance; they simply reflect the number of immunoglobulin heavy chain gene duplications a species has undergone.

Allotypes

In addition to subclass differences, individual animals have inherited variations in immunoglobulin amino acid sequences.

Thus the immunoglobulins of one individual may differ from those of another individual of the same species (Fig. 16.11). These allelic sequence variations in heavy chain genes are reflected in structural differences called allotypes.

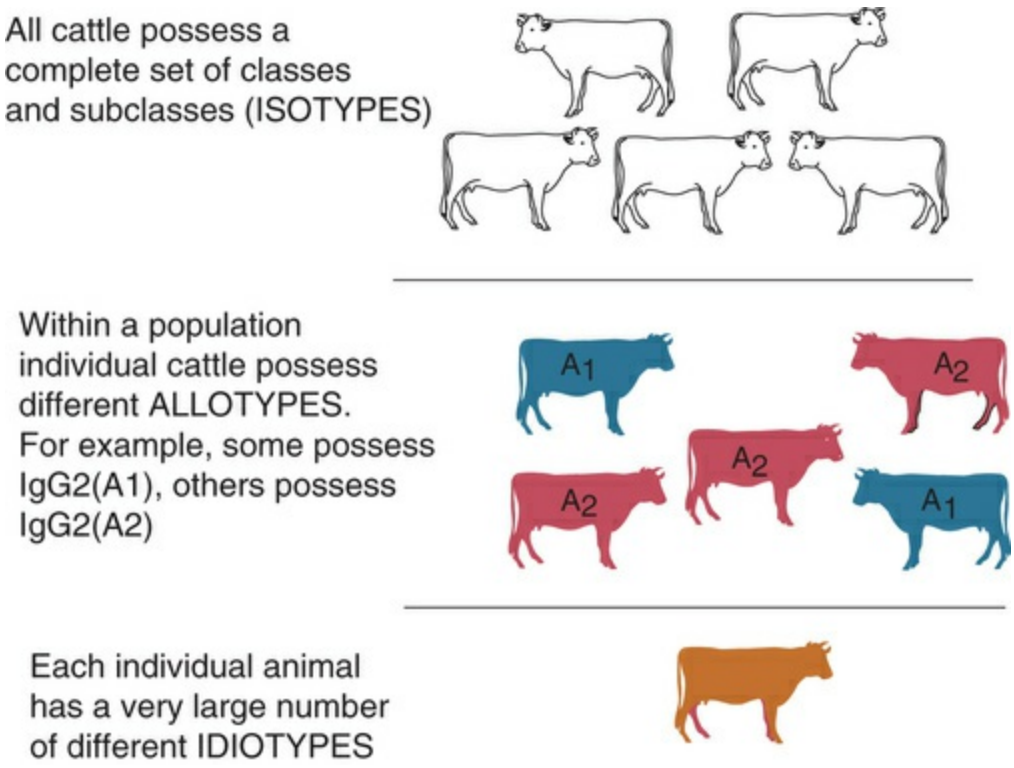


FIG. 16.11 A schematic diagram showing the differences among the inheritance of the major immunoglobulin variants.

Idiotypes

The third group of structural variants found in immunoglobulins results from the variations in the amino acid sequences within the variable domains on light and heavy chains. These variants are called idiotopes. The collection of idiotopes on an immunoglobulin is called its idiotype. Most idiotopes are located within the antigen-binding site.

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Production of Immunoglobulin Heavy Chains

Two genes code for each immunoglobulin heavy chain. One gene codes for the variable domain (and thus the antigen-binding site), whereas a separate gene encodes the constant domains. The way in which genes code for the variable domains is discussed in Chapter 17. The genes that code for the constant regions of immunoglobulin heavy chain (*IGH* genes) each consist of multiple expressed sequences or exons. One exon codes for each constant domain, and one codes for the hinge region (Fig. 16.12). A complete IgM constant region gene *(IGHM)* therefore consists of five exons, whereas an IgA constant region gene *(IGHA)* contains four exons. The heavy chain constant region genes are clustered on one chromosome. They are generally arranged in the order 5′-*IGHM-IGHD-IGHG-IGHE-IGHA*-3′. Thus the gene for the μ chain is followed by the gene for the δ chain, and these are followed by the γ chain genes and so on.

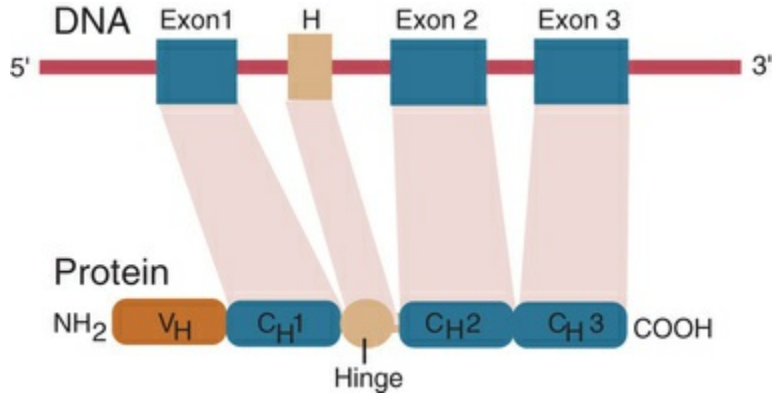


FIG. 16.12 A peptide chain such as an immunoglobulin heavy chain is coded for by a series of expressed sequences (exons) separated by intervening sequences (introns). Usually each exon codes for a single domain. When transcription occurs, the introns are spliced out and the exon sequences joined together in RNA.

As they mature, B cells undergo two DNA recombination events. The first, called V(D)J recombination, creates the antigen binding site of the BCR while the B cells are developing within the bone

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marrow. Once antigens activate the B cells, a second DNA recombination event occurs. This second event results in a switch in the class of BCR and thus antibodies produced by a B cell. This class switch recombination does not affect the antigen-binding site.

Class Switch Recombination

During the course of a B cell response, the class of immunoglobulin produced by a cell changes. This "class switch" can be explained by the way in which heavy chain genes are assembled.

During an antibody response, the immunoglobulins are synthesized in a standard sequence. Thus a responding B cell first uses the *IGHM* gene to make IgM BCRs. The remaining genes located 3′ to *IGHM* are ignored. In species that make IgD, the B cell also transcribes the *IGHD* gene and then expresses both IgM and IgD. As the immune response progresses, responding B cells switch to using *IGHG, IGHA,* or *IGHE* genes and become committed to synthesizing IgG, IgA, or IgE. The unwanted, unused *IGH* genes are excised as a DNA circle and are lost from the cell, so that the required *IGH* gene can be spliced directly to the *IGHV* gene.

For example, if IgM is to be synthesized, an *IGHV* gene is spliced directly to the *IGHM* gene (Fig. 16.13). On the other hand, if IgA is to be synthesized, the genes coding for Cμ to Cε inclusive are deleted, and the *IGHV* gene is then spliced directly to the *IGHA* gene. There are several ways by which these intervening genes can be excised. The simplest is called looping out–deletion. In this case the V region and C genes come together by looping out and then excising the intervening DNA using an enzyme called a recombinase. Two signals are needed to initiate class switching in a B cell. First, the B cell must receive an activation signal generated when CD40 on the B cell binds to CD154 on a helper T cell. Second, the specific class switch is determined by signals from cytokines, especially by IL-4, transforming growth factor-β (TGF-β), and interferon-γ (IFN-γ). Signals from CD40 and the antigen activate the recombinase in the B cell, while signals from the cytokine receptors, by activating specific promoter regions, target the recombinase to a specific heavy chain gene.

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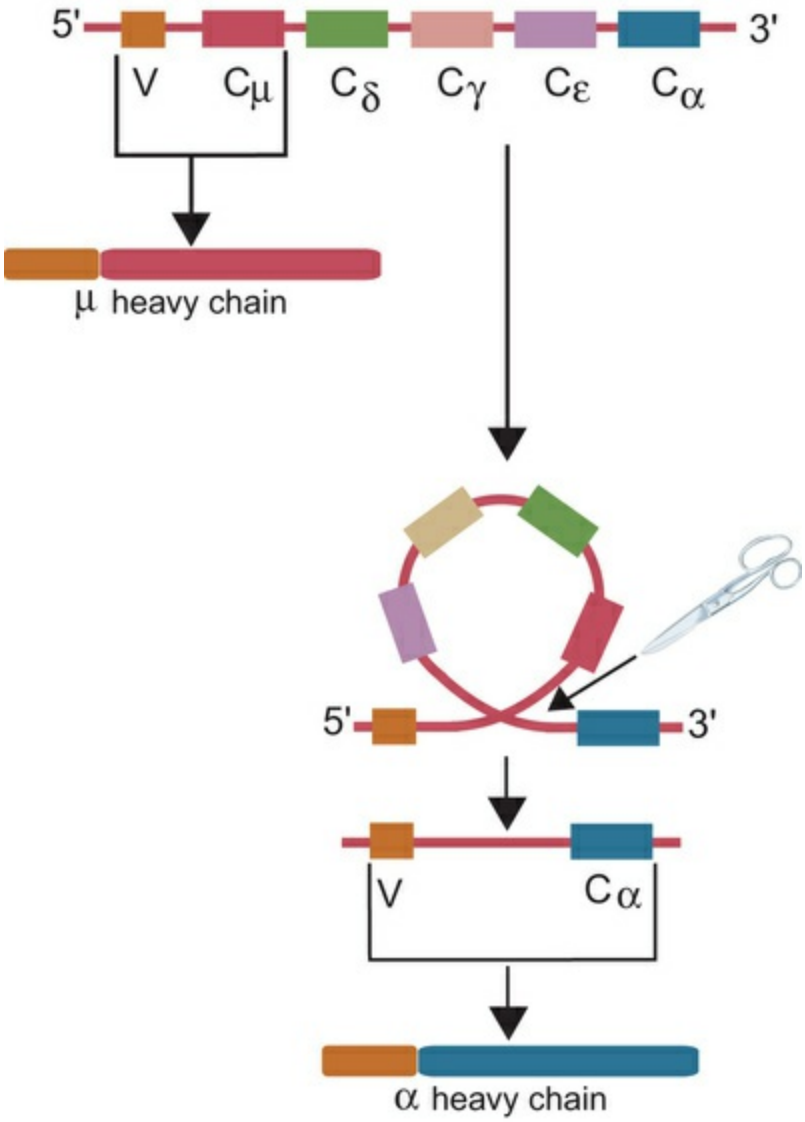


FIG. 16.13 The mechanism of class switching. In this example, a switch is made from IgM production to IgA production by deleting intervening heavy chain genes and joining the V genes to the appropriate heavy chain gene.

B Cell Antigen Receptors and Soluble Immunoglobulins

Immunoglobulins are first produced as cell-bound BCRs and are subsequently secreted as antibodies. The heavy chains of BCRs have a hydrophobic transmembrane C-terminal domain that attaches them to a B cell surface. This domain is absent from the secreted antibody. The switch between the two forms results from the differential splicing of exons. For example, the *IGHM* gene

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encodes two short exons, called CμS and CμM, located 3′ to Cμ4 (Fig. 16.14). CμS codes for the C-terminal domain of the secreted form, whereas CμM codes for the hydrophobic domain of the membrane-bound form. When IgM is made, all the Cμ exons are first transcribed to messenger RNA (mRNA). To produce membrane-bound IgM, the mRNA for the CμS exon is deleted, and the Cμ4 exon is spliced directly to the CμM exon. To produce secreted IgM, the exon coding for the CμM domain is deleted, and translation is stopped after Cμ4 and CμS are read.

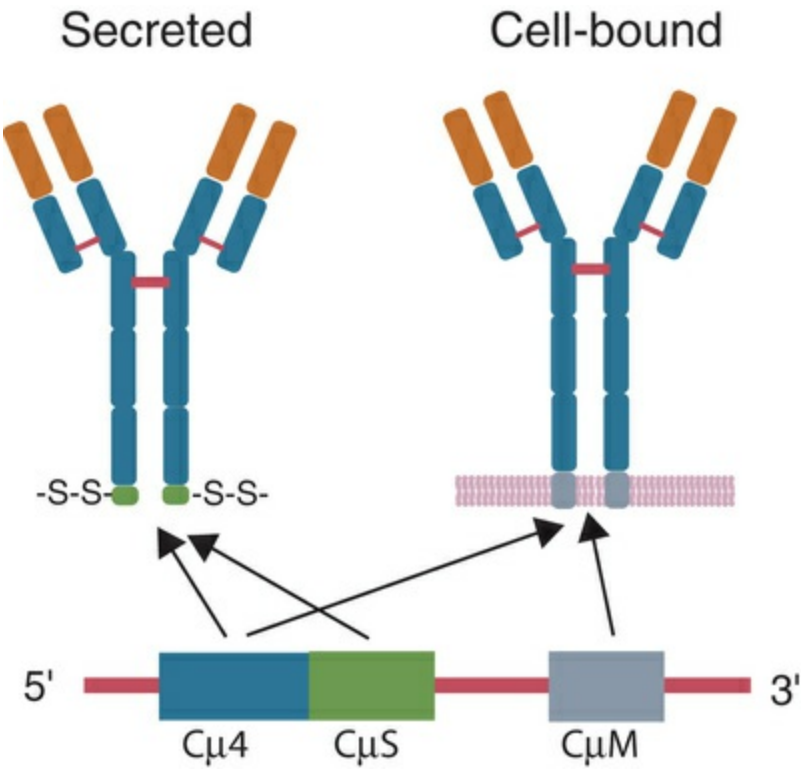


FIG. 16.14 IgM immunoglobulins serving as BCRs have a choice as to which C terminal domain they will use. The membrane-bound form uses a hydrophobic transmembrane domain (CμM). In contrast, the secreted form deletes this sequence and uses the CμS gene. The difference between the two forms is determined by RNA splicing following transcription.

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Immunoglobulins of Domestic Mammals

All mammals possess genes for and express four or five major immunoglobulin classes (IgG, IgM, IgA, IgE, IgD), although these may not have been formally identified in all species (Table 16.3). The basic characteristics of each of these classes are as described previously. However, during the course of evolution, as pointed out earlier, the *IGH* genes have duplicated, sometimes several times (Fig. 16.15). Over time, these duplicated genes mutate so that animals may produce several different subclasses of a specific immunoglobulin. If a duplicated gene mutates in such a way that it is no longer functional, it becomes a pseudogene. The number of duplications and hence the number of immunoglobulin subclasses and pseudogenes varies among species. In looking at these species differences, the reader might gain additional insight by examining the phylogeny of domestic animal species (Chapter 43).

TABLE 16.3

Immunoglobulin Classes and Subclasses in Selected Mammals

Species	IMMUNOGLOBULIN CLASSES				
	IgG	IgA	IgM	IgE	IgD
Horses	G1, G2, G3, G4, G5, G6, G7	A	M	E	D
Cattle	G1, G2, G3	A	M1, M2	E	D
Sheep	G1, G2, G3	A1, A2	M	E	D
Pigs	G1, G2a, G2b, G3, G4	A	M	E	D
Dogs	G1, G2, G3, G4	A	M	E1, E2	D
Cats	G1, (G2?)	A	M	(E1, E2?)	?
Mice	G1, G2a, G2b, G3	A1, A2	M	E	D
Chimpanzees	G1, G2, G3,	A	M	E	D
Humans	G1, G2, G3, G4	A1, A2	M1, M2	E	D

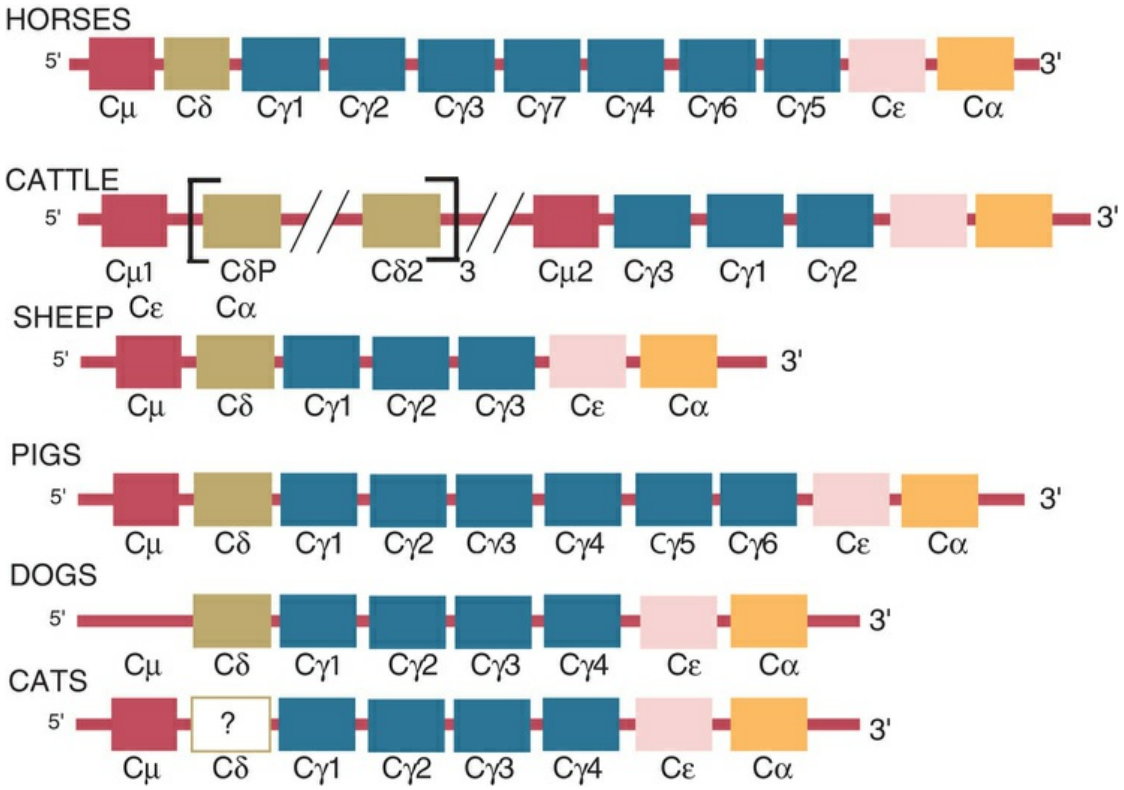


FIG. 16.15 The arrangement of the immunoglobulin heavy chain genes of the major domestic species.

Horses

The horse heavy chain gene locus is located on chromosome 24qtr. The locus contains seven *IGHG* genes, and all are expressed. Thus there are seven IgG subclasses: IgG1 thru IgG7. (The previous nomenclature for IgG1 thru IgG4 was IgGa, IgGc, IgG[T], and IgGb. IgG6 was previously called IgG[B]). The order of the Ig heavy chain genes in the horse is;

5′-*M-D-G1-G2-G3-G7-G4-G6-G5-E-A*-3′.

IgG7 is closely related to IgG4 and likely resulted from a recent duplication of the *IGHG4* gene. IgG1, IgG4, and IgG7 are produced in response to intracellular infections, while IgG3 and IgG5 are mainly produced in response to extracellular invaders. Horses also express IgM, IgD, IgA, and IgE. The horse *IGHD* gene is located downstream from *IGHM.* It appears to be expressed at least at the mRNA level. Horses have two IgG4 alleles ($IgG4^{a}$ and $IgG4^{b}$) and four IgE alleles ($IgE^{1\text{-}4}$).

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Cattle

Cattle are the only mammals known to possess two functional heavy chain loci. Most of these genes are located on chromosome 21, but a truncated μCH2 exon is present on chromosome 11. The genes on chromosome 21 are organized thus: (*n* denotes a variable number of these genes, *p* denotes a pseudogene).

5'-*Vn-Dn-Jn-M1- (D1p-V3-D1n)*$_3$*-Jn-M2-D-G3-G1-G2-E-A*-3'.

Thus there are internal duplications of DH, JH, and C region genes. Both IgM genes can be expressed independently or sequentially by class switching. Cattle have three *IGHG* genes corresponding to their three subclasses: IgG1, IgG2, and IgG3. IgG1 constitutes about 50% of the serum IgG and is the predominant immunoglobulin in cows' milk rather than IgA. IgG2 levels are highly heritable and its concentrations vary greatly among cattle. Cattle possess a unique Fc receptor on their macrophages and neutrophils that binds only IgG2. Since bovine IgG2 has a very small hinge region, this receptor might represent a special adaptation to the structure of this immunoglobulin.

Some cattle immunoglobulin molecules are unusually large because they use a long third hypervariable polypeptide loop (CDR3) that may contain up to 69 amino acids. This length is due to a very long germline Dh2 gene segment that encodes four cysteines that form interchain bonds with each other, together with repeated glycine, serine, and tyrosine residues. As a result, these heavy chain CDR3s fold into a long stalk supporting a disulfide-bonded "knob" domain located far from the antibody surface (Figs. 16.16 and 16.17). The knob folds into a mixture of minidomains generated by multiple mutations involving cysteine residues. It is this protruding knob and stalk that recognizes antigens. The benefits of this structure are unclear.

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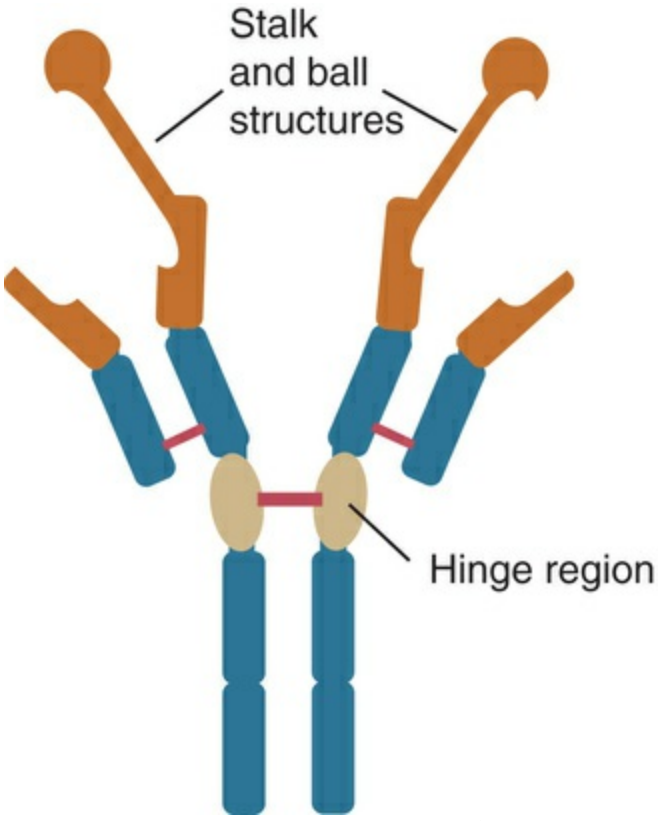


FIG. 16.16 The unusual variable regions of the bovine IgM with a stem and knob structure

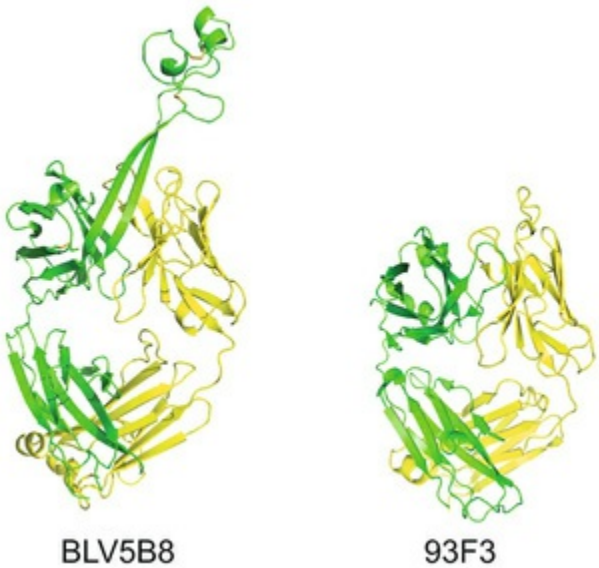


FIG. 16.17 A ribbon diagram showing the structure of a bovine Fab *(left)* as compared to a "normal" Fab showing the superlong β-stranded stalk protruding from the VH immunoglobulin domain. (From Wang F, Ekiert DC, Ahmed I, et al: Reshaping antibody diversity, *Cell,* 153: 1379-1393, 2013, Courtesy Elsevier Inc.)

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Two heavy chain allotypes (a and b) have been identified in all three IgG classes. Allotype B1 is found on light chains of some cattle but is relatively uncommon. IgA, IgM, and IgE also occur in cattle. Cattle IgD may be expressed on B cells. About 95% of bovine immunoglobulins have λ light chains.

Sheep

The immunoglobulin subclasses of sheep are similar to those of cattle with *IGHG* genes coding for IgG1, IgG2, and IgG3. Some sheep have an IgG1a allotype. An *IGHD* gene has been detected in sheep. Three IgA heavy chain allotypes have been identified, as have three IgE allotypes.

Pigs

Pigs have 10 immunoglobulin genes: *IGHM*, *IGHD*, six *IGHG*, *IGHE*, and *IGHA*. Eleven pig Cγ gene sequences have been described. These code for six IgG subclasses named IgG1 through IgG6. There are two allelic forms of all of these subclasses except IgG3. The differences between the alleles may be minor. For example, $IgG2^a$ and $IgG2^b$ differ by only three amino acids. IgG3 has an extended hinge, is structurally unique, and appears to be the most evolutionarily conserved porcine IgG. $IgG5^b$ differs most from its allele, and its C_H1 domain shares sequence homology with the C_H1 of IgG3. Some pigs may have two *IGHG5* and two *IGHG6* genes but no *IGHG2* or *IGHG4*. Other pigs may lack IgG4 or IgG6. IgG is the predominant serum immunoglobulin, accounting for about 85% of the total. IgM accounts for about 12% and dimeric IgA for about 3% of serum immunoglobulins. Pigs have a single *IGHA* gene with two alleles. IgA^b differs from IgA^a by a 12-nucleotide deletion in the hinge region owing to a mutation in its splice acceptor site. The consequences of this are unclear. An *IGHD* gene has been identified in pigs. Its first constant domain may be coded by either a C_H1 δ gene or by a C_H1 μ gene! Thus pig IgD heavy chain transcripts may contain either VDJ-CH1μ-CH2δ-CH3δ or VDJ-CH1δ-CH2δ-CH3δ. This pattern has not been reported in other mammals. These two genes, however, show almost 99% similarity, so the biological

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consequences are probably not great, especially since IgD has not been identified and may not even be expressed in pigs. Pig IgE has been identified. One IgM allotype has been reported (Box 16.2).

Box 16.2

Curious Case of the Camel

Members of the camel family from both the Old and New Worlds (camels and llamas) have three IgG subclasses: IgG1, IgG2, and IgG3. IgG1 has a conventional four-chain structure and therefore has a molecular weight of 170 kDa. In contrast, IgG2 and IgG3, which together account for 75% of camel immunoglobulins, are 100-kDa heavy-chain dimers that have no light chains! In addition, camel IgG2 heavy chains lack a CH1 domain but compensate for this by having a very long hinge region. Despite lacking light chains, these molecules can still bind to many antigens. It has been noted that these antibodies bind effectively to the substrate pockets of enzymes. Studies have also shown that the antigen-binding site on these heavy chains is very convex. This enables it to fit snugly into the concave active site on an enzyme. Thus these single-chain antibodies may have a structural advantage over conventional immunoglobulins in neutralizing enzyme activity.

Dogs and Cats

Dogs have four *IGHG* genes and hence four IgG subclasses, named IgG1, IgG2, IgG3, and IgG4, in order of abundance. In addition, dogs have IgA, IgM, IgD, and IgE. Four alleles have been identified in the dog *IGHA* gene. All are restricted to the hinge region. An IgM allotype has been described in the dog.

Cats have two *IGHG* genes, one IgM subclass, and possibly two IgA subclasses (IgA1 and IgA2), as well as two possible IgE subclasses. The two IgG genes appear to be allotypes of one subclass and have been designated IgG1a and IgG1b. They account for about 98% of serum IgG. About 2% of cat serum immunoglobulins belongs to a second uncharacterized IgG subclass.

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Primates

Humans have four *IGHG* genes coding for IgG1 to IgG4. Chimpanzees and rhesus macaques possess three *IGHG* genes coding for IgG1, IgG2, and IgG3. The chimpanzee IgG2 molecule contains epitopes also found on both human IgG2 and IgG4, suggesting that the *IGHG2* and *IGHG4* genes split after humans separated from chimpanzees. Baboons *(Papio cynocephalus)* have four *IGHG* genes, but they differ significantly from human IgG in their hinge region. Rhesus macaques may have two IgM subclasses. All the great apes, with the exception of the orangutan, have two IgA subclasses.

Other Mammals

Rats and mice have four or five functional *IGHG* genes. In contrast, rabbits have only one *IGHG* gene despite having 13 *IGHA* genes, at least 12 of which are functional! They appear to lack IgD. The expression of these IgA subclasses varies among tissues.

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How Antigen-Binding Receptors Are Made

CHAPTER OUTLINE

Receptor-Antigen Binding, 174
Antigen Receptor Genes, 174
Immunoglobulin Diversity, 175
Gene Recombination, 175
IGL Locus, 175
IGK Locus, 175
IGH Locus, 175
Generation of Junctional Diversity, 176
Gene Rearrangement, 176
Base Deletion, 177
Base Insertion, 177
Receptor Editing, 177
Somatic Mutation, 178
Gene Conversion, 179
Receptor Assembly, 180
Potential Immunoglobulin Diversity, 180
Species Differences, 181
Horses, 181

Cattle, 181
Sheep, 181
Pigs, 182
Dogs and Cats, 182
Humans and Mice, 182
Intestinal Bacteria and Expansion of the B Cell Repertoire, 182
T Cell Receptor Diversity, 183
T Cell Receptor Gene Structure, 183
α Chain, 183
β Chain, 183
δ Chain, 183
γ Chain, 183
Generation of T Cell Receptor V-Region Diversity, 183
Gene Rearrangement, 183
Base Insertion and Deletion, 184
Somatic Mutation, 185
Where Does This Happen? 185
T Cell Receptor Diversity, 185
γ/δ T Cell Diversity, 185
Epigenetic Regulation, 185

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LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- List the major noncovalent bonds that bind antibodies to antigens.
- Explain why the shape of the antigen-binding groove depends on the sequence of the amino acids that line the groove.
- Describe how V, D, and J genes act together to generate hypervariable antigen-binding site.
- Explain how an enormous number of different BCRs are generated through the use of a limited number of genes.
- Explain the generation of variable regions by means of gene recombination.
- Explain the process of gene conversion.
- Explain why the genes coding for antigen-binding sites in BCRs, but not TCRs, also undergo random somatic mutation.
- Define gene conversion, somatic mutation, gene recombination, base deletion, receptor editing, and junctional diversity.
- Explain the role of the microbiota in generating immunoglobulin diversity.
- Explain how TCR diversity is generated.
- Explain how the developing T cell produces either γ/δ or α/β antigen receptors.

Because bacteria and viruses have a short generation time, they have the ability to mutate and change rapidly. As a result, the immune system must be able to respond not only to existing organisms but also, within reason, to newly evolved organisms. The ability of the adaptive immune responses to respond specifically to an enormous number of foreign antigens implies the existence of an enormous number of lymphocytes, each with its own specific antigen receptors. This then raises the question, how do lymphocytes generate such an enormous diversity of these receptors?

The ability of a receptor to bind an antigen is determined by the shape of its binding site. This shape depends on the folding of its peptide chains, which is governed, in turn, by their amino acid sequences. Each amino acid in a peptide chain exerts an influence on its neighboring amino acids, which determines their relative

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configuration. The shape of a peptide chain therefore represents the contributions of all amino acids in the chain as the peptide assumes its most energetically favorable conformation. The folding of a protein is determined by its amino acid sequence, and that sequence is determined by the sequence of bases in the DNA coding for that protein. The diversity of antigen receptors implies either a corresponding diversity in the genes coding for these receptors or a mechanism that generates diversity from a limited pool of receptor genes. This second mechanism is now known to be the method used by the adaptive immune system.

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Receptor-Antigen Binding

When an antigen and its receptor bind, they interact through the chemical groups on the antigen and on the complementarity determining regions (CDRs) of the receptor. In classic chemical reactions, molecules are assembled through the establishment of firm, covalent bonds. These bonds can be broken only by the input of a large amount of energy; energy that is not readily available. In contrast, the formation of non-covalent bonds provides a rapid and reversible way of forming complexes and permits reuse of molecules in a way that covalent bonding would not allow. However, noncovalent bonds act over short intermolecular distances and, as a result, form only when two molecules approach each other very closely. The binding of an antigen to a BCR or TCR is exclusively non-covalent, so the strongest binding occurs when the shape of the antigen and the shape of the receptor perfectly match. This requirement for a close conformational fit has been likened to the specificity of a key for its lock.

The major bonds formed between an antigen and its receptor are hydrophobic (Fig. 17.1). When antigen and antibody molecules come together, they exclude water molecules from the area of contact. This frees some water molecules from constraints imposed by the proteins and is therefore energetically stable. (The bond can be likened to two wet glass microscope slides stuck together. Anyone who has tried to separate two wet glass slides can confirm the effectiveness of this type of bonding.)

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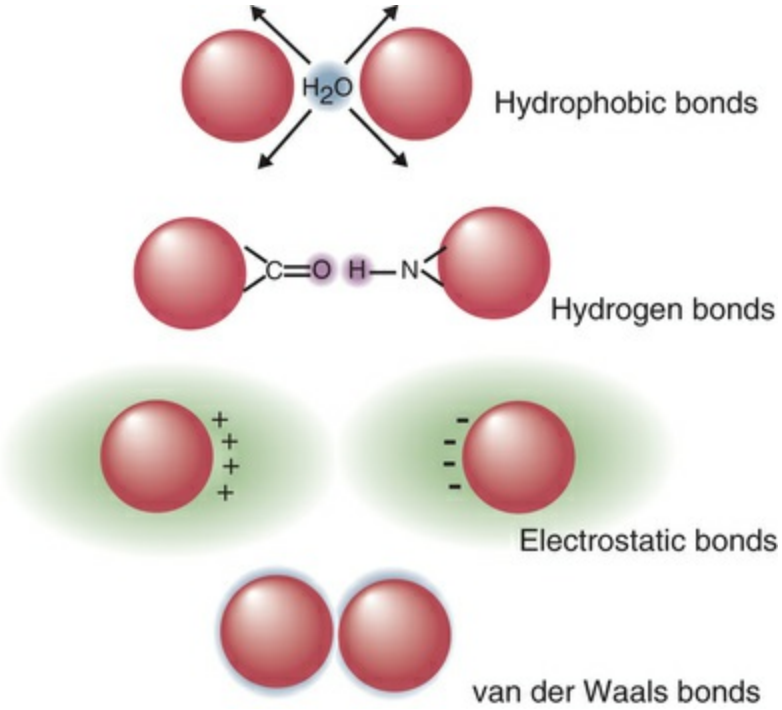


FIG. 17.1 Noncovalent bonds that link an antigen with its receptor arranged in order of relative importance. All these bonds are effective only over a very short distance. It is therefore essential that the shape of the antigen and its receptor site match very well if strong binding is to be achieved.

A second type of binding between an antigen and its receptor is through hydrogen bonds. When a hydrogen atom covalently bound to one electronegative atom, for example, an -OH group, approaches another electronegative atom such as an O═C- group, the hydrogen is shared between the two electronegative atoms. This situation is energetically favorable and is called a hydrogen bond. The major hydrogen bonds formed in antigen-receptor interaction are O–H–O, N–H–N, and O–H–N. Hydrogen bonds are already present between proteins and water molecules in aqueous solution, so the binding of an antigen to its receptor by hydrogen bonds requires relatively little net energy change.

Electrostatic bonds formed between oppositely charged amino acids may contribute to antigen-receptor binding, but the charge on many protein groups is commonly neutralized by electrolytes in solution. As a result, the importance of electrostatic bonding is unclear.

When two atoms approach very closely, a nonspecific attractive force, called a van der Waals force, becomes operative. It occurs as a result of a minor asymmetry in the charge of an atom because of the

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position of its electrons. This force, although very weak, may become collectively important when two large molecules come into contact. It can therefore contribute to antigen-receptor binding.

The binding of a receptor to its antigen is therefore mediated by multiple noncovalent bonds. Each bond is relatively weak in itself, but collectively they may have a significant binding strength. All these bonds act only across short distances and weaken rapidly as that distance increases. Electrostatic bond and hydrogen bond strengths are inversely proportional to the square of the distance between the interacting molecules; the van der Waals forces and hydrophobic bonds are inversely proportional to the seventh power of that distance. Thus the strongest binding between an antigen and its receptors occurs when their shapes match perfectly and multiple noncovalent bonds form. Antigens can bind to receptors when they fit less than perfectly, although the binding affinity will be much reduced.

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Antigen Receptor Genes

The information needed to make all proteins, including antigen receptors, is stored in an animal's genome. All that is required for the production of these molecules is that the necessary genes be turned on. Once the appropriate genes are activated, they can be transcribed into RNA and translated into the appropriate receptor protein on B or T cells. It has been estimated that mammals can produce up to 10^{15} different antigen receptors to be expressed on B and T cells. In order to produce this enormous diversity, they use fewer than 500 genes!

Multiple genes code for each receptor peptide chain. Several genes code for each variable region, whereas only one codes for a constant region. As a result, the single constant-region gene can be combined with any one of several variable-region genes to make a complete receptor chain (Fig. 17.2). Instead of having genes for all possible receptor chains, it is only necessary to have genes for all the variable regions and to join these to an appropriate constant-region gene as required. In addition, antigen receptor chains may be paired in different combinations to yield even greater diversity, a process called combinatorial association.

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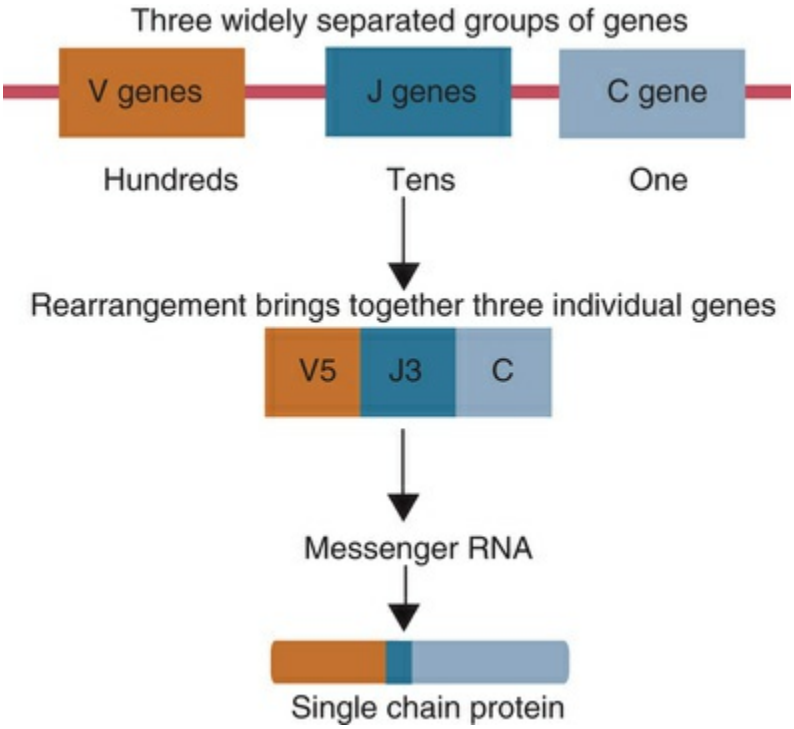


FIG. 17.2 Antigen receptor chains are coded for by three genes originating in three widely separated groups. The genes for a complete receptor chain are assembled by joining one gene selected from each group.

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Immunoglobulin Diversity

To make as many different antibodies as possible, it is necessary to diversify the amino acid sequences of the variable domains in both light and heavy chains. Since these amino acid sequences are determined by the nucleotide sequences, mechanisms must exist for generating this nucleotide sequence diversity. In practice, gene diversity is generated through three distinct mechanisms: gene recombination, somatic mutation, and gene conversion. The relative importance of each of these mechanisms differs among species, and the diversity-generating mechanisms that operate in humans and mice are not the same as those that operate in domestic mammals.

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Gene Recombination

Gene recombination results from the random selection of one gene from each of several groups of genes followed by recombining these selected genes to generate sequence diversity. It is well seen in the genes that code for immunoglobulins (Table 17.1).

TABLE 17.1

Examples of Different Gene Use in Mammals

Species	*IGKV*	*IGKJ*	*IGLV*	*IGLJ*	*IGHV*	*IGHJ*	*IGHD*
Horses	60	5	144	7	52	8	40
Bovine	22	3	25	4	36	6	14
Sheep	10	3	>100	1	7	6	>1
Pigs	14	5	23	4	20	1	2
Mice	169	9	14	3	161	7	18
Humans	48	9	69	8	215	27	30
Rats	163	7	3	2	174	5	21

Three gene loci code for immunoglobulin peptide chains, and each is found on a different chromosome (Fig. 17.3). One locus, called *IGL*, uses three genes to code for λ light chains; one, called *IGK*, uses three genes to code for κ light chains; and one, called *IGH*, uses four genes to encode heavy chains.

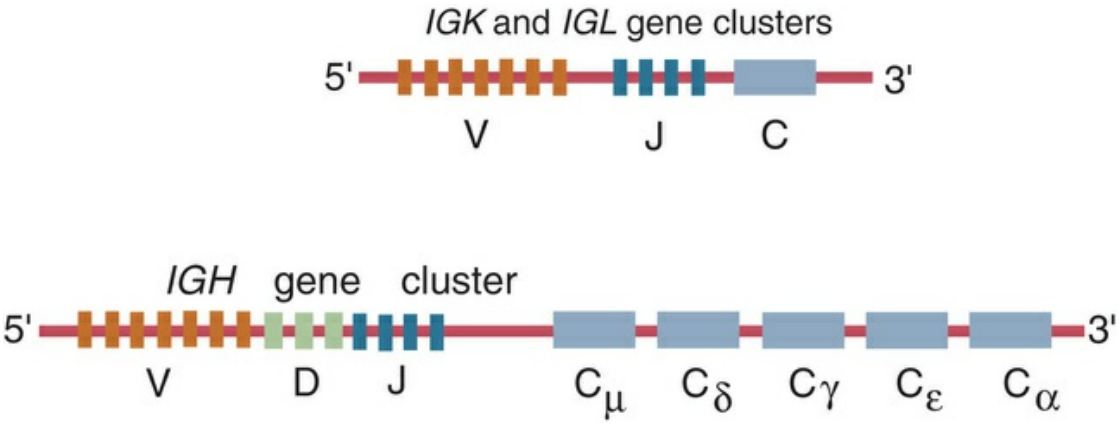


FIG. 17.3 Genes coding for immunoglobulin light chains and heavy chains. Note that there are two distinct light chain loci, one coding for kappa chains and one coding for lambda chains. These are located on different chromosomes. The precise number of V, D, and J genes varies among species.

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IGL Locus

Each lambda light chain is coded for by three genes. These are called *IGLV*, *IGLJ*, and *IGLC*. The *IGLV* gene codes for the variable region from the N-terminus to position 95. The *IGLC* gene codes for the constant region starting at position 110. The amino acids between 95 and 110 are coded for by *IGLJ*. The number of all three of these genes varies among domestic species.

IGK Locus

Kappa light chains are also coded for by three genes: *IGKV*, *IGKJ*, and *IGKC*.

IGH Locus

Four genes, *IGHV*, *IGHD*, *IGHJ* and *IGHC*, code for immunoglobulin heavy chains. The *IGH* locus contains close to 100 *IGHV* genes. It also contains multiple *IGHJ* genes situated 3′ to the *IGHV* genes. Several short genes, called *IGHD* genes (D for diversity), are located between the *IGHV* and *IGHJ* genes (see Fig. 17.3). The *IGHC* genes consist of a series of constant-region genes, one for each heavy chain class and subclass, arranged in the order 5′-Cμ-Cδ-Cγ-Cε-C*α*-3′ along the chromosome.

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Generation of Junctional Diversity

Gene Rearrangement

The most obvious way to generate V-region diversity is to select one V gene at random from the available pool and join it to one randomly selected J gene; a process called recombination. Since many V and J genes are available, the number of possible combinations can be very large. For example, if there are 100 V genes and 10 J genes, then 100 × 10 = 1000 different V regions can be constructed.

Light chain assembly requires the combination of one V, one J, and one C gene. During B cell development, the intervening genes must first be removed, and discarded. The first step in this process is to identify the sites where the DNA has to be cut. Thus V and J genes have sites called switch regions at each end that guide the process (Fig. 17.4). Cutting is the function of an "activation-induced" cytidine deaminase (AID). When a B cell receives the appropriate signals telling it to eliminate unwanted genes, AID deaminates the cytidines in the specific switch regions involved (Fig. 17.5). As a result, these cytidines are converted to uracils. This conversion results in DNA "damage" and, as a result, both strands of the DNA are cut by an endonuclease at these points. The looped-out genes are chopped off, and the free ends of the DNA are "rejoined" by a DNA ligase so that the V and J genes form a continuous sequence. Two sets of enzymes are used in this process. Endonucleases cut the DNA at two points, thus excising unwanted genes. Following this, DNA ligases join the free ends to form a continuous sequence.

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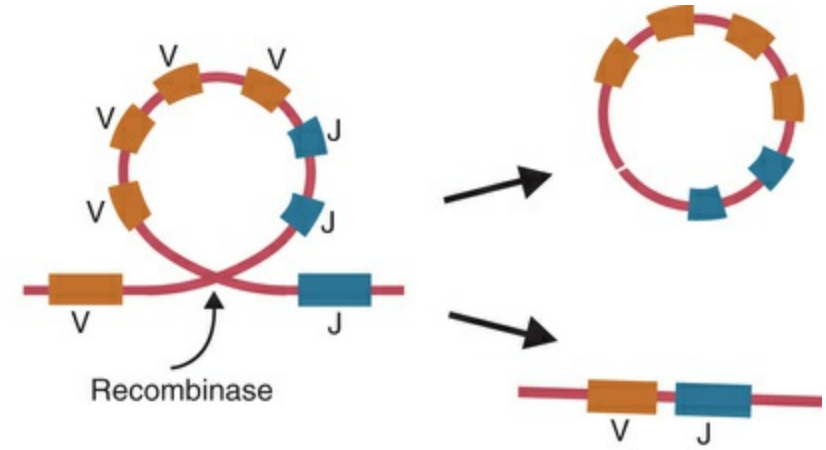


FIG. 17.4 One of the most important mechanisms of deleting unwanted genes is by looping out. In this case, unwanted V genes form a loop that is then cut off by a recombinase enzyme and the cut ends joined together. As a result, the desired V gene is linked directly to a J gene. The excised loop is destroyed.

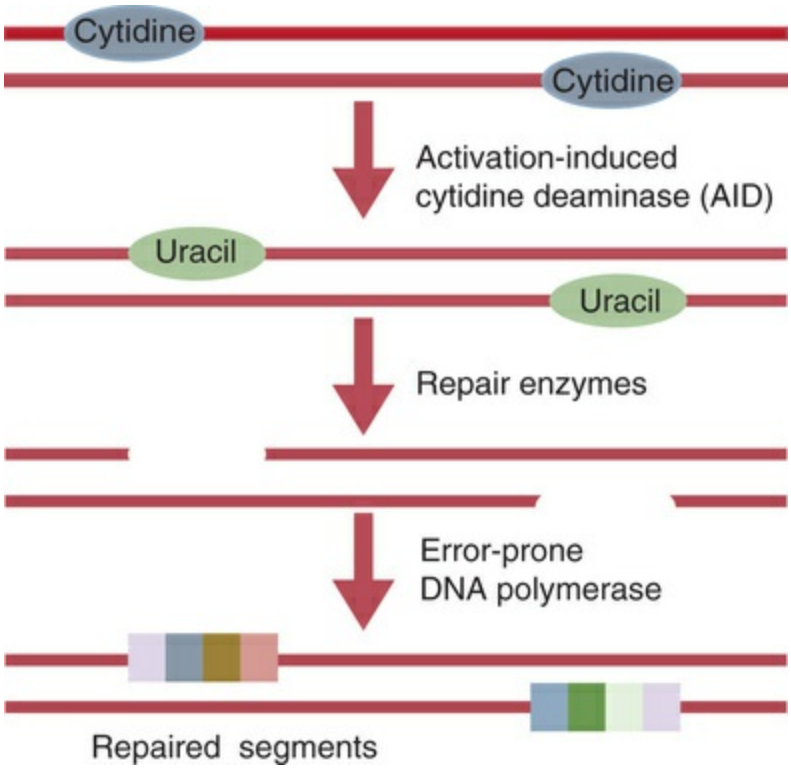


FIG. 17.5 The mechanism of somatic mutation. An "activation-induced" cytidine deaminase (AID) converts the cytidines in specific switch regions to uracils. Both strands of the DNA are cut by an endonuclease at these points. The free ends of the DNA are "rejoined" but the gaps are filled by randomly selected nucleotides.

Light chain gene recombination occurs in two steps. Randomly

selected V and J genes are first joined to form a complete V-region gene. The joined V-J genes remain separated from the C gene until messenger RNA (mRNA) is generated. At that time the unwanted J genes are excised, and the complete V-J-C mRNA is then translated to form a light chain (Fig. 17.6).

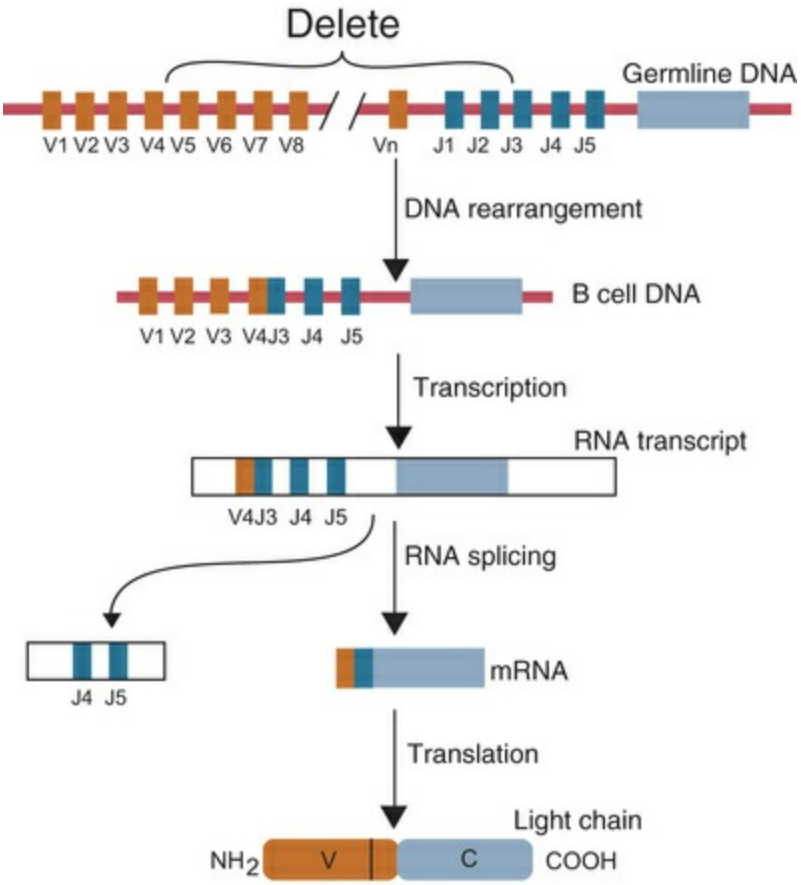


FIG. 17.6 Construction of an immunoglobulin light chain. Selected V and J genes are first joined as the intervening genes are deleted. The VJ and C genes remain separated until RNA splicing occurs. At that time, the intervening RNA segments are deleted, leaving the V, J, and C genes together in the mRNA. DNA rearrangement occurs during early B cell development so that each individual B cell is committed to making a single form of light chain for its antigen receptor.

When a heavy chain V region is assembled, its construction requires the splicing together of *IGHV, IGHD,* and *IGHJ* genes (Fig. 17.7). This use of three randomly selected genes enormously increases the amount of variability. For example, if a pool of 100 V, 10 J, and 10 D genes are recombined, then 100 × 10 × 10 = 10,000 different V regions can be constructed. The recombination of these

genes also occurs in a specific order. Thus *IGHD* is first joined to *IGHJ*, and then *IGHV* is attached to make a complete V-region gene. After transcription, any unwanted J genes are deleted, the *IGHC* gene mRNA is attached, and the completely assembled V-D-J-C mRNA is translated to form a heavy chain.

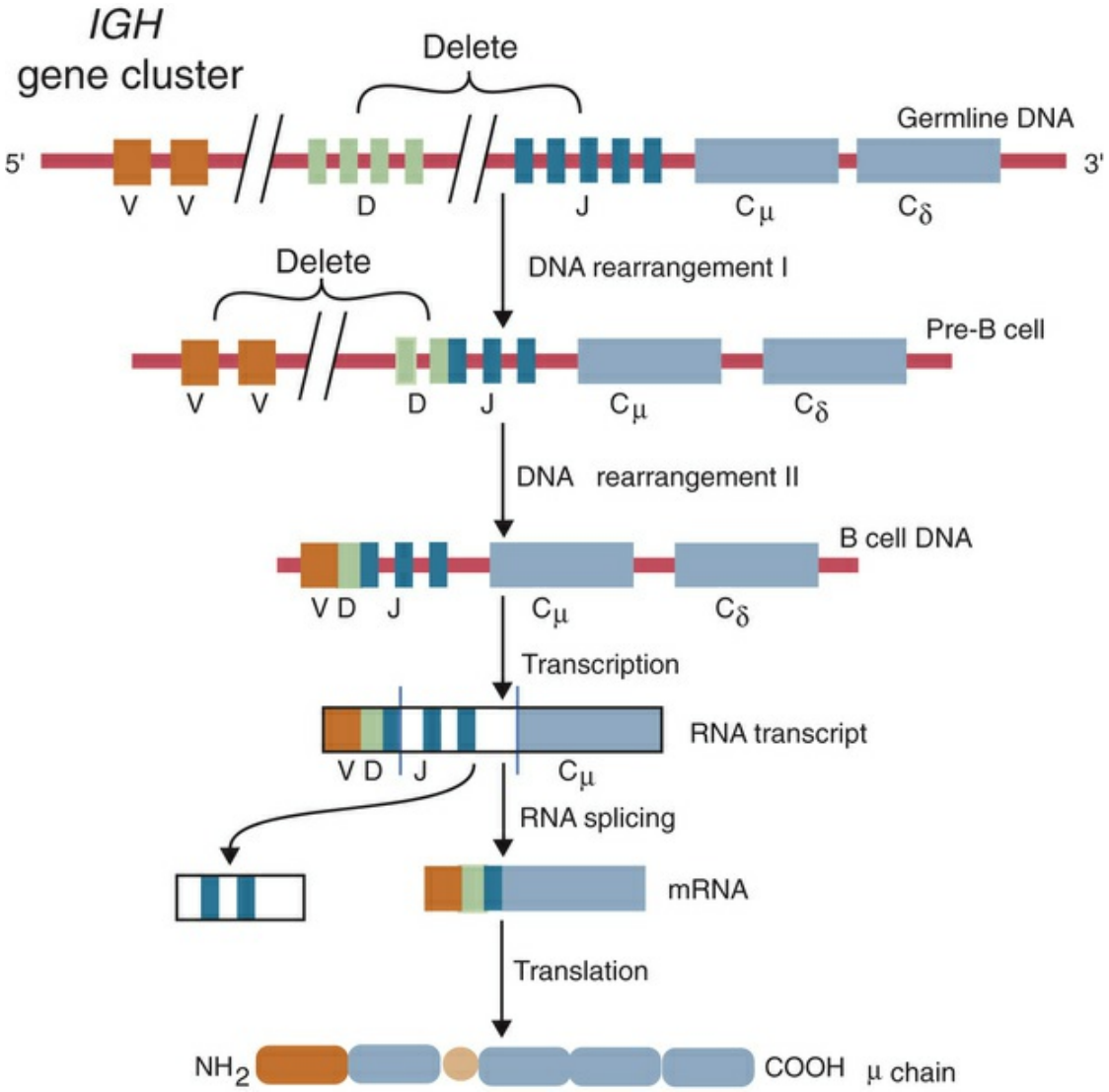


FIG. 17.7 Construction of a complete immunoglobulin heavy chain gene. Two DNA rearrangement events are required to link V, D, and J genes together. The first event joins selected D and J genes; the second event adds a selected V gene. Finally, unwanted J genes are excised and VDJ joined to C in the mRNA.

Base Deletion

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Although random recombination of two or three genes generates much V-region diversity, additional mechanisms can increase this diversity still further. For example, endonucleases can remove nucleotides randomly from the cut ends of the genes. As a result, the precise nucleotide at which V and J genes join varies, leading to changes in the nucleotide sequence at the splice site and variations in the amino acid sequence in the V region.

Base Insertion

In immunoglobulin heavy chain gene processing, additional nucleotides may also be inserted at the V-D and D-J splice sites. Some of these nucleotides (N-nucleotides) are added randomly by an enzyme called terminal deoxynucleotidyltransferase (TdT). Up to 10 N-nucleotides may be inserted between V and D and between D and J.

Although the random selection of genes from two or three pools generates a huge number of combinations, not all of these combinations produce usable antibodies. Some combinations may generate sequences that cannot be translated into protein. These are called nonproductive rearrangements. For example, nucleotides are read as triplets called codons, each of which codes for a specific amino acid. If the codons are to be read correctly, then the sequence must be in the correct reading frame. If nucleotides are inserted or deleted so that the codon reading frame is changed, the resulting gene may code for a totally different amino acid sequence. If this "frameshift" results in inappropriate splicing, translation is prematurely terminated.

It is probable that nonproductive rearrangements are produced in two out of three attempts during B cell development. When this happens, the B cell has several additional opportunities to produce a functional antibody. For example, immature B cells initially rearrange one of the *IGK* genes (Fig. 17.8). If this fails to produce a functional light chain, they switch to the other *IGK* allele for a second attempt. If this does not work, the B cell will use one of the *IGL* alleles, and if this fails, the second *IGL* allele represents the last resort. If all these efforts fail to produce a functional light chain, the B cell cannot make a functional immunoglobulin. It will undergo

apoptosis without participating in an immune response.

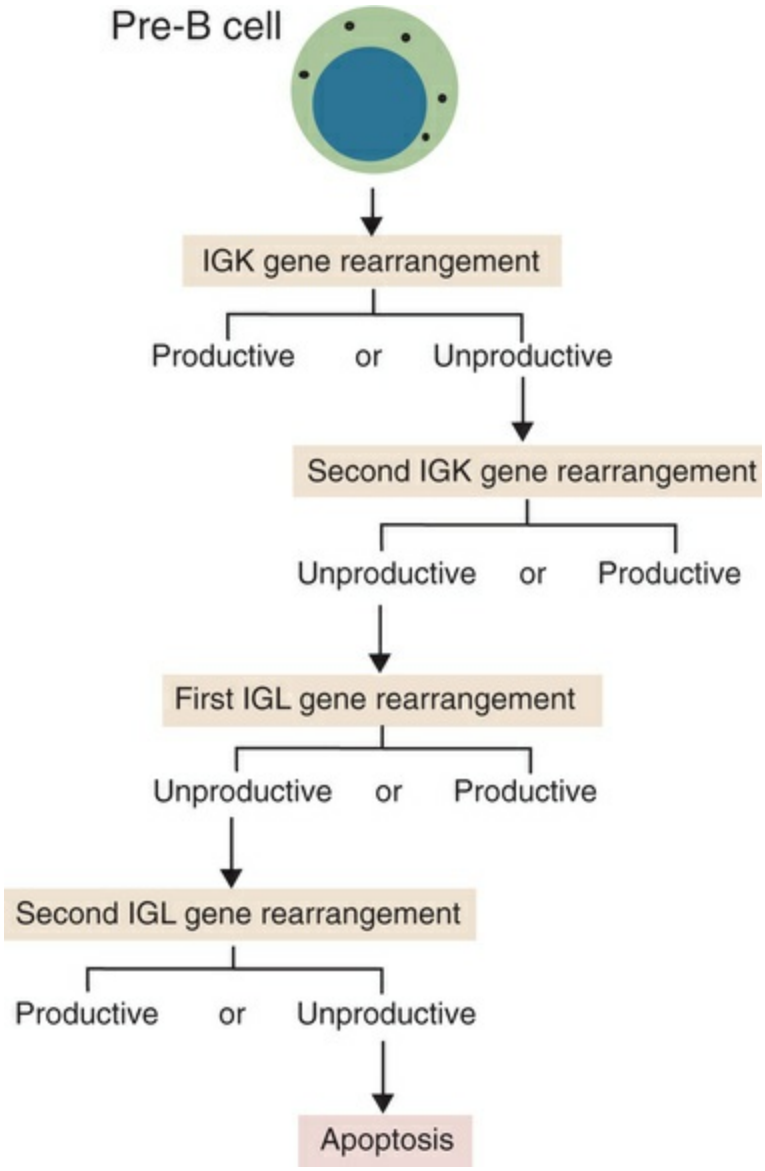


FIG. 17.8 During its development, each B cell has four attempts to make a productive gene rearrangement coding for a functional immunoglobulin. If it fails in all four attempts, the cell undergoes apoptosis.

The sequence of events described above has been worked out in mice and humans; the details differ in domestic mammals. One obvious difference lies in the use of κ and λ light chains. In mice, rabbits, pigs, and humans, κ chains are preferentially used. In the other domestic species, λ light chains predominate. The reasons for these differences are unknown.

It should also be pointed out that immunoglobulin gene

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rearrangement is not entirely random. For example, in rabbits, mice, and humans the most 3′ *IGHV* genes tend to be used most often. This preferential use of certain genes results from a combination of factors, including the recombination signal sequences, the accessibility of the genes to the recombinase enzyme, sequences at the splicing sites, and way in which DNA folds.

Receptor Editing

Although each new B cell expresses a specific antigen receptor, developing B cells may continue to rearrange their V, D, and J genes even after exposure to antigen. Thus a B cell expressing a specific κ chain may restart V gene rearrangement by switching to the other *IGKV* genes or even switching to either of the *IGLV* genes. The cell can continue to rearrange upstream non-rearranged V genes or downstream non-rearranged J genes. This receptor editing, which occurs within germinal centers, may be a method of eliminating receptors that bind to self-antigens (Chapter 36).

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Somatic Mutation

Recombination cannot account for all the sequence variability seen in immunoglobulin V regions. For example, there are three hypervariable areas (CDRs) within a V region (Fig. 17.9). One of these, CDR3, is located around position 96 and is generated by recombination between V and J genes. However, CDR1 and CDR2 are located far from V-J or V-D-J splice sites. Other mechanisms of generating antibody variability must therefore exist (Box 17.1). In fact, gene recombination is only the first step in generating antibody diversity. It is followed by somatic mutation that generates antibodies that bind much more strongly and specifically to antigens.

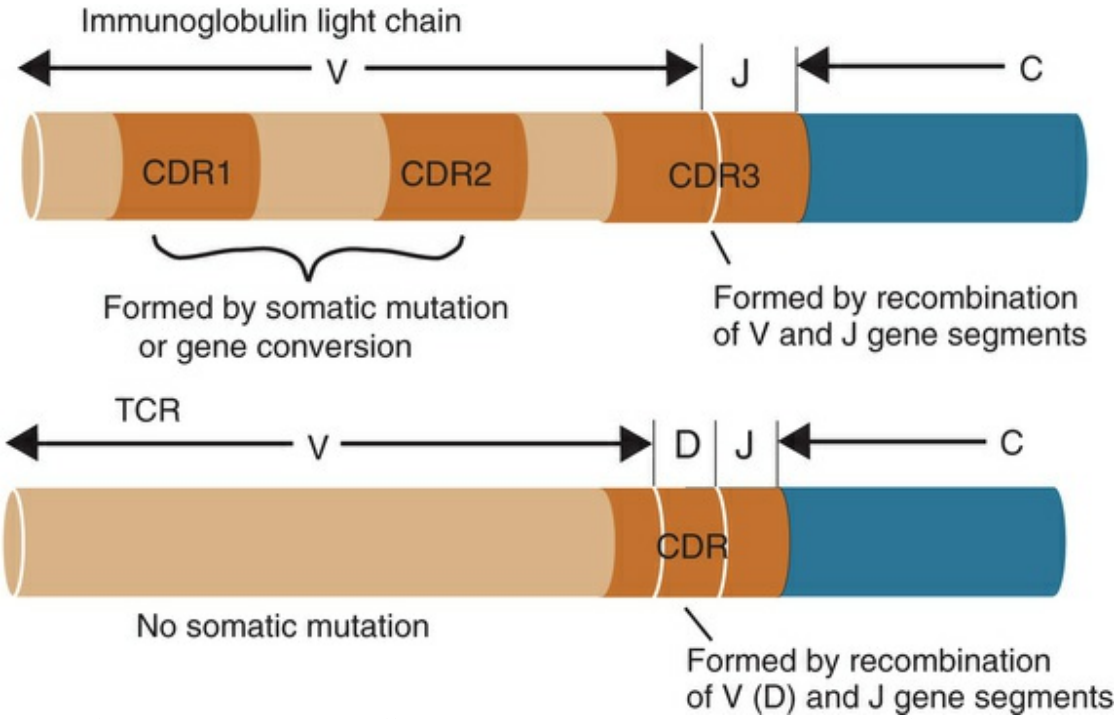


FIG. 17.9 The major difference between the variable regions of the TCR and immunoglobulins is in the formation of CDRs. Immunoglobulins have three CDRs. CDR1 and CDR2 are generated by somatic mutation. CDR3 is generated by gene conversion. This option is not available to the TCR, in which somatic mutation is stringently avoided to prevent self-reactivity.

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Box 17.1

Methods of Generating Antibody Diversity

VJ and *VDJ* gene recombination

Base deletion

Base insertion

Somatic mutation

Combinatorial association

Gene conversion

Receptor editing

Following initial exposure to an antigen, B cells proliferate and undergo antigen-driven selection in the dark zones of germinal centers (Chapter 12). The mutations in immunoglobulin V genes are generated by the same enzymes used for class switch recombination. They are triggered by antigen cross-linking of two BCRs, by the binding of CD40 to CD154, and by the binding of CD80 to CD28. These signals activate cytidine deaminase (AID), which deaminates the cytidines in V gene DNA and converts them to uracils. These uracils are recognized as errors (after all, uracil is not normally found in DNA), and their appearance therefore triggers repair processes. Other enzymes delete the uracils and leave a gap that is repaired by DNA polymerases using randomly selected nucleotides. The gaps are "patched" by short nucleotide sequences. As a result of this "repair," V gene sequences gradually change as the B cells respond to antigens. On average, one amino acid changes each time a B cell divides (see Fig. 17.5).

The degree to which a B cell responds to antigen is directly related to the strength (affinity) with which its receptors bind that antigen. The better the fit between antigen and receptor, the greater will be the stimulus received by the B cell. If a BCR cannot bind an

antigen, the B cell will not be stimulated and will die. In contrast, those B cells whose receptors bind antigen with a high affinity survive and proliferate (Fig. 17.10). Thus as B cells respond to an antigen, successive cycles of mutation and selection of the highest affinity receptors eventually generate populations of B cells producing very-high-affinity antibodies.

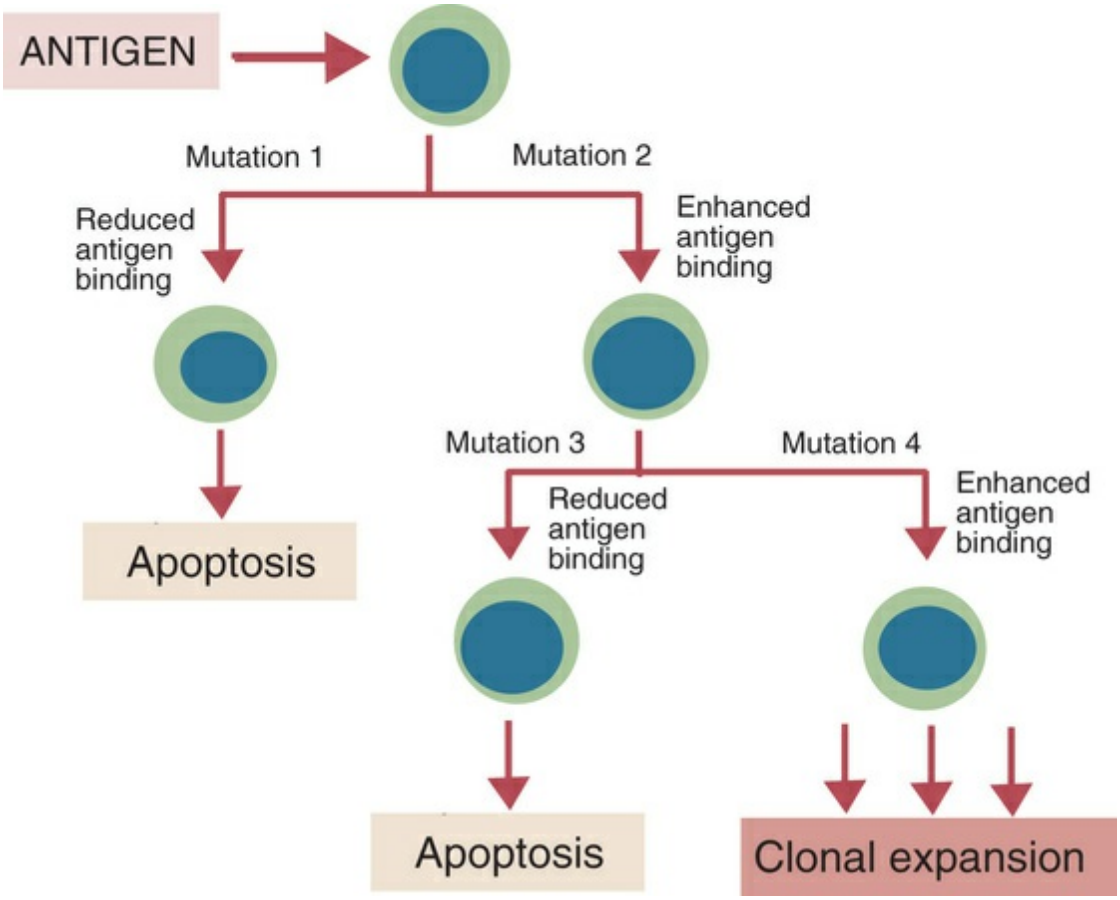


FIG. 17.10 The selection of somatic mutants. Spontaneous mutation during the expansion of a B cell clone results in the development of cells with antigen receptors that differ in their affinity for antigen. Cells that bind antigen strongly will be more intensely stimulated than cells that bind it weakly. As a result of this selection pressure, the B cell population gradually increases its binding affinity during the course of an antibody response.

Somatic mutation does not begin until after B cells have switched from making immunoglobulin M (IgM) to making either IgG or IgA. This suggests that the mutation mechanism is not activated until after a responding B cell had committed to utilizing a specific heavy chain V gene. As a result, the affinity of IgM antibodies does

not increase during an immune response, whereas the affinity of IgG antibodies does.

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Gene Conversion

Mammals other than the human and mouse may have only a few V genes, and gene recombination cannot explain their immunoglobulin diversity. In these species, V-region diversity is generated by gene conversion (Fig. 17.11). Species that employ gene conversion must have available a supply of multiple V genes or pseudogenes. (Pseudogenes are segments of DNA that are defective and so cannot be transcribed.) During gene conversion, B cell cytidine deaminase inserts a uracil, which is then removed, leaving a gap in the nearest V gene. This gap is then filled by randomly selected short segments of DNA obtained from an upstream V-region gene or pseudogene. The "repaired" V gene will therefore have a different sequence than its precursor. Some of these gene conversion events may not generate a functional V region. In these cases, these defective B cells are eliminated.

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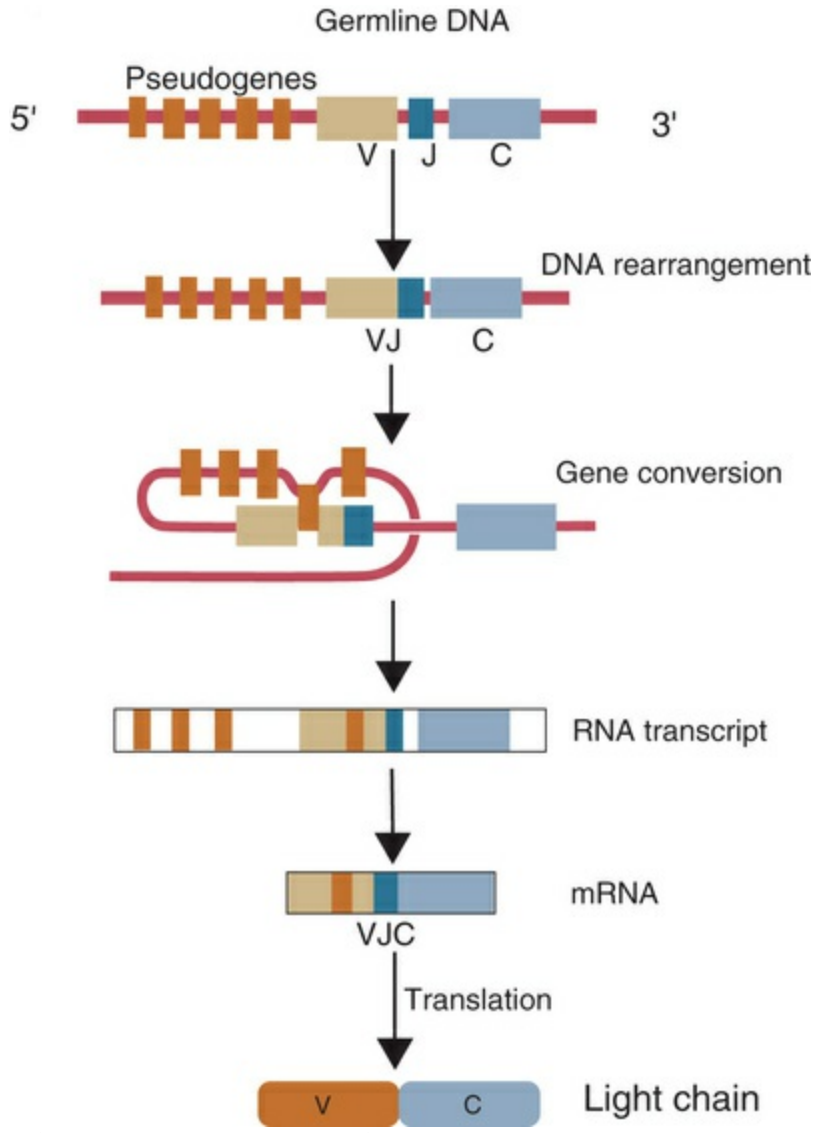


FIG. 17.11 The process of gene conversion. In this process, segments of upstream genes or pseudogenes are inserted into a single V region to generate sequence diversity.

Receptor Assembly

When B cell antigen receptors are generated, the first chain to be assembled is the heavy chain. This chain is capable of generating much more junctional and combinatorial diversity than the light chain and is the major contributor to antigen binding. This heavy chain is linked to signal transduction molecules, and a surrogate partner chain is provided so that the pre-B cell can respond in a limited way to antigens. As a result, a small clone of B cells expressing only the heavy chain is formed. Signaling through this pre-receptor triggers limited proliferation. This is followed by the

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assembly of a partner chain. In B cells this is the light chain. The partner chain is the one that uses only V and J genes and thus contributes much less diversity to the antigen receptor, although it tends to "fine-tune" its antigen-binding abilities. Once a complete heavy chain has been formed using V, D, and J genes, further recombination and rearrangement of its genes are stopped thus preventing assembly of the second heavy chain allele.

Potential Immunoglobulin Diversity

Gene rearrangement generates enormous V-region diversity and antigenic specificity in several ways. In humans, for example, only one of a possible 80 *IGKV* genes is selected for transcription, as is only one of the five *IGKJ* genes. Random joining of these will generate 400 (80 × 5) different light chain V regions. With 300 *IGHV*, 5 *IGHD*, and 2 *IGHJ* genes available, as many as 3000 (300 × 5 × 2) different heavy chain V regions can be generated. Since paired heavy and light chains are used to form the antigen-binding site, the total number of possible combinations is 1.2 million (400 × 3000). In addition, the presence of two splice sites multiplies the potential for diversity generated as a result of base deletion and insertion. However, as pointed out previously, many of the gene combinations so formed may be of little functional use.

Taking all possible mechanisms into account, the number of antigen-binding sites and hence binding specificities generated in humans is about 1.8×10^{16} without accounting for somatic mutation. (This figure may be compared with the estimated 1×10^{7} antigenic determinants that the immune system may recognize.)

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Species Differences

Antigen receptor diversity is generated in different ways depending on species. Some mammals rely on gene recombination followed by somatic mutation. In these species, immunoglobulin diversity is continuously generated from B cell precursors throughout an animal's life. Other mammals, in contrast, use gene conversion for a short period early in life. After initial B cell diversity is generated, this pool of B cells expands by a self-renewing mechanism with little somatic mutation.

Horses

The equine IGH locus contains 40 D genes, 8 J genes and 52 V genes as well as 11 C genes. The IGK locus contains a single C gene, 5 J genes and 60 V genes. The IGL locus contains 7 C genes, each preceded by a single J gene and 144 V genes located downstream of the J-C cluster. Horses therefore predominantly employ gene recombination. Lambda light chains constitute 92% of the antibody repertoire in horses.

Cattle

Cattle likely employ recombination for their light chains and a combination of recombination and conversion for their heavy chains. Initial diversification occurs in lymphoid organs followed by somatic mutation in ileal Peyer's patches. They have at least 36 IGHV genes divided into three subgroups. Subgroup IGHV1 contains at least 11 and perhaps as many as 20 functional genes. Subgroups IGHV2 and IGHV3 consist entirely of pseudogenes (see Table 17.1). Heavy chain diversity in older cattle is generated primarily by somatic mutation. IGHJ contains six genes of which two are functional. IGHD contains 14 long and short genes. As a result, their heavy chain CDR3 regions range from 31 to 154 nucleotides. In addition, conserved short nucleotide sequences of 13 to 18 nucleotides may be inserted at V-D junctions encoding IgM, so that the CDR3 loop is exceptionally long and may contain as many as 61 amino acids. As pointed out in the previous chapter, this leads

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to the generation of a stem and knob structure, where the knob shows significant structural heterogeneity as a result of somatic mutation. This knob can fold into multiple mini-domains and can bind diverse antigens (see Fig. 16.17).

The bovine IGL locus contains 25 V genes, of which 17 are functional, organized in three subclusters 5′ to four J-C genes. The predominantly expressed IGL*V*1 genes are found in two 5′ subclusters, whereas the rarely expressed *IGLV*2 and *IGLV*3 genes are proximal to the J-C genes. Cattle have more than one *IGLJ* gene, but only one is expressed. Many of the pseudogenes are fused to *IGLJ* in the germline. Cattle also have four *IGLC* genes. Two of these (*IGLC2* and *IGLC3*) are functional, whereas the other two (*IGLC1* and *IGLC4*) are pseudogenes. *IGLC3* is preferentially expressed. Over 90% of bovine antibodies use lambda light chains.

Sheep

Sheep also use both recombination and conversion. Immature B cells first diversify their V (D) and J genes in lymphoid tissues such as the spleen or bone marrow. The immature cells then migrate to follicles in the ileal Peyer's patches where somatic mutation occurs (Fig. 17.12). Sheep light chain genes have more than 90 *IGLV* genes and a single *IGLJ* gene, so these are diversified by recombination. On the other hand, sheep have only 7 functional *IGHV* genes and therefore use conversion to diversify their heavy chains. They have 6 I*GHJ* genes, 2 of which are pseudogenes. One of the active genes, *IGHJ1*, is used in 90% of heavy chains, suggesting that recombination is minimal. More than 98% of all rearrangement events are in frame, and there are few N- or P-nucleotides. Unlike rabbit, human, or mouse, stimulation by the intestinal microbiota is not absolutely necessary for V gene diversification in sheep.

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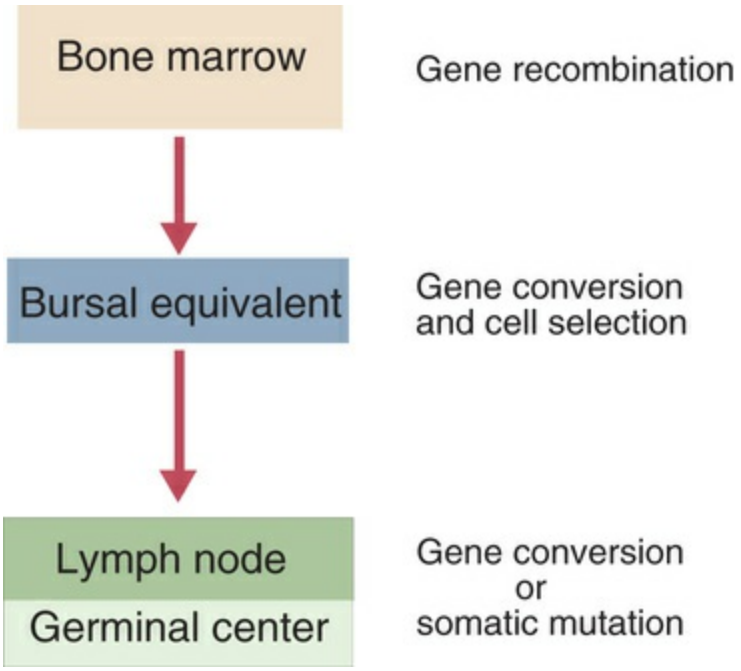


FIG. 17.12 The lymphoid organs where gene recombination, gene conversion, and somatic mutation occur.

Pigs

Pigs have about 20 *IGHV* genes, two *IGHD* genes, and a single germline *IGHJ* gene. Early in fetal life, the pig uses only four or five *IGHV* genes, and their early repertoire consists of only 8 to 10 combinations. Later in fetal life, this restricted repertoire is compensated for by early TdT activity and extensive, in frame, N-region addition leading to significant junctional diversity. V_H use is independent of gene position, but three *IGHV* genes account for 40% of the pre-immune repertoire and six genes for 70%. The neonatal piglet has very little diversity available at birth.

Pigs produce kappa and lambda light chains in approximately equal amounts. They have 23 *IGLV* genes of which 10 are functional. The constant loci consist of three tandem *IGLJ-IGLC* cassettes plus a fourth downstream *IGLJ* gene. The kappa locus contains at least 14 *IGKV* genes of which nine are functional, five *IGKJ* genes and one *IGKC* gene.

Unlike the great combinatorial diversity seen in humans, the early pre-immune antibody repertoire in pigs is relatively limited with only 224 variants accounting for almost 100% of the immunoglobulin variation. Most of this diversity is accounted for

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by heavy chain rearrangements.

The presence of the intestinal microbiota significantly assists the development of pig B cells, whose numbers increase greatly during the first 2 weeks of age, although receptor diversity may not increase until 4 to 6 weeks of age. Germ-free pigs have serum immunoglobulin levels 20- to 100-fold less than conventional pigs. Conventional pigs exhibit much greater diversity in their mucosal IgM and IgA V genes than germ-free pigs.

Dogs and Cats

Dogs have 43 functional and 37 pseudogenes in their IGHV region. Cats have 42 and 22 respectively.

Humans and Mice

Humans and mice use recombination to generate most of their antibody diversity (Table 17.2). Additional diversity is generated by base deletion and insertion and by somatic mutation. In these species, B cells with diverse antigen receptors are produced throughout life.

TABLE 17.2

Immunoglobulin Diversity Among Mammals

Species	C_H GENE PRODUCTS					C_L GENES		V_H And V_L FAMILIES		
	IgM	IgD	IgG	IgE	IgA	λ	κ	H	λ	κ
Horses	1	1	7	1	1	7	1	7	1	?
Bovine	2	1	3	1	1	4	1	1	2	?
Sheep	1	1	3	1	2	>1	1	1	6	3
Pigs	1	1	8-12	1	1	1?	1	1	?	?
Dogs	1	1	4	2?	1					
Rabbits	1	0	1	1	13	8	2	1	?	?
Mice	1	1	4	1	1	3	1	14	3	4
Humans	1	1	4	1	2	7	1	7	7	7

From Butler JE: Immunoglobulin gene organization and the mechanism of repertoire development, *Scand J Immunol* 45:455-462, 1997, and other sources.

Intestinal Bacteria and Expansion of the B Cell Repertoire

As pointed out previously, some mammals, including the large

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domestic herbivores, develop their B cell antibody repertoire in two stages. The first diversification involves rearrangements of a small number of V, D, and J genes. These early B cells then migrate to the intestinal lymphoid tissue, where they greatly increase their numbers of B cells as well as the diversity of their B cell repertoire. This second phase of B cell diversification takes place in intestinal lymphoid organs in direct contact with the intestinal microbiota. The importance of the microbiota is supported by the failure of germ-free pigs to develop significant B cell diversity. Intestinal bacteria play an especially critical role in this process. For example, in rabbits, normal intestinal lymphoid tissue development can take place in the presence of both *Bacteroides fragilis* and *Bacillus subtilis* but not with either alone. Other bacterial combinations are also effective, suggesting that some form of bacterial interaction is needed for optimal effect.

Analysis of the expansion of intestinal B cells by commensal bacteria also shows that it tends to affect B cells with certain V_H domains. This expansion is not simply a specific response to microbial antigens but rather a polyclonal, non–antigen-specific response. It may be directed through the toll-like receptors or be a result of microbial superantigen binding to the BCR, or some combination thereof.

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T Cell Receptor Diversity

TCR and immunoglobulin gene rearrangements are specific in that immunoglobulin genes are not rearranged in T cells, and TCR genes are not rearranged in B cells. Like immunoglobulins, the four peptide chains, α, β, γ, and δ, that make up the two types of TCR can bind many specific antigens. They are able to do this because each consists of a variable region attached to a constant region. The diversity of TCR V regions is generated exclusively by gene recombination in contrast to the diverse mechanisms employed by B cells.

T Cell Receptor Gene Structure

The four TCR peptide chains are coded for by three gene loci. The TRA/D locus codes for both α and δ chains since the *TRD* genes are embedded within the TRA locus. The TRB locus codes only for β chains, and the TRG locus codes only for γ chains. All three TCR loci contain V, J, and C genes, and the TRB and TRD loci also contain D genes (Fig. 17.13).

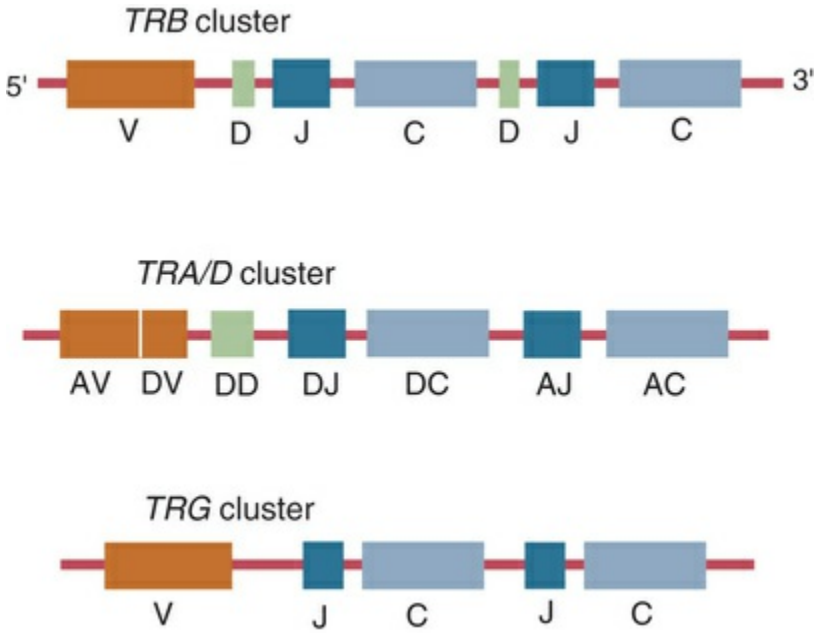


FIG. 17.13 The basic structure of the three gene loci that code for the four TCR chains. The genes for δ chains are embedded within

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Each of the three TCR loci contains two or more C genes. In the TRA/D locus, one C gene codes for *TRAC* and the other for *TRDC*. The TRB and TRG loci, in contrast, may contain multiple C genes. For example, there are eight *TRGC* genes in dogs.

Cells with α/β TCRs rearrange and express *TRA* and *TRB* V genes, whereas γ/δ T cells express *TRG* and *TRD* genes. α/β T cells and γ/δ T cells arise from a common precursor, and the TCR class switch is mediated by signals within the thymus. Developing T cells committed to the α/β TCR lineage delete their *TRD* genes by looping out and switch to using their *TRA* genes. Some of the V genes in the TRA/D locus may be used by either α or δ TCR chains.

α Chain

The number of *TRAV* genes varies ranging from four and five in the horse and sheep, 34 in the dog, and 33 in the pig to more than 300 in cattle. Likewise, *TRAJ* gene numbers ranges from five in the horse to 61 in pig, mouse, and human. Only one *TRAC* gene has been identified in the mammals investigated so far.

β Chain

The TRB locus contains a cluster of V genes located upstream of two D-J-C cassettes, each containing several functional J genes. The D genes are all similar in sequence and length and their use is optional. Any of the *TRBV* genes may be joined to either of the two D-J-C cassettes, and a V gene may join to either a D or a J gene. Dogs have about 21 *TRBV* genes, but about one-third of these make up 90% of the T cell repertoire. *TRBV* gene use may in fact be restricted to a single V gene family in the dog. In the pig, 10 *TRBV* genes and three Dβ-Jβ-Cβ cassettes have been identified. Other mammals may have very diversified *TRBV* genes, ranging from 16 in the horse to 134 in cattle.

δ Chain

The TRD locus contains between 8 and 100 V genes depending on species, 2 to 10 J genes, two to six D genes, and only one C gene in all species examined. As mentioned previously, D gene use is

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optional. Pigs have at least six *TRDD* genes compared to three and two in human and mouse respectively. These pig genes may form transcripts with up to four joined *TRDD* domains.

γ Chain

The TRG locus contains 4 to 17 V genes, from 4 to 16 J genes, and from two to eight C genes depending on species. In the dog, the TRG locus is organized into eight cassettes, each containing a basic V-J-J-C unit, except for a J-J-C cassette at the 3′ end. It contains a total of 40 genes (16 V, 16 J, and 8 C). Eight of the 16 canine *TRGV* genes, 7 of 16 *TRGJ* genes, and six of eight *TRGC* genes are functional. The existence of these multiple *TRGC* genes suggests that the TCRs they generate may have diverse biological properties.

Generation of T Cell Receptor V-Region Diversity

There are three hypervariable regions (CDRs) in each TCR V region. The first two, located within the V genes, have probably arisen through selection. The third is by far the most variable and is located in the region where V, D, and J genes recombine (Table 17.3). Neither somatic mutation nor gene conversion occurs in TCR genes. The genes that are separate in the germline are brought together by DNA rearrangement and are then modified by base insertion or deletion as the T cells differentiate (Fig. 17.14).

TABLE 17.3

TCR Diversity Among Mammal Germlines*

Species	*TCRA*			*TCRD*				*TCRB*				*TCRG*		
	V	J	C	V	J	D	C	V	D	J	C	V	J	C
Horses	5	5	1	8	3		1	16	1	14	2			2
Bovine	>300	52	1	>100	3	5	1	134	3	21	3	17	8	6
Sheep	4			28				120	3	18	3	13	13	5
Pigs	33	61	1	31	10	6	1	10	3	21	3			6
Dogs	34							37	1	6	1	16	16	8
Cats												4	8	6
Mice	100	61	1	10	2	2	1	52	2	13	2	7	4	4
Humans	54	61	1	8	4	3	1	88	2	14	2	15	5	2

*The numbers in this table have been drawn from multiple references. They represent the number of germline genes reported at each locus. Not all these genes will be expressed,

and many are pseudogenes. When sources differ, the author has chosen the highest number of genes reported since as genetic analysis proceeds, more and more genes are being identified, and these numbers may be expected to rise.

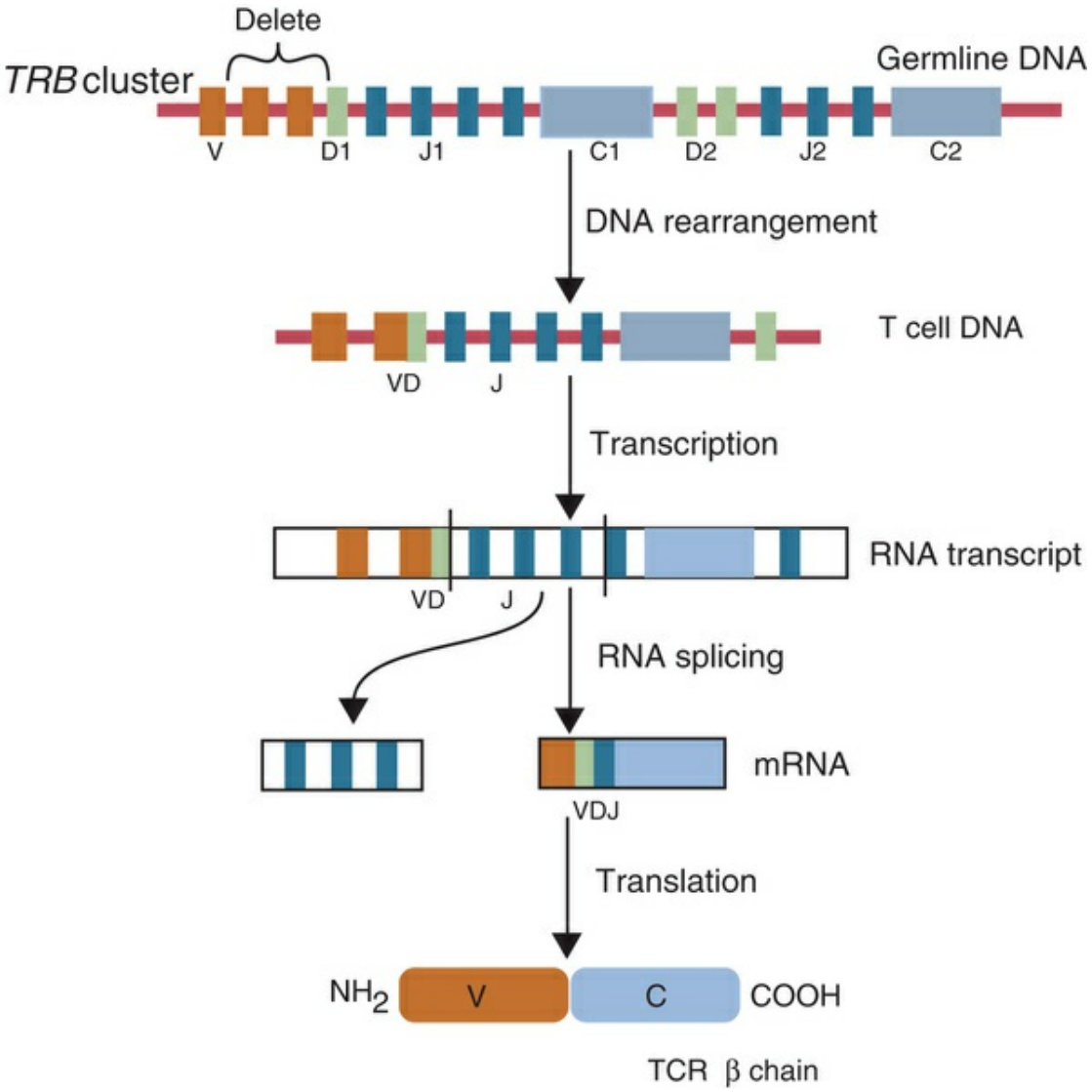


FIG. 17.14 The production of a complete TCR peptide chain. Note the similarities between this and Fig. 17.7.

Gene Rearrangement

TCR α and γ chains are constructed using only V, J, and C genes. TCR β and δ chains use V, D, J, and C genes and can use multiple D genes. As a result, V-D-D-J or longer constructs can be formed. This amount of recombination also means that the reading frame of the D genes may change and can yield productive rearrangements.

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Looping out and deletion account for more than 75% of TCR rearrangements. The rest of the rearrangements are due to either unequal sister chromatid exchange or inversion. That is, moving an inverted segment of gene into a position beside a segment in the opposite orientation.

Base Insertion and Deletion

Although, in general, TCRs are constructed from a smaller V-, D-, and J-gene pool than immunoglobulins, their diversity is greater as a result of junctional diversity (Box 17.2). Random N-nucleotides may be inserted at the V, D, and J junctions using TdT. Up to five nucleotides may be added between V and D and four between D and J genes. Likewise, random nucleotides may be removed by nucleases. This insertion of N-nucleotides and base deletion is probably the most significant component of TCR junctional diversity.

Box 17.2

Methods of Generating TCR Diversity

VJ, VJJ, VDJ, and VDDJ gene recombination

Base deletion

Base insertion

Combinatorial association

Somatic Mutation

Somatic mutation does not occur in TCR V genes. Although T cells must be able to recognize a foreign antigen in association with the presenting major histocompatibility complex (MHC) molecule, it is essential that they do not respond to self-antigens. If random somatic mutation were to occur, it would carry the unacceptable risk of altering MHC restriction and rendering the foreign antigen unrecognizable. It might also lead to the production of TCRs able to bind self-antigens and thus trigger autoimmunity.

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Where Does This Happen?

TCR genes are rearranged and expressed in the thymus. Although both types of T cell arise from a common precursor stem cell, the TCR class switch depends on the signals received by the developing T cell within the thymus. Immature thymocytes begin to rearrange *TRB, TRG,* and *TRD.* If *TRB* is productively rearranged, *TRG* is silenced and *TRD* is deleted so that the cell commits to using *TRA* and expressing α/β TCR. Because of the geometry of the TRA/D locus, joining of a *TRAV* gene to *TRAJ* inevitably deletes the *TRD* genes on that allele. Thus α-chain rearrangements eliminate any possibility of δ-chain expression. Alternatively, the cell may successfully rearrange *TRG* and *TRD* and so express γ/δ TCR.

T Cell Receptor Diversity

In the human TRA locus there are at least 61 *TRAJ* genes and 54 *TRAV* genes, giving more than 3000 possible combinations. N-region addition and base deletion also occur, resulting in great junctional diversity. After correction for codon redundancy and correct reading frame, the number of potentially different TCR α chains is about 10^6. In the human TRB locus, there are at least 88 *TRBV* genes, 2 *TRBD* genes, and 14 *TRBJ* genes giving $88 \times 2 \times 14 = 2464$ possible combinations. In addition, there is junctional diversity and the use of many *TRBD* combinations. After corrections there are about 5×10^9 possible *VDJ* β sequences. Thus, the number of possible TCR α/β combinations is about 5×10^{15}.

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γ/δ T Cell Diversity

The function of γ/δ T cells differs among mammals. For example, in "γ/δ-low" species such as humans and mice there are relatively few V genes in the TRD and TRG loci, and the combinational repertoire is therefore small. In addition, the cells bearing these receptors use only a few V gene combinations. In contrast, human α/β T cells show a much wider range of binding specificities. Thus there is a marked difference between the size of the α/β and γ/δ TCR repertoires. Human γ/δ T cells probably have a limited role in adaptive defense but recognize conserved PAMPs.

The situation in "γ/δ-high" ruminants is very different. In these, γ/δ T cells form a large proportion of total T cells. In young lambs or calves, they account for up to 60% of T cells. In addition, ruminant γ/δ T cells show a considerably greater receptor diversity. In the sheep, γ/δ V-region diversity results from the use of 28 *TRDV* genes and 13 *TRGV* genes that contain two distinct hypervariable segments similar to the CDRs seen in immunoglobulin V genes. In addition, there are multiple TCR γ/δ isoforms generated by the association of a single Cδ chain with up to six or eight Cγ chains. All this suggests that γ/δ T cells in domestic mammals recognize a very wide diversity of antigens and mount adaptive rather than innate responses.

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Epigenetic Regulation

While every cell in an animal body contains a complete copy of the genome, they only employ the genes needed for their specific function. Thus a hepatocyte, for example, only uses those genes required for liver cell function. How do they do that? Processes that collectively control gene expression determine which genes are transcribed in each cell. This is called epigenetic regulation and has been likened to the software in a computer. Like software, a cell selects the most appropriate components (genes) for the task at hand. The importance of epigenetic regulation is well seen in the regulation of immunoglobulin production. Consider, for example, how somatic hypermutation and class switch recombination are regulated.

There are three major mechanisms of epigenetic regulation; DNA methylation is one. By adding a methyl group to the 5-position in certain cytosines within a gene, a gene can be turned off. Conversely demethylation results in gene activation. A second epigenetic mechanism involves histone modification. DNA in the nucleus is closely bound to the nuclear proteins called histones. These histones can be subjected to many chemical modifications such as methylation, phosphorylation, or acetylation. These histone modifications are introduced or removed by histone modifying enzymes. The modifications influence the interactions between the histones and DNA and as a result may activate or inhibit gene transcription. The third major epigenetic mechanism involves the production of microRNAs (miRNAs). These are small, noncoding RNAs that regulate gene expression by binding to messenger RNAs and influencing their functions. Collectively, these three epigenetic mechanisms determine which genes are active and which are inactive in any given cell type.

Thus B cell activation and differentiation is associated with genome-wide hypomethylation, an increase in histone acetylation and the appearance of a specific set of miRNAs. In the case of immunoglobulin synthesis, class switch recombination and somatic hypermutation are regulated by all three of these epigenetic processes. In effect, the production of the appropriate recombinases,

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polymerases, AID, TdT, and repair enzymes is turned on by activating histone modifications and DNA hypomethylation, triggered by exposure to antigen and the presence of appropriate cytokines. These epigenetic pathways regulate immunoglobulin somatic hypermutation, class switch DNA recombination as well as the differentiation of B cells into plasma cells, and long-lived memory B cells. Histone modifications affect the class switch and possibly somatic hypermutation. DNA methylation and miRNAs influence the activities of cytidine deaminase as well as plasma cell differentiation. These epigenetic pathways influence the normal immune response and, if dysregulated, also influence the development of abnormal B cell responses and autoimmunity.

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18

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T Cells and the Destruction of Cell-Associated Invaders

CHAPTER OUTLINE

Endogenous Antigens, 187
Apoptosis, 188
Cell Cooperation, 189
Cytotoxic T Cell Responses, 190
Perforin Pathway, 191
Adhesion Phase, 191
Lethal Hit, 192
CD95 Pathway, 192
Cytotoxic T Cell Subsets, 193
Other Mechanisms of Cellular Cytotoxicity, 193
Macrophage Activation, 194
Classical Macrophage Activation, 194
Alternative Macrophage Activation, 196
Delayed Hypersensitivity Reactions, 197
Effector T Cell Memory, 197

LEARNING OBJECTIVES

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After reading this chapter, you will be able to:

- Define apoptosis, death receptor, caspase, perforin, granzyme, CD95.
- Describe the processing of endogenous antigens.
- Describe and compare the two major pathways of apoptosis.
- Explain how T cell–mediated immune responses eliminate abnormal cells and intracellular organisms.
- Describe how the elimination of abnormal cells involves the forced apoptosis of target cells by cytotoxic T cells.
- Describe how cytotoxic T cells kill their targets.
- Describe the characteristic features of cytotoxic T cells.
- List the signals required to activate a cytotoxic T cell.
- Discuss how some bacteria and parasites may evade destruction by living within the endosomes or cytosol of phagocytic cells.
- Explain how the elimination of intracellular organisms is mediated by activation of macrophages.
- Discuss the role of M2 macrophages in wound healing and recovery from infection.
- List other methods by which the immune system can eliminate unwanted cells.
- Describe how effector memory T cells are generated.

Antibodies bind to invading organisms in blood or tissue fluids, hastening their destruction. However, not all foreign organisms are found outside cells. All viruses and some bacteria grow inside cells at sites inaccessible to antibodies. Antibodies are therefore of limited use in defending the body against such invaders. Viruses and other intracellular organisms must be eliminated by other mechanisms. For this, the body uses two different cell-mediated processes. Either infected cells are killed rapidly by T cells so that the invader has no time to grow, or alternatively, infected macrophages develop the ability to destroy the intracellular organisms. In general, organisms such as viruses that enter the cell cytosol or nucleus are killed by cell destruction (cytotoxicity), whereas organisms such as bacteria or parasites that reside within endosomes are destroyed by macrophage activation. T cells mediate both processes. The antigens that trigger these immune responses

arise from intracellular locations and are thus called endogenous antigens.

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Endogenous Antigens

As described in Chapter 10, every time a cell makes a protein, a sample is processed and peptides are carried to the cell surface bound to major histocompatibility complex (MHC) class I molecules (Fig. 18.1). If these endogenous peptides are not recognized by T cells, no response is triggered. If, however, the peptide-MHC complex can trigger a T cell's antigen receptors (TCR), then that T cell will respond. For example, when a virus infects a cell, T cells may recognize the viral peptides expressed on the cell surface. The T cells that respond to these endogenous antigens are $CD8^+$. They use their CD8 to bind to MHC class I molecules on the infected cells, thus promoting intercellular signaling and eventually the killing of the infected cells.

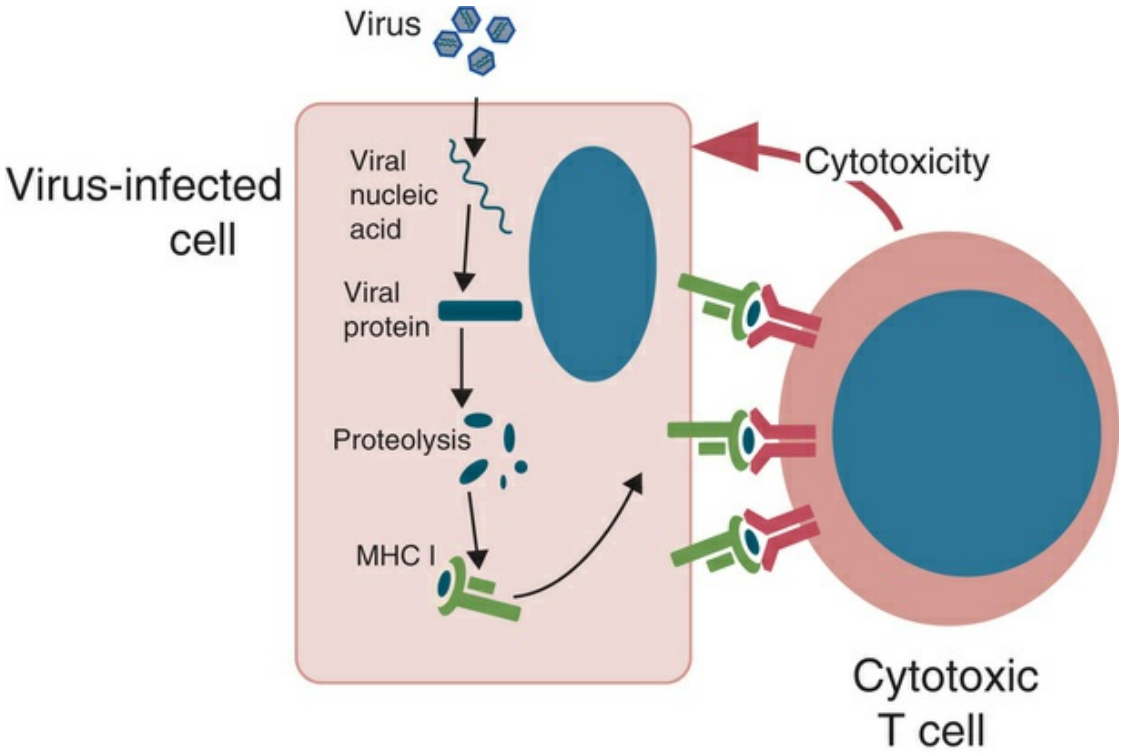


FIG. 18.1 A simplified view of the processing of endogenous antigen. Endogenous antigen is first broken down into small peptides and inserted into the antigen-binding groove of MHC class I molecules. When presented on a cell surface, antigen bound to MHC class I molecules triggers a cytotoxic T cell response.

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Apoptosis

Cells can kill themselves. Old, surplus, damaged, or abnormal cells that would otherwise interfere with normal tissue functions can be persuaded to die as necessary. This cell suicide is called apoptosis. Apoptosis is carefully regulated and must only be triggered when a cell must die. Structurally, apoptosis is characterized by membrane blebbing, nuclear fragmentation, and cell death without lysis. These dying cells are usually phagocytosed by macrophages.

There are two major pathways of apoptosis: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. The extrinsic pathway is triggered by cytokines such as tumor necrosis factor-α (TNF-α) acting through specific death receptors such as CD95 (Fas). Death receptors are a family of cell surface receptors that when activated trigger apoptosis. They all possess an 80-amino acid cytoplasmic sequence called a death domain. The most important of these death receptors are CD95 and the tumor necrosis factor receptors (TNFRs). Death receptors are activated by ligands expressed on cytotoxic cells. The ligands bind to the receptors and cause the target cells to assemble multiple adaptor proteins into a signaling complex. This complex then activates initiator caspase-8 and -10 (Fig. 18.2).

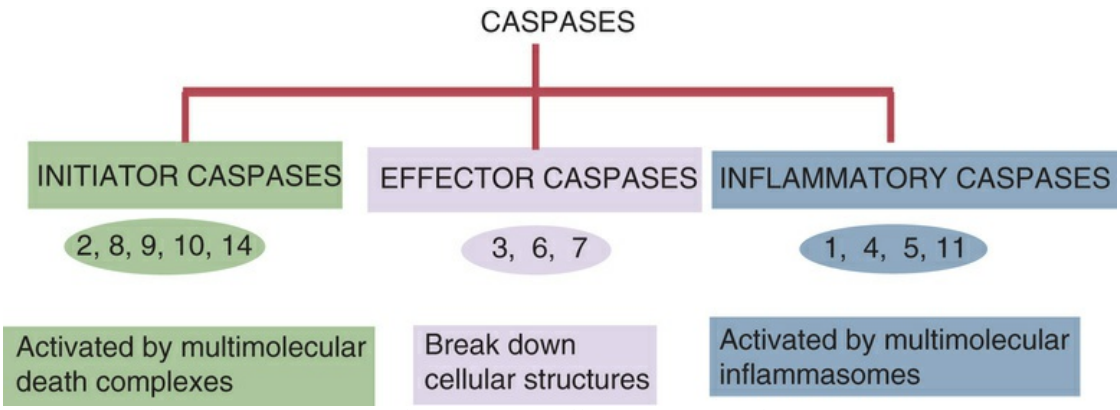


FIG. 18.2 The role of the three different types of caspase in inflammation and apoptosis. The inflammatory caspases are described in Chapter 3.

The mitochondrial pathway, in contrast, is triggered by noxious

stimuli that cause mitochondrial injury. The damaging stimuli (e.g., oxidants, radiation) activate pro-apoptotic proteins, which then release cytochrome C from mitochondria (Fig. 18.3). The cytochrome C triggers the formation of a large multiprotein complex called an apoptosome. The apoptosome then activates initiator caspase-9.

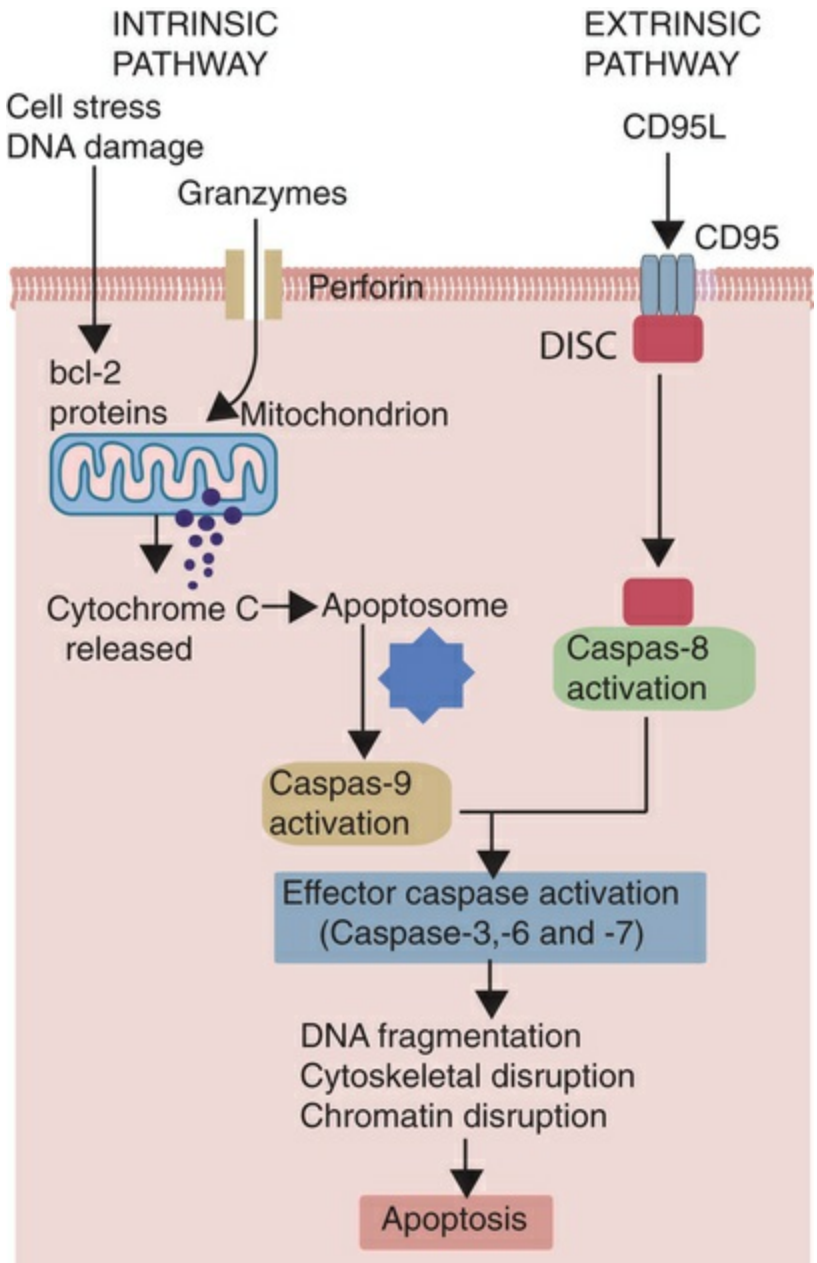


FIG. 18.3 The two pathways, intrinsic and extrinsic, by which apoptosis may be triggered. Both lead to caspase activation, DNA fragmentation, and cell death. The extrinsic pathway is activated by ligation of death receptors such as CD95 and formation of the death-inducing signaling complex *(DISC)*. The intrinsic pathway is

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initiated by multiple damage signals, including injection of granzymes, and leads to the release of cytochrome C from mitochondria, the formation of an apoptosome, and activation of caspase-9.

The initiator caspases activated by either pathway activate a cascade of "effector caspases" (caspase-3, -6, and -7) that degrade cell proteins, activate endonucleases, break down organelles, and result in cell death and disassembly. The DNA of apoptotic cells is broken into many low-molecular-weight fragments. This fragmentation may be responsible for the characteristic way in which the nuclear chromatin condenses against the nuclear membrane (Fig. 18.4). Affected cells shrink and detach from the surrounding cells. Eventually nuclear break-up and cytoplasmic budding form cell fragments called apoptotic bodies (Fig. 18.5).

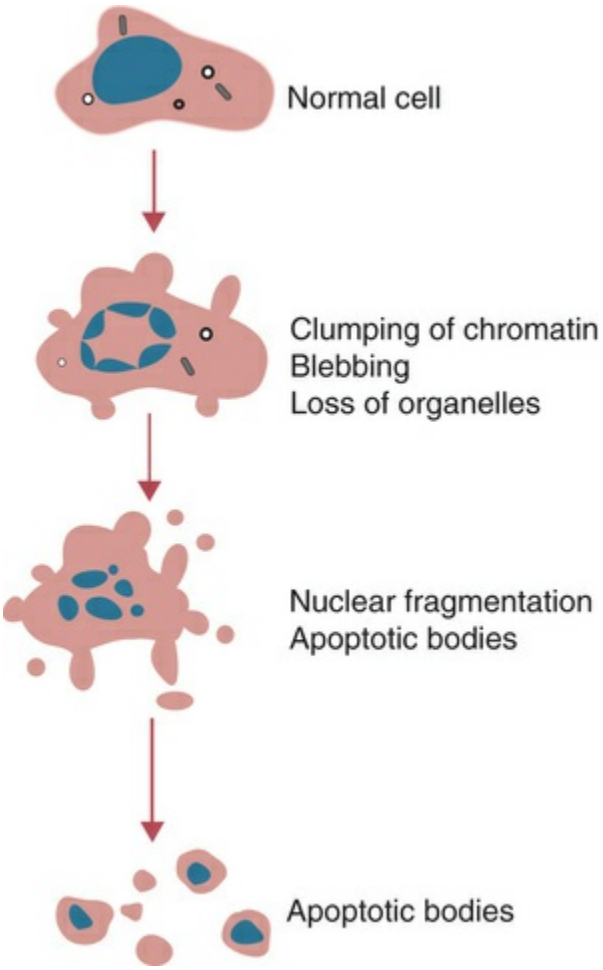


FIG. 18.4 Major morphological features of cell death by apoptosis.

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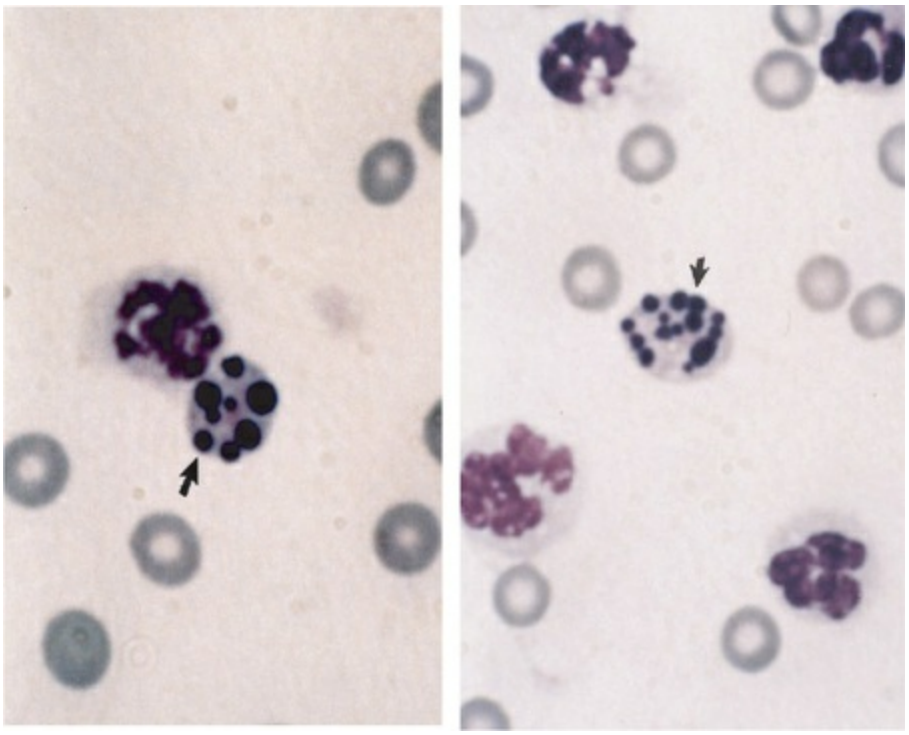


FIG. 18.5 Two rat neutrophils showing nuclear condensation and fragmentation characteristic of apoptosis *(arrows)*. (Courtesy Ms. K. Kennon.)

As cells undergo apoptosis, their cell membrane "flips" so that the lipid phosphatidylserine is exposed on their surface. This lipid binds to receptors on macrophages and dendritic cells and triggers phagocytosis of the dying cell. It also triggers the release of antiinflammatory cytokines such as TGF-β while minimizing the release of proinflammatory cytokines such as TNF-α.

If cells are severely damaged as a result of trauma, toxicity, or microbial invasion, they may die as a result of necrosis. This has been believed to be a largely unregulated process, although a molecular signaling network regulating the process (necroptosis) has been partially defined. Cells killed by necrosis will trigger inflammation. Thus HMGB-1 escaping from necrotic cell nuclei is a potent inflammatory mediator. Likewise, when dendritic cells engulf necrotic cells, they not only process their proteins into MHC-antigen complexes but also express co-stimulatory molecules. T cells that recognize this antigen are therefore activated. Thus a cell killed by a virus through necrosis triggers inflammation and a T cell response to the viral antigens.

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Cell Cooperation

During a primary immune response, $CD8^+$ cytotoxic T cells cannot respond to infected cells alone. There are about 10^{13} nucleated cells in a human-sized body and possibly several hundred naïve T cells with receptors for each individual viral antigen. Clearly it would be almost impossible for these T cells to find and kill all the cells expressing corresponding viral antigens without help. In practice, dendritic cells link processed antigens to MHC class I molecules and carry them to lymphoid organs, where they are presented to $CD8^+$ T cells. To respond fully, the $CD8^+$ cells must also be co-stimulated by $CD4^+$ helper cells. Co-stimulation is only effective when both the $CD8^+$ and $CD4^+$ T cells recognize antigen on the same antigen-presenting cell. Thus a helper T cell first interacts with an antigen-presenting dendritic cell through CD40 and CD154. The helper T cells activate the dendritic cells, upregulate their expression of MHC class I, and stimulate their production of interleukin-12 (IL-12). Once activated, dendritic cell MHC class I–linked peptides bind to $CD8^+$ T cells. For complete activation, the cytotoxic T cells require three key signals. The first is IL-12 from activated dendritic cells. The second is an antigen-specific signal from the antigen–MHC class I complex on their target cell. The third signal comes from IL-2 and IFN-γ produced by the Th1 cells. Only after all three signals are received will the CD8 T cells respond.

Different levels of stimulation trigger different activation responses in $CD8^+$ T cells. As with helper cells, the duration of the stimulus is important. Although activated cytotoxic T cells can be triggered by brief exposure to antigen, naïve T cells must be stimulated for several hours before responding. The required stimulation time may be shortened by increasing TCR occupancy or by providing additional co-stimulation. Once activated, naïve $CD8^+$ T cells begin to divide. They undergo multiple rounds of division to generate large numbers of cytotoxic effector cells. Calculations have suggested that they undergo as many as 19 cell divisions in the days following antigen exposure and they probably divide every 4 to 6 hours. All this is regulated by extracellular signals from the

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TCR, co-stimulatory molecules, and cytokines such as IL-2, -12, -21, and -27. These activated cells migrate to peripheral sites and differentiate into effector and memory cells. Short-lived effector cells form the bulk of the population and will mostly die once infections are cleared. Cells that may have received less stimulation survive and become long-lived memory cells. The differentiation into these two cell populations is probably due to asymmetric cell division.

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Cytotoxic T Cell Responses

Once fully activated, $CD8^+$ T cells leave lymphoid organs and seek out infected cells by themselves. When they recognize an antigen expressed on another cell, the T cells will induce apoptosis and kill their target (Fig. 18.6).

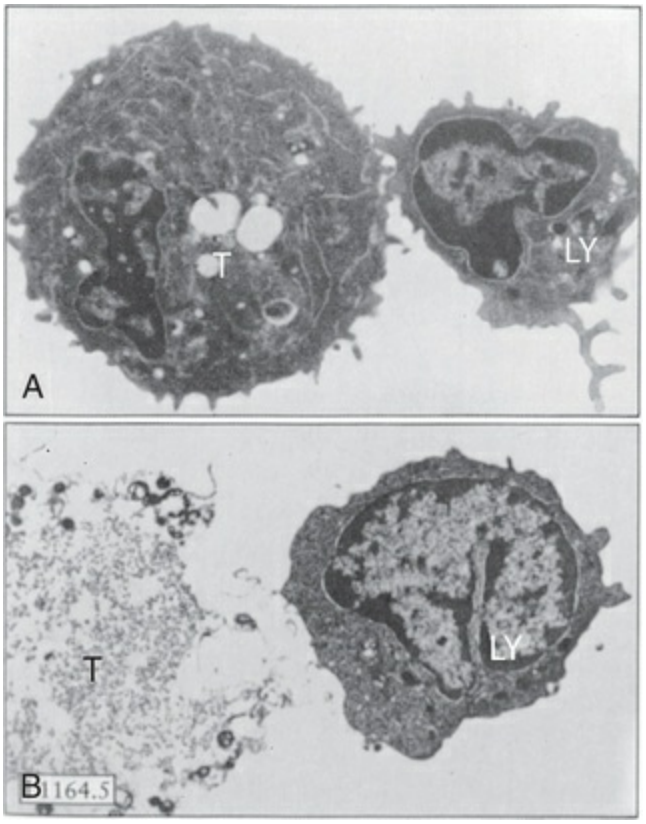


FIG. 18.6 Destruction of target cells by cytotoxic T cells. **A,** Conjugation between a peritoneal exudate lymphocyte (the small cell on the right) and a target cell. Note the lysosome-like bodies *(LY)* and the nuclear fragmentation of the target cell *(T)*. **B,** A lymphocyte with the remains of a lysed target cell. (From Zagury D, Bernard J, Thierness N, Feldman M and G Berke: Isolation and characterization of individual functionally reactive cytotoxic T lymphocytes, conjugation, killing and recycling at the single cell level, *Eur J Immunol* 5:881-822, 1975.)

The density of peptide-MHC complexes on a target cell required to stimulate T cell cytotoxicity is much lower than that needed to stimulate cytokine production. Thus T cell binding to a single peptide-MHC complex may be sufficient to trigger killing, whereas binding to 100 to 1000 complexes is required to stimulate cytokine

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production and clonal expansion. Presumably cytotoxic T cells need to be highly sensitive to viral peptides so that they can kill infected cells as soon as possible. These differences in signal thresholds are probably due to the structure of the immunological synapses formed when a cytotoxic T cell encounters a target.

When cytotoxic T cells encounter a target cell, a synapse forms at the point of contact (Fig. 18.7). This synapse has two "centers." One part of the central zone contains clustered TCR-CD8 complexes. The other attracts secretory lysosomes that release their contents into the synaptic space and so destroy the target cell. Both are surrounded by a pSMAC rich in adhesion molecules that forms a "gasket," preventing the accidental escape of cytotoxic molecules. Once the synapse forms, cytotoxic T cell killing is efficient. Within seconds after contacting a T cell, the organelles and the nucleus of the target show apoptotic changes, and the target is dead in less than 10 minutes. Cytotoxic T cells can disengage and move on to kill other targets within 5 to 6 minutes. In addition, several cytotoxic cells may join in killing a single target.

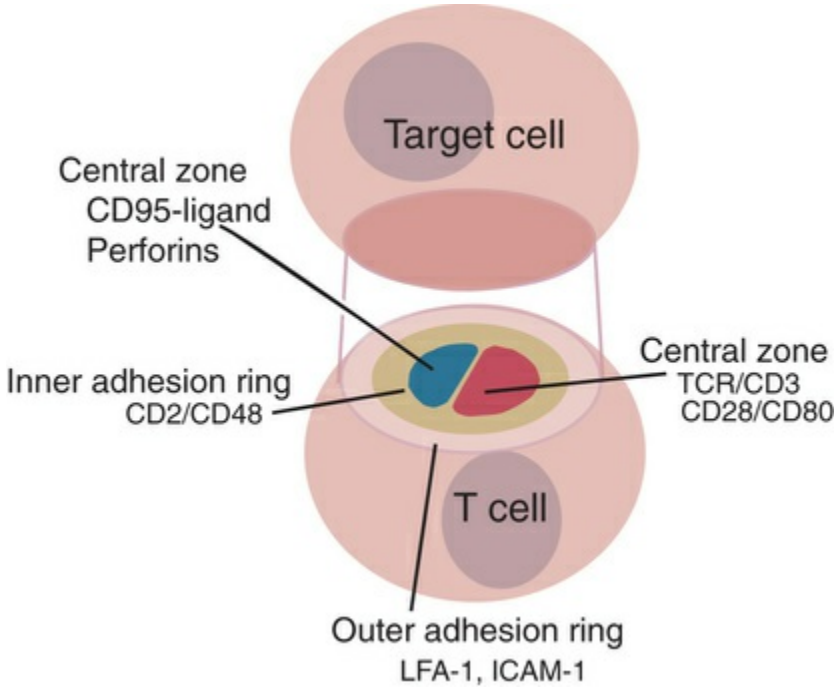


FIG. 18.7 Structure of the immunological synapse that forms between a cytotoxic T cell and its target. The outer ring of adhesive proteins forms an effective "gasket" that prevents leakage of cytotoxic molecules into tissue fluid. There are, however, two central SMACs. One is dedicated to signaling and contains the TCR

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together with accessory molecules and co-stimulators. The other is dedicated to cytotoxic mechanisms. It is through this cSMAC that perforins, granzymes, and the CD95-CD95L signals are transmitted.

Cytotoxic T cells kill their targets through two pathways. The perforin pathway involves the secretion of perforins and granzymes from secretory lysosomes (Fig. 18.8). These kill cells through intrinsic apoptotic mechanisms. The second pathway kills cells by signaling through the CD95 death receptor. The perforin pathway is used to destroy virus-infected cells, whereas the CD95 pathway is mainly used to kill unwanted surplus T cells.

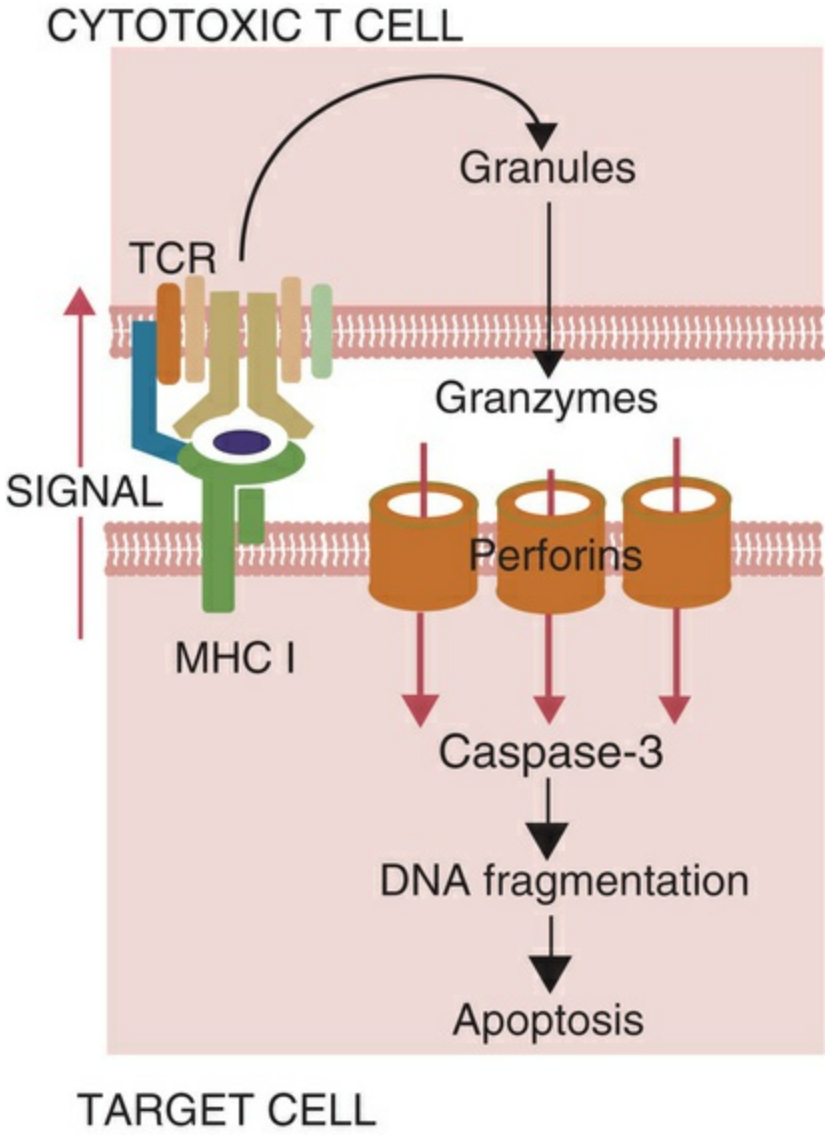


FIG. 18.8 The perforin pathway by which T cells kill targets.

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Perforin Pathway

The killing process can be divided into three phases: adhesion, lethal hit, and cell death (see Fig. 18.8).

Adhesion Phase

When TCRs on cytotoxic T cells bind to antigen-MHC complexes on the target cell, an immunological synapse rapidly forms around the area of contact. The TCRs and other signaling molecules cluster at one of the centers of the complex. The CD8 molecules bind target cell MHC class I and strengthen T cell–target binding. If the TCR has a very high affinity for the target antigen, co-stimulation through CD8 may not be necessary.

In addition to receiving signals from antigen-MHC-CD8 complexes, cytotoxic T cells need co-stimulation. As with CD4 helper T cells, CD8 cytotoxic cells require signals from CD28 bound to CD86 on the target cell. Additional adhesion between cytotoxic T cells and their targets is mediated by T cell CD2 binding to target cell CD58 (in non-rodents) or CD48 (in rodents) and T cell CD11a/CD18 (LFA-1) binding to target cell CD54 (ICAM-1).

Lethal Hit

Within a few minutes of binding to a target, the T cells orientate their microtubule organizing center, their Golgi complex, and their granules toward the target cell. The cytoplasmic granules migrate to the center of the synapse. Here they fuse with the T cell membrane in such a way that the toxic granule contents are inserted into the target. Cytotoxic T cell granules contain several lethal molecules, of which the most important are perforins, granzymes, and granulysin.

Perforins are pore-forming glycoproteins produced by cytotoxic T cells and natural killer (NK) cells. Perforins insert themselves into the target cell membrane and oligomerize to form tubular transmembrane channels (Fig. 18.9). Between 19 and 24 perforin monomers aggregate to form a circular membrane attack complex that forms large (130-200 A) pores in target cell membranes. These perforins are related to and act in a similar manner to C9, the molecule that forms the terminal complement complex (Chapter 4).

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Although the size of the central pore of the polyperforin permits granzyme monomers and dimers to enter target cells, killing also occurs at low perforin concentrations. It is believed that perforins may also release granzymes from target cell endosomes.

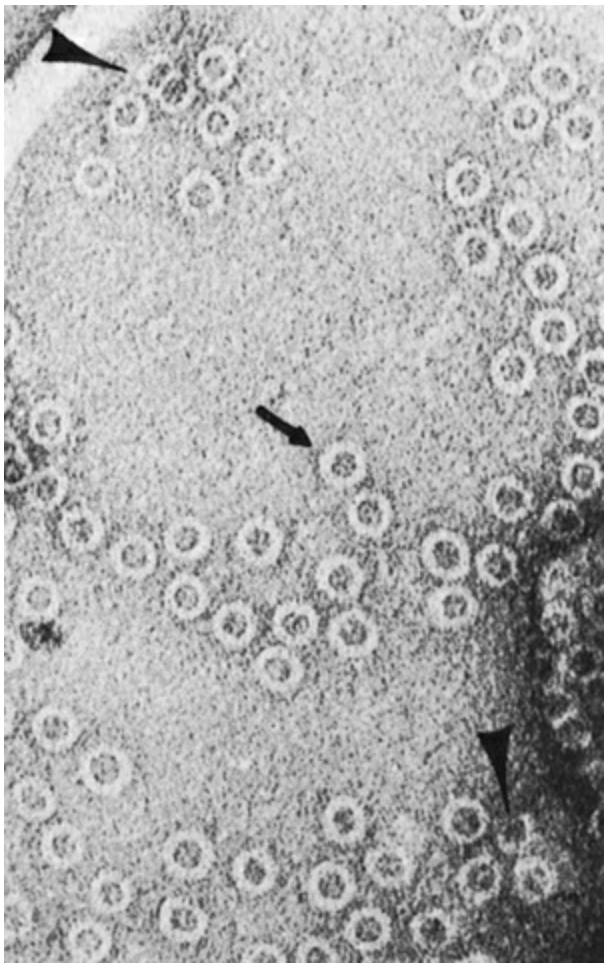


FIG. 18.9 Perforins from human natural killer cells on the surface of a rabbit erythrocyte target. The arrowheads point to incomplete rings and double rings. (From Podack ER, Dennert G: Assembly of two types of tubules with putative cytolytic function by cloned natural killer cells, *Nature* 301:442-445, 1983.)

Granzymes are serine proteases found in T cells, where they account for about 90% of the total granule contents. Granzyme A is most abundant and triggers apoptosis of target cells. It destroys histones and releases a nuclear deoxyribonuclease. It is this enzyme that causes the DNA damage. Granzyme B then enters the target cell, either by injection through the central pore of the perforin complex or by endocytosis. It activates pro-apoptotic bcl-2 proteins,

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triggering the release of mitochondrial cytochrome C. As described previously, the cytochrome C generates an apoptosome that in turn activates caspase-9 and the effector caspase cascade. The effector caspases activate endonucleases that cause DNA fragmentation and cell death. Cytotoxic T cells possess a granzyme inhibitor that ensures that they are not killed during this process.

Granulysin is an antibacterial lipid-disrupting peptide found in the granules of cytotoxic T cells and NK cells in primates and ruminants. A related molecule (Bo-lysin) is expressed in bovine T cells. Granulysin can kill target cells as well as a wide variety of extracellular bacteria, fungi, and parasites. It shares homology with other proteins that attack lipid membranes called saposins. Saposins do not form pores but activate lipid-degrading enzymes such as sphingomyelinases. An increase in saposins therefore increases ceramide content, and ceramide can induce apoptosis. For example, cytotoxic T cells can control *Listeria monocytogenes* and *Mycobacterium tuberculosis* infections simply by killing infected cells. It is possible that living bacteria released from these killed cells might infect healthy cells. To avoid this, the T cell–derived granulysin kills not only infected macrophages but also any bacteria that happen to be inside them.

TNF-β (also called lymphotoxin-α [LT-α]) is secreted by some cytotoxic T cells and has a similar mode of action to CD95L. Structural changes are seen by 2 to 3 hours, and by 16 hours more than 90% of target cells exposed to TNF-β are dead.

CD95 Pathway

The second mechanism of T cell–mediated cytotoxicity involves the binding of a T cell surface protein called CD95L (Fas-ligand or CD178) to a target cell death receptor called CD95 (Fas) (Fig. 18.10). CD95L is expressed on activated $CD8^+$ T cells and NK cells. It binds to CD95 on target cells. When the cells touch, CD95L binds to CD95, and the CD95 trimerizes. This leads to the formation of a death-inducing signaling complex (DISC) that activates initiator caspase-8 and -10. These in turn activate caspase-3 and trigger the apoptosis cascade. The CD95L-CD95 system regulates T cell survival. Unwanted surplus or self-reactive T cells are conveniently

eliminated once they have served their functions. For example, when activated T cells have completed their task of killing their targets, they themselves undergo CD95-mediated apoptosis.

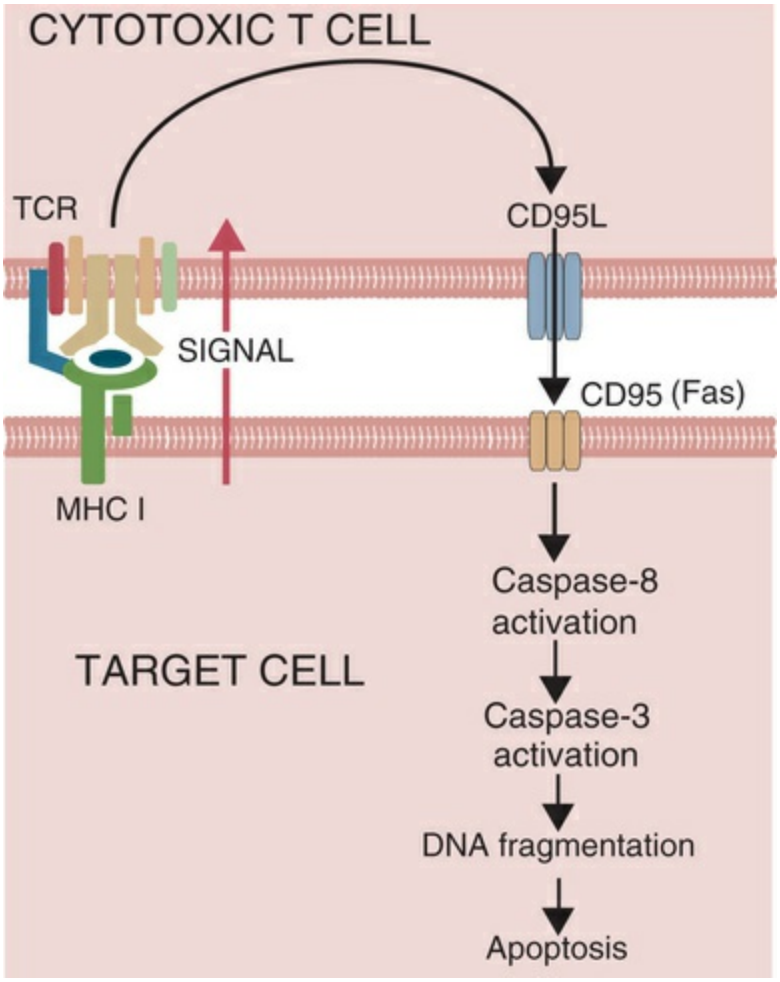


FIG. 18.10 The CD95 pathway of T cell–mediated cytotoxicity.

In mice, *lpr* (lymphoproliferation) and *gld* (generalized lymphoproliferative disease) are loss-of-function mutations in the genes encoding CD95 and CD95L, respectively. Both mutations permit activated T cells to accumulate and accelerate autoimmune diseases. For example, *lpr* mice do not express CD95 on their thymocytes. As a result their thymocytes do not undergo apoptosis (negative selection), and they escape into the secondary lymphoid organs. Here they proliferate, resulting in a gross increase in the size of their lymphoid organs (lymphadenopathy). Many of these cells respond to self-antigens and *lpr* mice develop an autoimmune disease similar to systemic lupus erythematosus (Chapter 38).

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Cytotoxic T Cell Subsets

Subsets of $CD8^+$ T cells have been identified in rodents, where they are called Tc1 and Tc2. Tc1 cells secrete IL-2 and IFN-γ, whereas Tc2 cells secrete IL-4 and IL-5. A third subset, Tc0, has an unrestricted cytokine profile. Unlike helper cells that can differentiate readily into Th1 or Th2 cells, $CD8^+$ T cells show a strong preference for the Tc1 phenotype. Differentiation into Tc2 requires exposure to large amounts of IL-4. All three subsets are cytotoxic.

As described in Chapter 14, T cell interactions with their targets are regulated by positive co-stimulation from CD28 and negative signals from CTLA-4. In some cancers, where T cell cytotoxicity is insufficient to kill the cancer cells, blockage of CTLA-4 by monoclonal antibodies will enhance T cell cytotoxicity and induce long-term tumor remission.

Another molecule that limits T cell cytotoxicity is called programmed cell death-1 (PD-1, CD279). This is a T cell receptor that binds to ligands (PD-L1 and PD-L2) on target cells and then inhibits signaling from the TCR. Upregulation of PD-1 is a normal consequence of T cell activation and is required to terminate an immune response. Persistent viral infections and some cancers induce strong stable expression of PD-1 on activated T cells. This leads to T cell exhaustion, failure of activation, and a loss of T cell function. When PD-L1 is expressed on tumor cells, it protects them from attack by cytotoxic T cells. Conversely, inhibition of PD-1 signaling will enhance T cell–mediated destruction of certain cancers. Both CTLA-4 and PD-1 are regarded as immune checkpoint molecules and their inhibition by monoclonal antibodies may result in successful cancer treatments. You can read more about these checkpoint inhibitors in Chapter 35.

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Other Mechanisms of Cellular Cytotoxicity

T cell–mediated cytotoxicity is not the only way by which the immune system can destroy abnormal cells (Table 18.1; Fig. 18.11). For example, cells that possess the antibody receptors FcγRI or FcγRII may bind to target cells or bacteria through specific antibodies and then kill them. These cytotoxic cells may include monocytes, eosinophils, neutrophils, B cells, and NK cells (Chapter 19). The mechanism of this antibody-dependent cell-mediated cytotoxicity (ADCC) is unclear. However, neutrophils and eosinophils probably release lethal oxidants and toxic granule contents. ADCC is slower and less efficient than direct T cell–mediated cytotoxicity, taking 6 to 18 hours to occur.

TABLE 18.1

Comparison of the Three Major Mechanisms of Cell-Mediated Cytotoxicity

Cytotoxic Cells	Time	Mechanism	MHC Restricted	Antigen Specific
NK cells	24 hr	NK-mediated cytotoxicity	No	No
Normal lymphocytes or macrophages with FcγRIII with specific antibody	6 hr	ADCC activity	No	Yes
Primed T cells	10 min	T cell–mediated cytotoxicity	Yes	Yes

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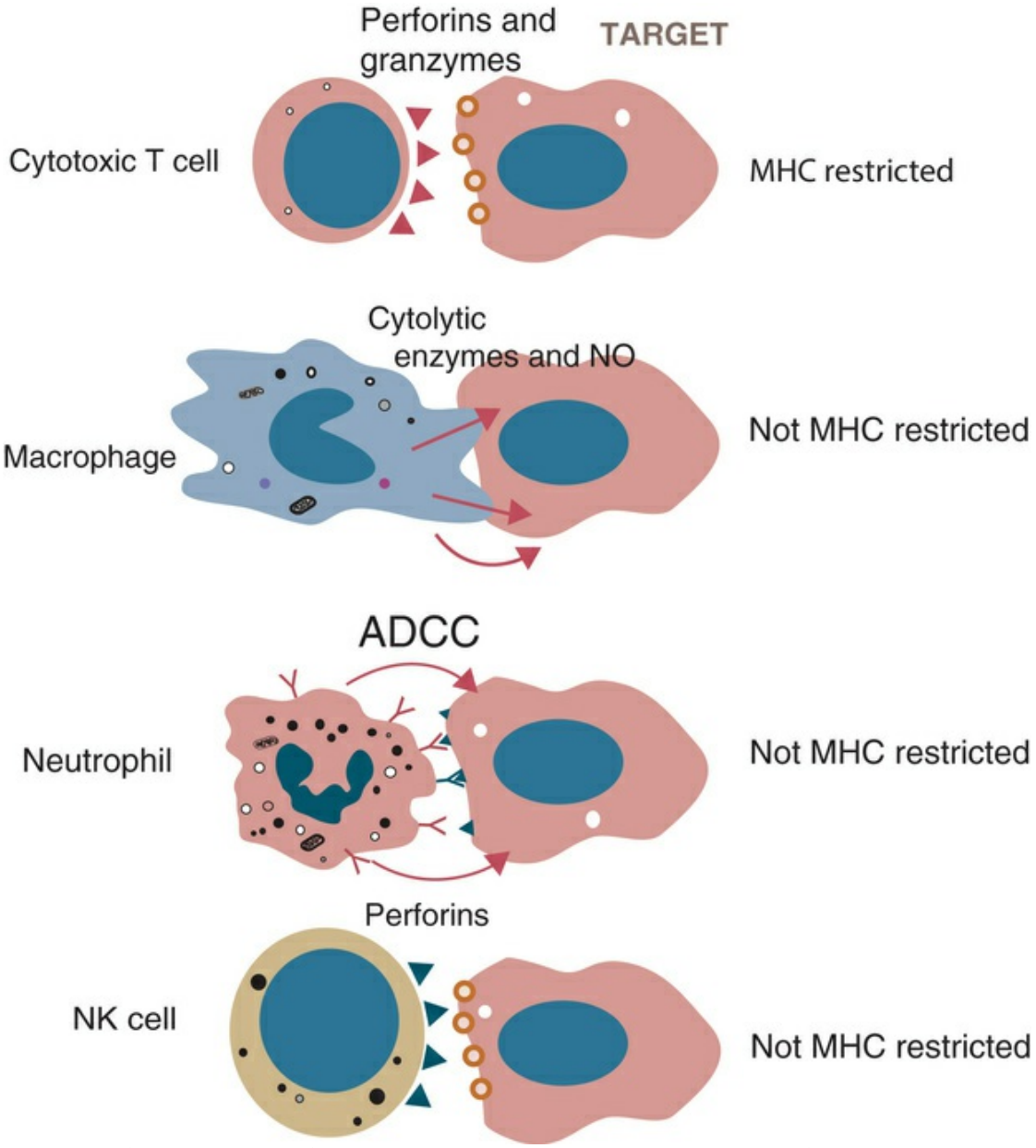


FIG. 18.11 The four major pathways by which the cells of the immune system can kill nucleated target cells. These targets would normally be tumor cells or virus-infected cells. T cells and NK cells are directly cytotoxic. Macrophages secrete NOS and enzymes that kill nearby cells. Cells with Fc receptors act through ADCC mechanisms.

Whether a macrophage participates in ADCC depends on its Fc receptors and its degree of activation. Macrophage-activating cytokines such as IFN-γ or granulocyte-macrophage colony-stimulating factor (GM-CSF) promote ADCC. Macrophages may also destroy target cells in an antibody-independent process. For example, when they ingest bacteria or parasites, macrophages release nitric oxide, proteases, and TNF-α. The nitric oxide will kill

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nearby bacteria and cells, whereas the TNF-α is cytotoxic for some tumor cells.

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Macrophage Activation

When macrophages attack and ingest invading bacteria, they produce enzymes and oxidants that assist in the killing process. This response, however, may be insufficient to kill some invaders. For example, bacteria such as *L. monocytogenes, M. tuberculosis,* and *Brucella abortus,* and protozoa such as *Toxoplasma gondii,* can survive and multiply inside normal macrophages. Antibodies are ineffective against these organisms, so protection against this type of infection requires additional macrophage activation (Fig. 18.12). Activated macrophages are functionally polarized. Classically activated or M1 macrophages are proinflammatory effector cells. Alternatively activated or M2 macrophages have antiinflammatory effects and play a role in tolerance induction and in resolving inflammation.

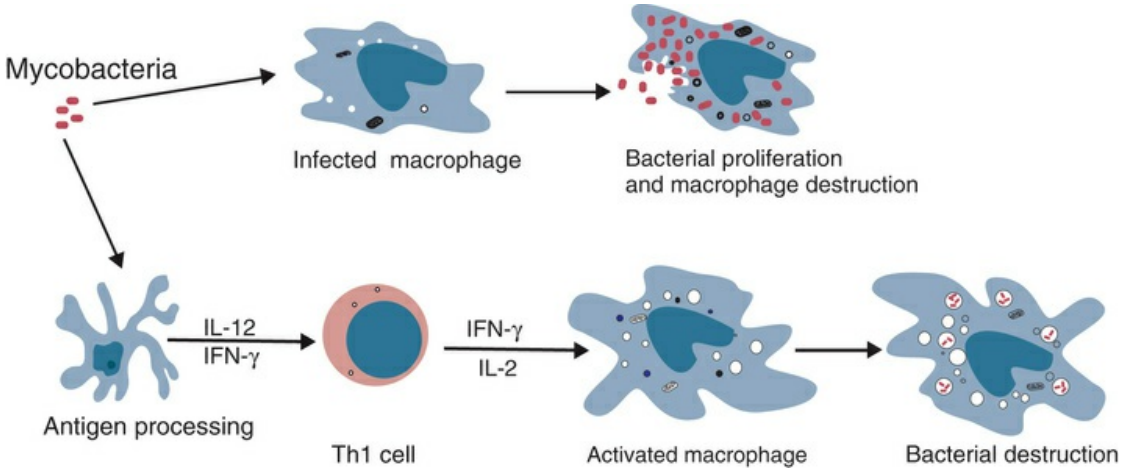


FIG. 18.12 Normal macrophages are killed by growing intracellular bacteria. IFN-γ and IL-2 released by Th1 cells can activate macrophages and enable them to kill otherwise resistant intracellular bacteria.

Classical Macrophage Activation

Macrophages become fully activated in two stages. Initial activation is triggered by exposure to PAMPs and DAMPs from invading bacteria and damaged tissues as described previously. Dendritic

cells subsequently produce IL-12 and induce IFN-γ production by NK and Th1 cells. Complete activation to the M1 phenotype requires exposure to this IFN-γ (Fig. 18.13). It is likely that the NK cell–mediated pathway works in the early stages of an infection, whereas the Th1-mediated pathway comes into operation later (Fig. 18.14).

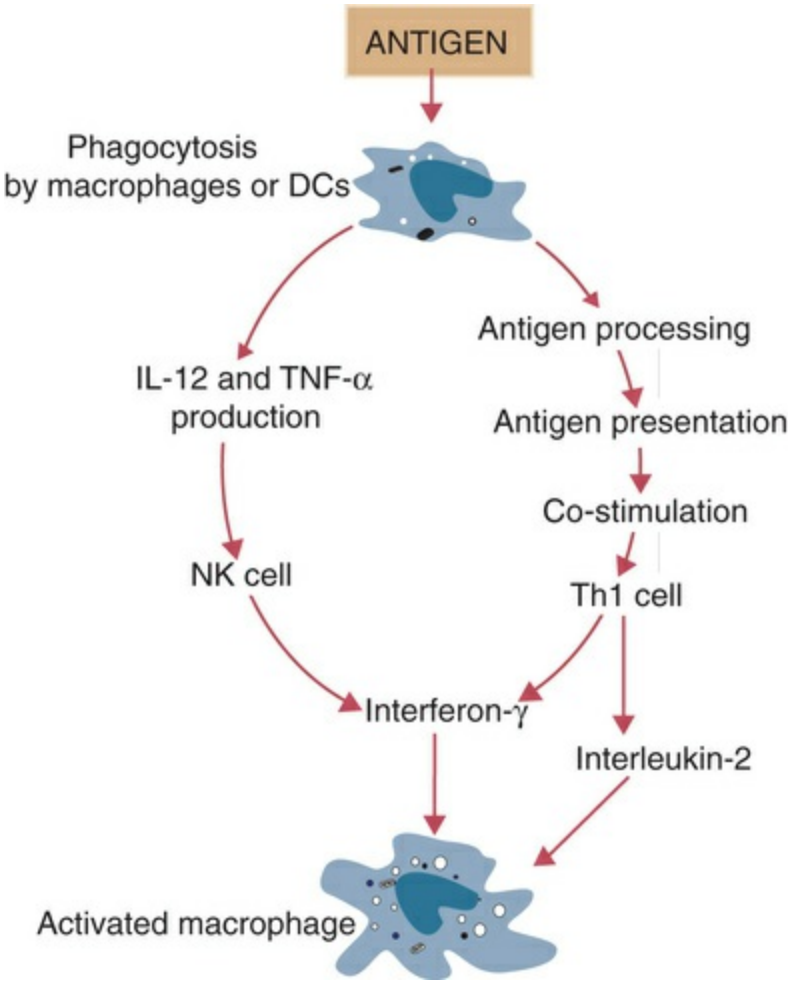


FIG. 18.13 The two pathways by which macrophages can be activated. One involves IFN-γ production by natural killer cells and is thus an innate pathway. The other is mediated by IFN-γ from Th1 cells and is an adaptive response.

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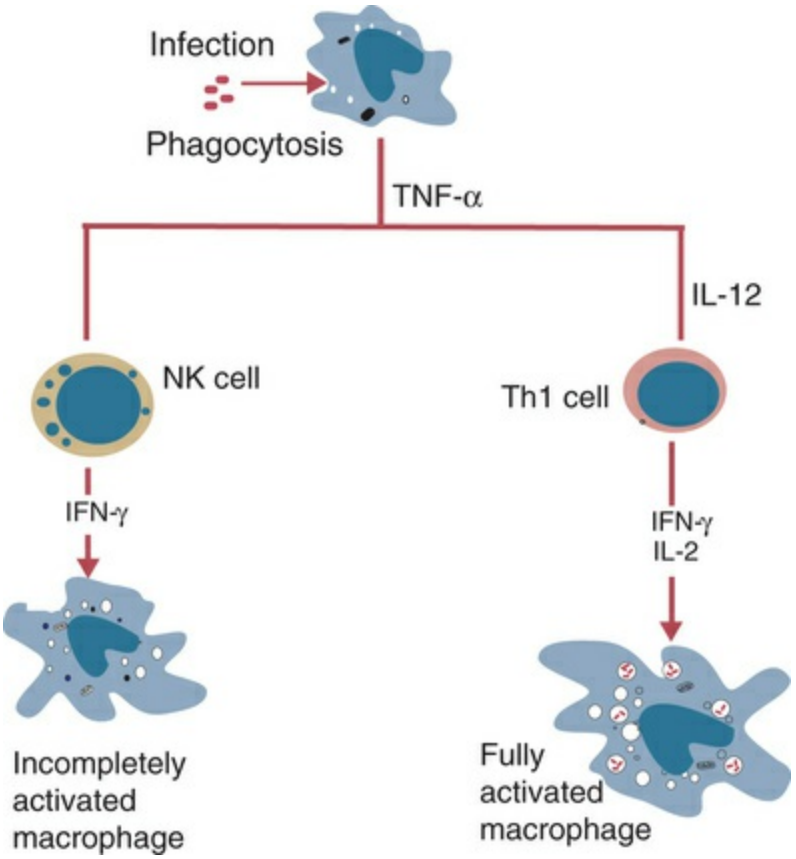


FIG. 18.14 M1 macrophage activation probably develops in stages. Thus IFN-γ produced by NK cells probably activates macrophages in the early stages of an immune response. If this is insufficient, then Th1 cells are activated, and the combination of IFN-γ and IL-2 that they produce causes maximal M1 activation and polarization.

When macrophages encounter bacteria or viruses that activate their TLRs, they produce cytokines. Two of these, TNF-α and IL-12, act on NK cells, causing them to produce large amounts of IFN-γ. Some TLR ligands can also activate pathways that result in IFN-β production. This endogenous IFN-β also promotes M1 cell polarization. Stimulation of Th1 cells by IL-12 will also trigger IFN-γ production. These Th1 cells also produce IL-2, which further promotes M1 polarization and complete cell activation.

Macrophages are activated by IFN-γ through the JAK-STAT pathway and IFN-γ exposure alters the expression of more than 1000 macrophage genes. Activated macrophages secrete proteases that activate complement. They secrete interferons as well as thromboplastin, prostaglandins, fibronectins, plasminogen activator, and the complement components C2 and FB. They increase expression of MHC class II, delay production of

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phagosomal proteases, and promote antigen loading onto MHC molecules, so enhancing antigen presentation. M1 macrophages are enlarged and show increased membrane activity (especially ruffling), increased formation of pseudopodia, and increased pinocytosis (uptake of fluid droplets) (Fig. 18.15). They move more rapidly in response to chemotactic stimuli and they are attracted by many different chemokines. They contain increased amounts of lysosomal enzymes and respiratory burst metabolites, and they are more avidly phagocytic than normal cells. M1 cells produce greatly increased amounts of nitric oxide synthase 2 (NOS2). As a result, they kill intracellular organisms or tumor cells by generating high levels of nitric oxide. The nitric oxide can destroy nearby tumor cells and intracellular bacteria such as *L. monocytogenes* (Fig. 18.16). IFN-γ-activated macrophages can also inhibit the growth of intracellular bacteria by down-regulating their transferrin receptors (CD71), degrading ferroportin, and by reducing the concentration of intracellular ferritin, their major iron storage protein (Chapter 7).

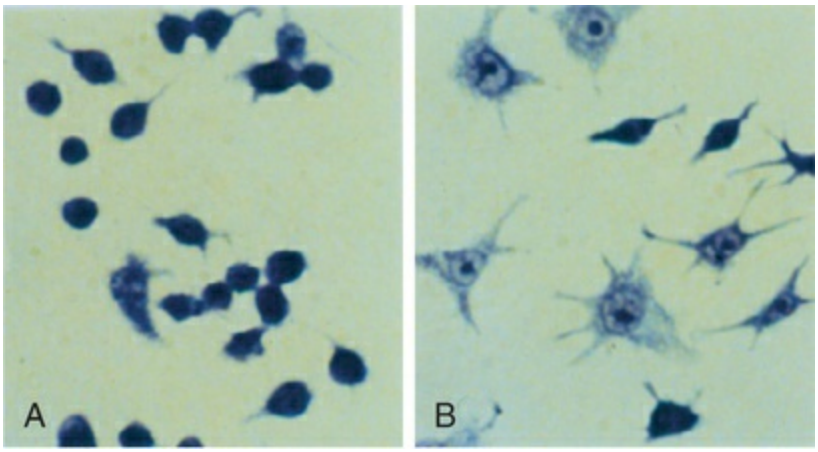


FIG. 18.15 Stained cultures of mouse macrophages grown under identical conditions: *Left,* Normal unstimulated macrophages. *Right,* Macrophages activated by exposure to IFN-γ and acemannan. Note the cytoplasmic spreading of the activated cells. These cells secrete large quantities of cytokines and nitric oxide. Original magnification ×400. (Courtesy Dr. L. Zhang.)

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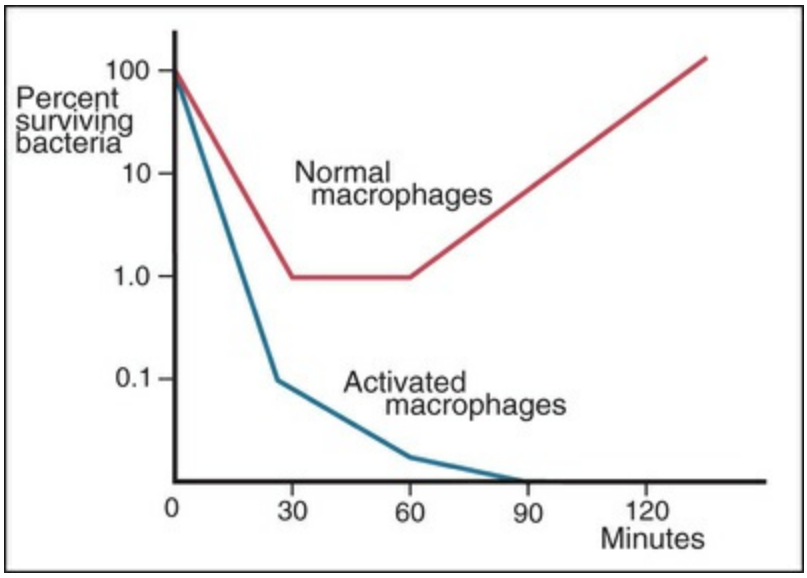


FIG. 18.16 The destruction of *Listeria monocytogenes* when mixed in vitro with cultures of normal macrophages and "activated" macrophages from *Listeria*-infected mice.

Alternative Macrophage Activation

Macrophages activated under the influence of Th2 cytokines such as IL-4, IL-10, and IL-13 become M2 cells. M2 cells differ in receptor expression, cytotoxic function, and cytokine production from M1 cells (Fig. 18.17). M2 cells are regulatory and antiinflammatory and they promote the resolution of inflammation and wound repair. They are protective in some parasitic diseases by walling off parasites such as schistosomes. Instead of producing nitric oxide, M2 cells use arginase to produce ornithine. They secrete large quantities of IL-10 and IL-1RA.

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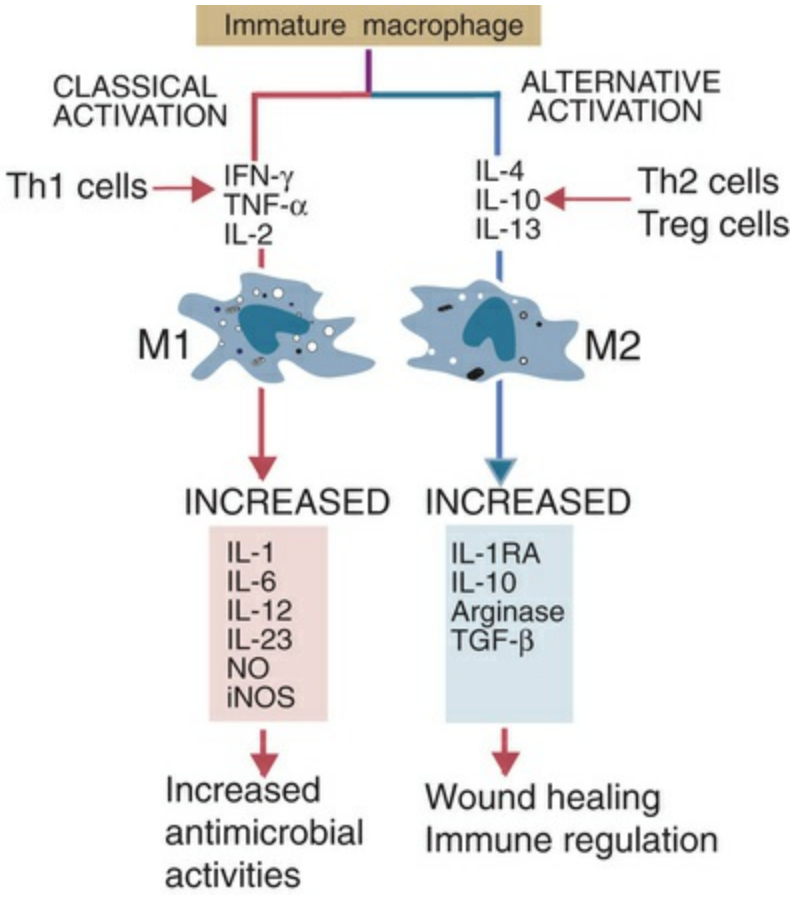


FIG. 18.17 Depending on their cytokine exposure, macrophages may be classically activated (M1 cells) or become alternatively activated (M2 cells). M2 cells have a major regulatory role and are critical to granuloma formation and wound healing. They produce very different cytokine mixtures.

TGF-β from M2 cells stimulates extracellular matrix (ECM) production by fibroblasts. M2 cells also secrete the matrix component fibronectin. They secrete transglutaminase, which promotes ECM cross-linking, and osteopontin, which promotes cell-binding to the ECM. Arginase is involved in proline and polyamine synthesis. Proline is required for ECM construction, whereas polyamines are required for cell proliferation. M2 cells secrete platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), and TGF-β, all of which promote cell proliferation. They secrete fibroblast-like growth factor-β (FGF-β), TGF-α, and vascular endothelial growth factor (VEGF), which promote angiogenesis. Thus the cytokines secreted by M2 cells promote resolution of inflammation and wound repair and have antiinflammatory, fibrotic, proliferative, and angiogenic properties.

The importance of macrophage activation can be seen in

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tuberculosis. Thus mycobacteria that enter the lungs are readily phagocytosed by alveolar macrophages that then mount a respiratory burst and secrete proinflammatory cytokines. These cytokines act on NK cells, triggering IFN-γ production and limited macrophage activation. This rapid response can slow mycobacterial growth significantly. Nevertheless, these macrophages cannot destroy the bacteria by these mechanisms alone. After several days, however, recruitment of T cells occurs. The T cells are stimulated by mycobacteria-infected dendritic cells secreting IL-12, TNF-α, and IFN-α. In response, the Th1 cells secrete more IFN-γ and fully activate the macrophages (Table 18.2). In most individuals, this activation to the M1 level is sufficient to control the infection.

TABLE 18.2

Effects of Cytokines on Macrophage Function

Cytokine	Major Source	Effect
IL-2	Th1 cell	Activates
IFN-γ	Th1 cell, NK cell	Activates
IFN-α/β	Macrophages, T cells	Activates
TNF-α	Macrophages, Th1 cells	Activates
TNF-β	Th1 cells	Activates
GM-CSF	Many cell types	Activates
IL-4	Th2 cells	Suppresses
IL-10	Th2 cells, macrophages	Suppresses
IL-13	Th2 cells	Suppresses
TGF-β	T cells	Suppresses

Cytotoxic T cells generated in cattle infected with *M. bovis* will kill infected macrophages. This cytotoxicity is mediated by both $WC1^+$ γ/δ and $CD8^+$ T cells. Presumably any *Mycobacteria* released are killed by granulysin.

Delayed Hypersensitivity Reactions

When certain antigens are injected into the skin of a sensitized animal, an inflammatory response, taking many hours to develop, may occur at the injection site. This is a T cell–mediated response called delayed hypersensitivity. Delayed hypersensitivity reactions are classified as type IV hypersensitivity reactions (Chapter 33). An important example of a delayed hypersensitivity reaction is the tuberculin response, the skin reaction that follows an intradermal injection of tuberculin.

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Effector T Cell Memory

In contrast to the prolonged antibody response, the effector phase of T cell responses is relatively brief. Indeed, cytotoxicity is seen only in the presence of antigen. This is logical. Sustained cytotoxic activities or overproduction of cytokines could cause severe tissue damage.

Naïve $CD8^+$T cells are long-lived resting cells that continuously recirculate between the bloodstream and lymphoid organs. Once they encounter antigen, they multiply rapidly in an effort to keep pace with the growth of invading pathogens. They reach a peak 5 to 7 days after infection when pathogen-specific, cytotoxic T cells can make up 50% to 70% of the total $CD8^+$ T cells. As with other lymphocytes, asymmetric division results in two daughter cells with different fates. In the case of CD8+ cells it results in the generation of effector and memory effector T cells. The effector cells are derived from the daughter cell closest to the antigen-presenting cell. The memory T cells are derived from the daughter distal to the APC. The proximal cell has increased glycolytic activity and increased expression of effector molecules. The distal cell has increased lipid metabolism, increased expression of anti-apoptotic molecules, and lives very much longer.

Once the infection has cleared, most of the effector cells are superfluous. Therefore up to 95% of them undergo apoptosis 1 to 2 weeks after infection. Elimination of these excess T cells is a tightly controlled process involving the CD95 pathway.

The number of surviving memory cells is directly related to the intensity of the primary response. In general only 5% to 10% of the peak number of cytotoxic T cells survive as memory T cells. Survival may be a function of duration of exposure to antigen. Cells exposed to antigen for prolonged periods may die, whereas cells exposed only briefly may live. The observation that chronic viral infections can exhaust T cells and impair both cytotoxicity and memory is consistent with this idea.

Memory T cells can be distinguished from naïve T cells by their phenotype, by secreting a different mixture of cytokines, and by their behavior. For example, memory T cells are $CD44^+$ and express

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high levels of IL-2Rβ, a receptor that binds both IL-2 and IL-15. They express increased amounts of adhesion molecules, so they can bind more efficiently to antigen-presenting cells. They produce more IL-4 and IFN-γ and respond more strongly to stimulation of their TCR. They continue to divide very slowly in the absence of antigen. This division requires cell-bound IL-15 and is inhibited by soluble IL-2. IL-15 is a unique cytokine that persists for very long periods attached to its receptor on T cells. It thus acts as a persistent stimulus for the memory cell microenvironment and stimulates nearby cells by cell-cell contact. The balance between IL-15 and IL-2 regulates the persistence of memory T cells. In the absence of IL-15, memory cells undergo apoptosis. In humans the $CD8^+$ memory cell half-life is 8 to 15 years.

Over an animal's lifetime, immunological memories accumulate. Older animals have more memory cells than young animals and are thus much better prepared to respond to antigens than younger animals. Repeated vaccination generates new memory cells. However, the size of the memory cell compartment expands to accommodate them. Previously generated memory cells are not removed to make space for the newcomers.

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19

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Innate Lymphoid Cells

CHAPTER OUTLINE

Innate Helper Cells, 199
Group 1 Innate Lymphoid Cells, 199
Group 2 ILCs, 199
Group 3 ILCs, 199
Innate Cytotoxic Cells, 200
Natural Killer Cells, 200
Origins and Location, 200
Target Cell Recognition, 200
Receptors, 201
KIR Receptors, 201
Ly49 Receptors, 203
NKG2 Receptors, 203
Fc Receptors, 203
Effector Mechanisms, 203
Functions, 204
Memory NK Cells, 204
NK Cell Subsets, 204
Species Differences, 204

VetBooks.ir

Horses, 204
Cattle, 204
Sheep, 205
Pigs, 205
Dogs, 205
Cats, 206
NKT Cells, 206
Species Differences, 206

LEARNING OBJECTIVES

After you read this chapter, you should be able to:

- Understand that T cell subpopulations have innate counterparts.
- Describe how innate lymphoid cells (ILCs) use antigen receptors encoded by germline genes.
- Explain that ILCs can be divided into two major groups based on their functions: the helper innate cells and cytotoxic innate cells.
- List and describe the functions of the three types of helper-like innate cells.
- Explain the major properties of NK cells.
- Explain how NK cells can kill virus-infected target cells, tumor cells, stressed cells, and some bacteria without prior activation.
- Understand why NK cells act as a first line of defense against pathogens such as viruses.
- Explain how NK cells employ a “missing-self” recognition strategy.
- Describe how NK cells can also recognize and attack target cells that express stress-associated molecules.
- Recognize that the destruction of some tumors probably involves killing by NK cells.
- Define the importance of CD1 molecules in the processing of lipid antigens.
- Define ILC, NK cell, NKT cell, KIR, Ly49, and NKG2.

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- Explain how KIR receptors can regulate NK cell function and hence immunity to infectious agents.
- Explain the functions and properties of NKT cells.

The structure of lymphocytes reveals few clues as to their functions. The major cell types involved in adaptive immunity, the T and B cells, as described in previous chapters, employ millions of highly diverse, randomly generated antigen receptors. T and B cells, however, require time to develop in sufficient numbers to affect the outcome of an infection. They also undergo clonal selection as cells with self-reactive receptors must be eliminated or suppressed.

Some lymphocytes are, however, able to respond to foreign antigens or destroy abnormal cells immediately after they are produced. These are innate lymphoid cells (ILCs) and they are critical components of the body's defenses. Innate lymphoid cells fall into two functional groups (Fig. 19.1). One group serves as helper cells. They regulate the responses to the intestinal microbiota and play important roles in allergic disease, autoimmunity, and obesity. The second group consists of cytotoxic cells called "natural killer" (NK) cells. They serve as a first line of defense against viruses, some intracellular bacteria, fungi, and parasitic worms. They eliminate stressed or damaged cells. Some play major roles in immunity to tumors.

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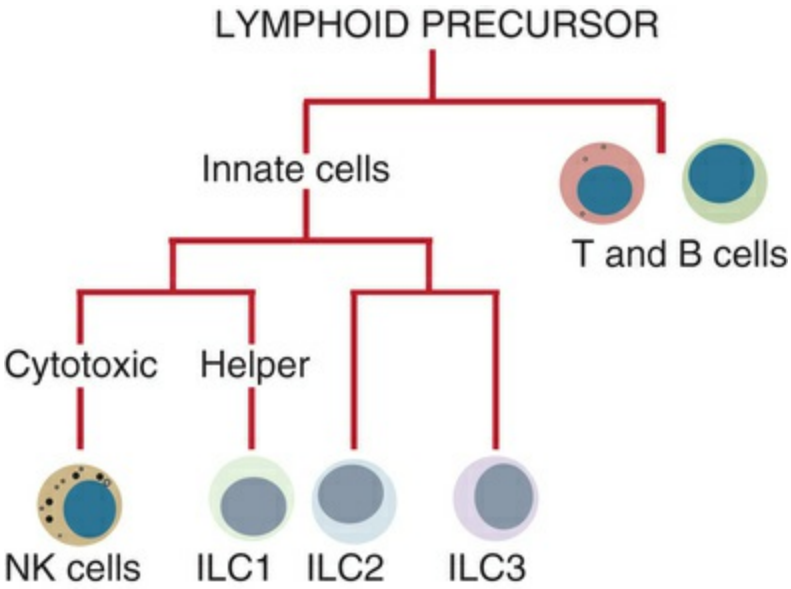


FIG. 19.1 A classification of innate lymphoid cells.

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Innate Helper Cells

Innate helper cells perform functions that were once thought to be performed only by helper T cells. Thus there are innate counterparts of Th1 cells, Th2 cells, and Th17 cells. Each population is characterized by their cell surface antigens, the signals that trigger their production, by their transcription factors, as well as their products and their effects. These innate lymphoid cells play an important role in the very early stages of antimicrobial immune responses. They also contribute to tissue repair and defense and the maintenance of epithelial integrity on body surfaces. They are classified into three groups. Group 1 defends against viruses, intracellular bacteria and parasites. Group 2 defends against helminths, and group 3 promotes immunity to intracellular bacteria.

Group 1 Innate Lymphoid Cells

Group 1 ILCs are found under body surfaces such as the intestinal mucosa, where they are scattered through the lamina propria. They arise from lymphoid stem cells through the use of the transcription factor T-bet. Group 1 ILCs produce large amounts of Th1-associated cytokines such as interferon-γ and TNF-α in response to activation by IL-12, -15 and -18 from dendritic cells (cDC1 cells) (Fig. 19.2). As a result, they activate macrophages. They differ from natural killer (NK) cells in that they cannot produce perforins and so are not cytotoxic. ILC1 cells are essential for defense against viruses, intracellular bacteria and protozoa, and some cancers through their production of IFN-γ and TNF-α. As a result, they also antagonize type 2 responses.

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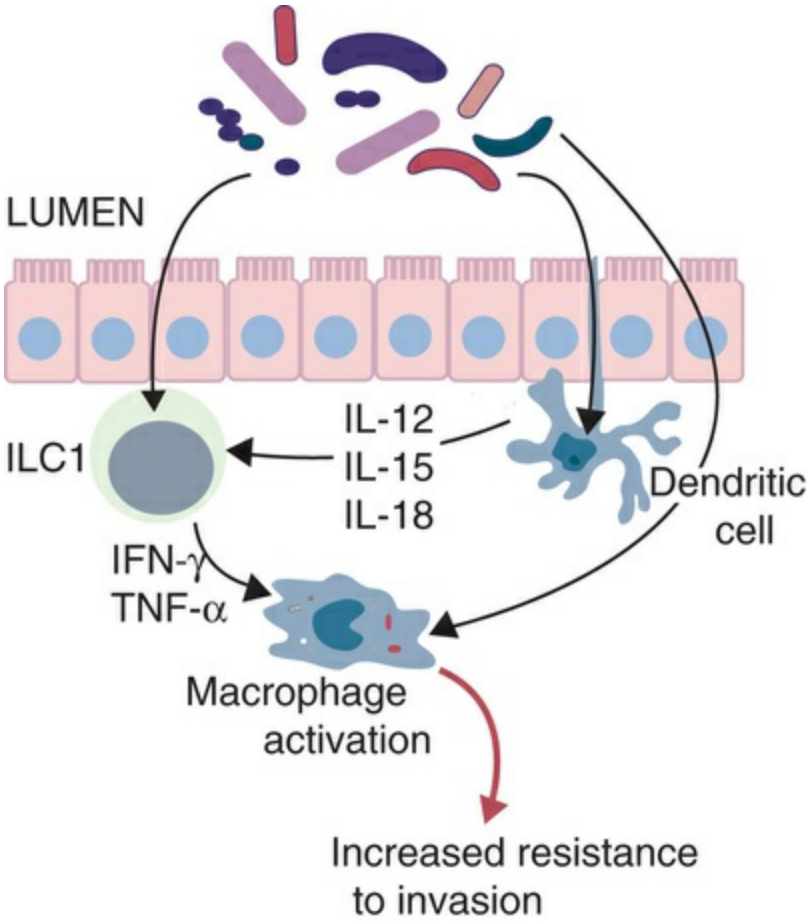


FIG. 19.2 The role of Group 1 innate lymphoid cells. Located in the intestine, they are activated by IL-12 generated by dendritic cells. They respond by producing type 1 cytokines and activating macrophages. They can be considered the functional partners of Th1 cells.

ILC1 cells can turn into ILC3 cells on exposure to IL-23, IL-1β, and retinoic acid. Conversely, ILC3 cells may turn into ILC1 cells under the influence of IL-12, IL-15 and IL-18. NK cells are closely related to group 1 ILCs since they too produce IFN-γ and rely on the T-bet transcription factor.

Group 2 ILCs

Group 2 ILCs are found in the lung, skin, bone marrow, liver, mesenteric fat, and in the small intestine. They arise from lymphoid stem cells and use two unique transcription factors, GATA-binding protein-3 (GATA3) and retinoic acid receptor-related orphan receptor-α (RORα). ILC2s produce large amounts of the Th2-associated cytokines IL-5 and IL-13 and smaller amounts of IL-4, and -9 in response to thymic stromal lymphopoietin (TSLP), IL-25,

and IL-33 from epithelial cells (Fig. 19.3). ILC2 cells are required for the development of early innate immunity to parasitic helminths as well as regulating some type 2 inflammatory responses such as asthma and allergic disease (Chapter 30). They play a key role in regulating type 2 immunity by acting on macrophages, mast cells, and eosinophils. Thus they control eosinophil production and induce an eosinophila. They also induce mucus production by goblet cells, alternative activation of macrophages, and tissue repair. ILC2s can be readily converted into ILC1s by IL-12, and this can be reversed by IL-4.

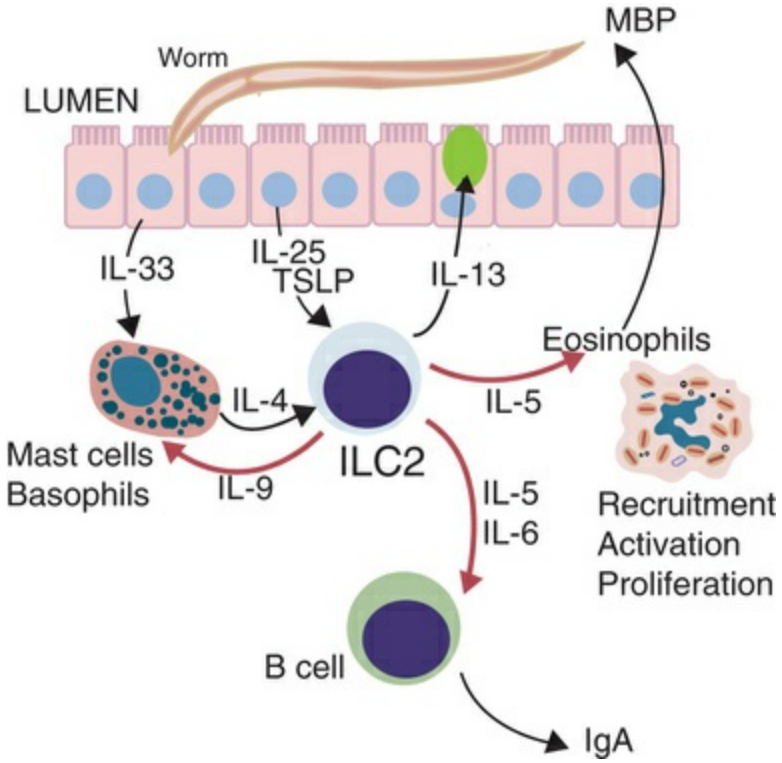


FIG. 19.3 The role of Group 2 innate lymphoid cells. Activated by the presence of intestinal helminths and mast cell products, they generate type 2 cytokines and hence stimulate both eosinophil and IgA production. They can be considered functional partners of Th2 cells.

Group 3 ILCs

Group 3 ILCs are found in the gastrointestinal tract (lamina propria, tonsils, Peyer's patches, and appendix) as well as in the lung. They

use RORγt as their transcription factor and their maintenance and function depend on signals from the aryl hydrocarbon receptor (AhR) (see Box 21.1).

ILC3s produce IL-17 and IL-22 in response to stimulation by TSLP and IL-23 and thus resemble Th17 cells in their cytokine profile (Fig. 19.4). They play a central role in immunity on mucosal surfaces by resisting extracellular bacteria and fungi. They do this by producing IL-22. On exposure to IL-22, enterocytes produce antimicrobial proteins that protect the intestinal epithelium. ILC3-derived IL-22 also inhibits T cell-mediated intestinal inflammation by inactivating those that respond to antigens from commensal bacteria. This IL-22 from ILC3s also inhibits the growth of segmented filamentous bacteria (Chapter 21).

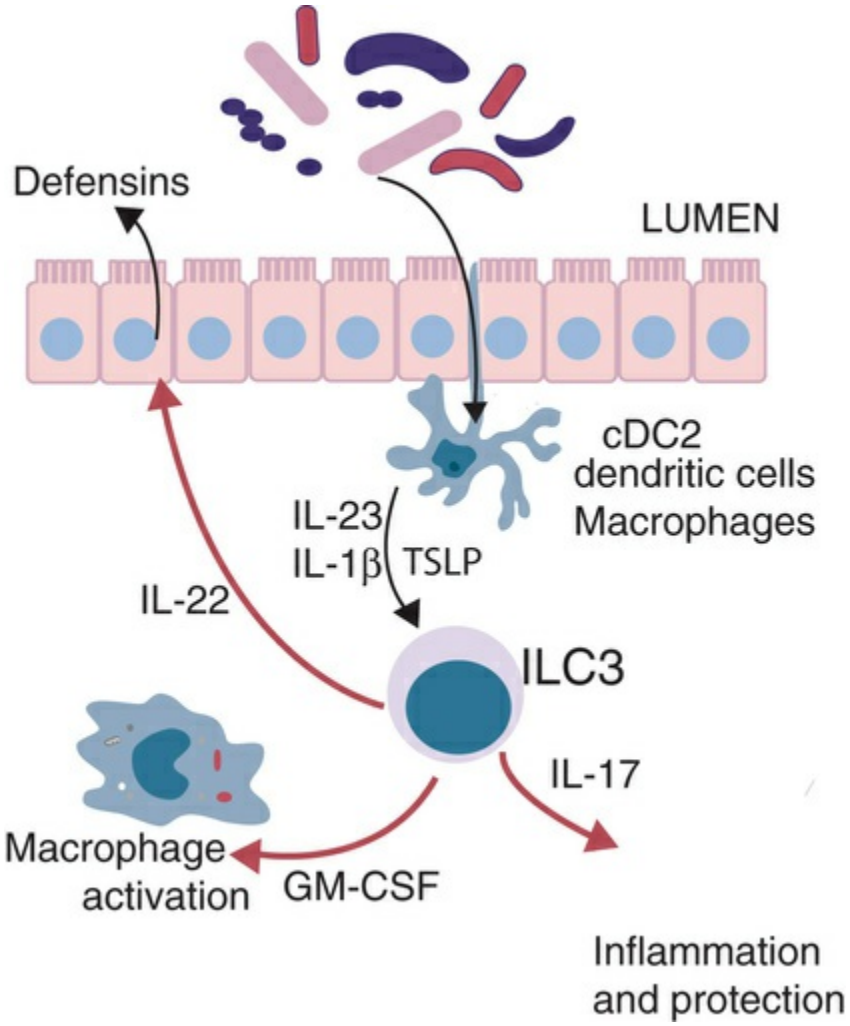


FIG. 19.4 The functions of Group 3 innate lymphoid cells. Microbial products stimulate cDC2 cells to produce IL-23 and IL-1β.

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Activated by these cytokines, they release IL-22, IL-17, and GM-CSF. This causes inflammation, macrophage activation, and the production of defensins.

Because they are required for the development of lymph nodes, Peyer's patches, and isolated lymphoid follicles, ILC3s are also called lymphoid tissue inducer (LTi) cells. They regulate differentiation of B cells. They activate dendritic cells (cDC2 cells) and so influence IgA class switching on surfaces. In the spleen, ILC3s express the B cell growth factors BAFF, APRIL, and CD40L, and stimulate IgM production (see Box 15.2).

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Innate Cytotoxic Cells

Natural Killer Cells

The first ILCs identified were called natural killer (NK) cells because they were able to kill virus-infected and tumor cells without requiring prior activation. NK cells can be considered to be cytotoxic ILC1s. In most mammals, NK cells are large, granular lymphocytes. In cattle, NK cells are large cells, but may not contain large cytoplasmic granules (Fig. 19.5). There is debate about NK cell morphology in the pig. Some investigators claim that they are large granular lymphocytes, whereas others believe that they are small lymphocytes without obvious cytoplasmic granules.

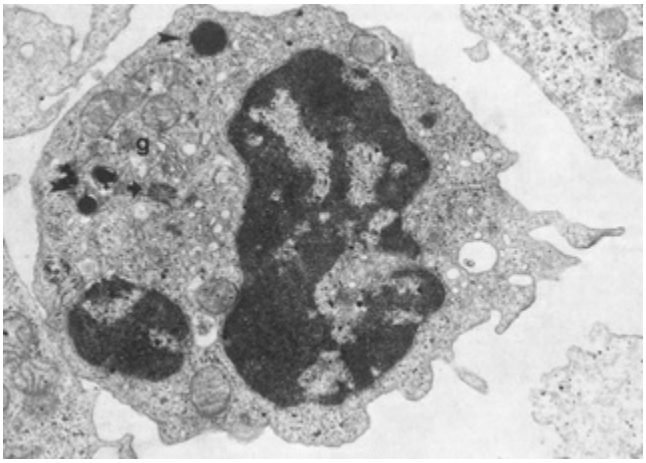


FIG. 19.5 A transmission electron micrograph of a human NK cell. The nucleus is indented and rich in chromatic. The cytoplasm is abundant and contains many granules. Numerous mitochondria, centrioles, and a Golgi are visible. Original magnification ×17,000. (From Carpen O, Virtanen I, Saksela E: Ultrastructure of human natural killer cells: nature of the cytolytic contacts in relation to cellular secretion, *J Immunol* 128:2691, 1982.)

Origins and Location

NK cells are produced by bone marrow stem cells and are found in peripheral blood, lymph nodes, spleen, and bone marrow but not in the thymus. They range from 2% of the lymphocytes in mouse

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spleen to 15% of the lymphocytes in human blood. Their characteristic cell surface phenotype is $CD3^-$, $CD56^+$, and $NKp46^+$.

Target Cell Recognition

NK cells do not make the enormous diversity of antigen receptors employed by T and B cells. Instead, they use two types of receptors to distinguish normal from abnormal cells. One type monitors the expression of MHC class I antigens by cells; the other detects "stress" molecules on unhealthy cells such as tumor or infected cells.

NK cells thus recognize abnormal cells using two different strategies. One is a "missing-self" strategy by which NK receptors bind MHC class I molecules expressed on healthy cells, and as a result prevent NK cell killing of normal MHC class I^+ cells (Fig. 19.6). If, however, a cell fails to express MHC class I, then inhibitory signals are not generated, and it will be killed. This occurs, for example, when a virus suppresses cellular MHC class I expression to avoid destruction by cytotoxic T cells. Likewise, tumor cells that fail to express MHC class I are also killed by NK cells.

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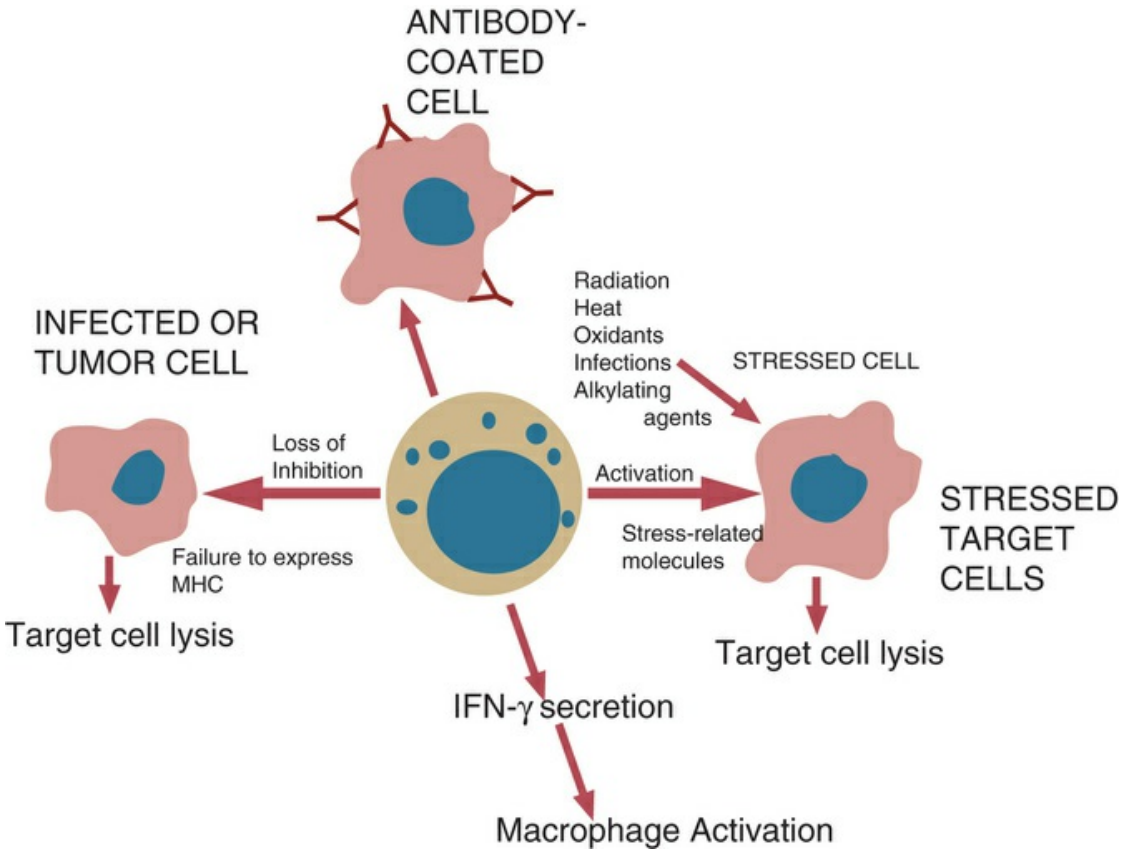


FIG. 19.6 The activities of NK cells. They kill stressed cells, cells not expressing MHC class I, and antibody-coated targets. By releasing IFN-γ, they also activate macrophages.

The second strategy uses NK receptors that recognize stress-induced proteins on cells. When the NK cells detect these stress proteins, an activating signal is generated and the NK cells kill the stressed cells (Fig. 19.7). Cells infected by viruses and intracellular bacteria, as well as some cancer cells, express these stress proteins.

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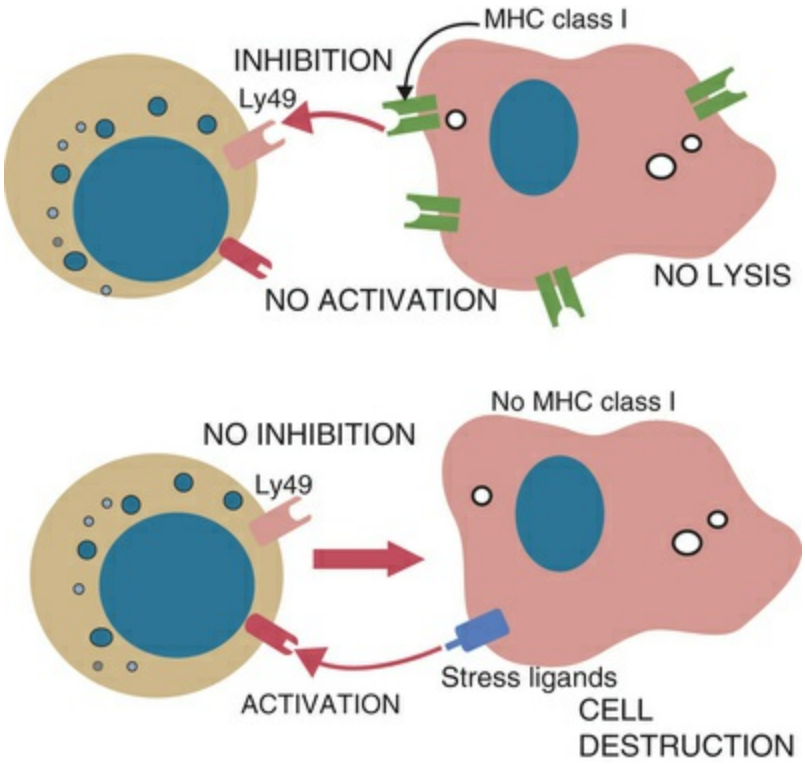


FIG. 19.7 The activation of NK cells is triggered by two situations. Target cells may fail to express MHC class I molecules. As a result, NK cells lose their inhibitions about attacking such cells. Alternatively, NK cells may be activated by the expression of stress-related proteins on target cells.

Receptors

The most important NK cell receptors belong to three multigene families. One is the killer cell immunoglobulin (Ig)-like receptor (KIR or CD158) family expressed in humans and cattle. The other two families consist of C-type lectins; one, called Ly49, is expressed in rodents and horses, and the other, called NKG2D, is expressed in rodents and primates. All three receptor families include both inhibitory and activating receptors (Fig. 19.8). Other important NK cell receptors include CD2, CD16 (FcγRIII), CD178, CD95L, CD40L (CD154), toll-like receptors (TLR3 and TLR9), and leukocyte function-associated antigen-1 (LFA-1) (Fig. 19.9). NK cells do not express conventional antigen receptors such as the B cell antigen receptors (BCRs) or T cell antigen receptors (TCRs), nor do they express a CD3 complex (Chapter 14).

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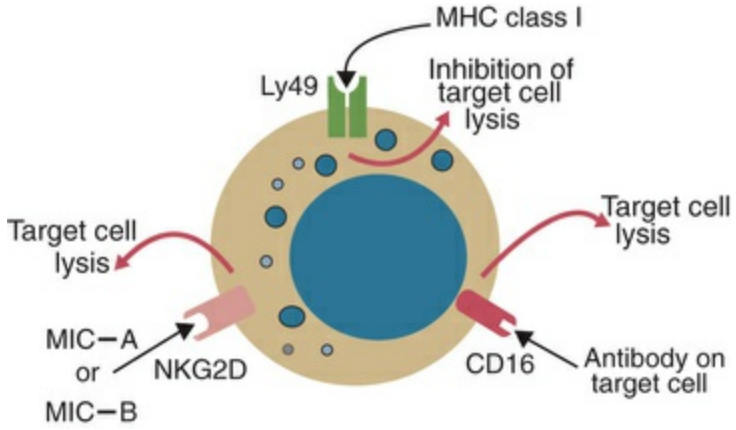


FIG. 19.8 The three major receptor types found on mouse NK cells. Ly49 recognizes MHC class I molecules and suppresses NK cytotoxicity. NKG2D is a receptor for molecules such as MIC-A and MIC-B. These molecules are commonly expressed on stressed, virus-infected, or tumor cells. CD16 binds immunoglobulins and triggers target cell death by antibody-dependent cellular cytotoxicity. Remember that in other mammals, Ly49 may be replaced by KIR receptors.

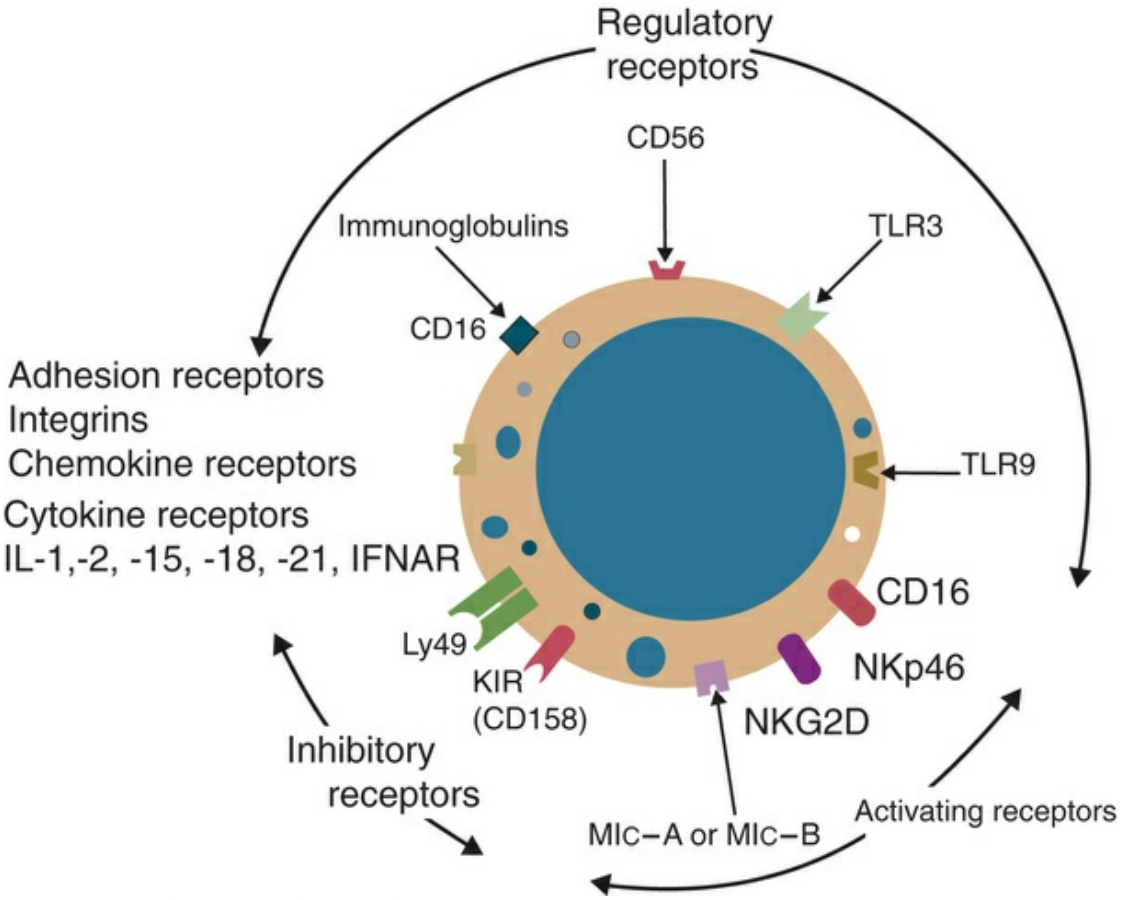


FIG. 19.9 Some of the receptors expressed on NK cells.

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KIR Receptors

On the NK cells of humans, other primates, and cattle, the receptors for MHC class I belong to a family of proteins called killer inhibitory receptors (KIRs or CD158). KIRs may be either inhibitory or stimulatory, but inhibitory receptors predominate. When these inhibitory KIRs bind to MHC class I molecules on a target cell, cytotoxicity is inhibited and healthy cells are not destroyed. Other members of the KIR family may stimulate NK cell cytotoxicity, but these receptors tend to have low binding affinities compared to the inhibitory receptors, so this contributes to the dominance of inhibition.

The human *KIR* gene locus is extremely polymorphic. Indeed, allelic polymorphism is so extensive in this locus that it is difficult to find unrelated individuals with identical *KIR* haplotypes. *KIR* gene polymorphism affects sequences in the MHC class I binding sites of these receptors, and as a result, it determines their binding specificity. *KIR* gene expression patterns also vary clonally, so that individual NK cells can express random combinations of KIR receptors. This extreme diversity at the primate KIR locus results from selection pressure analogous to that seen in MHC loci. In other words, resistance to specific infections conferred by the KIR locus will depend on an animal's haplotype. In addition to possessing KIRs, cattle NK cells also have a diverse receptor repertoire based on C-type lectin-like receptors, but it is unclear how this influences *KIR* expression.

Although primate *KIR* genes vary in their number and diversity, four are present in virtually all haplotypes and are called framework loci. The total number of *KIR* genes expressed by a single individual ranges between 7 and 12, depending on the presence or absence of activating KIR loci. Other members of the KIR family include the leukocyte immunoglobulin-like receptors (LILRs) and NCR1 (NKp46, CD335). NCR1 (natural cytotoxicity triggering receptor 1) is only expressed on NK cells, while the LILRs are expressed on other leukocytes.

Ly49 Receptors

In rodents and horses, in contrast to humans and cattle, the predominant NK cell MHC-binding receptors belong to the Ly49

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family of glycoproteins. They have the same function as KIR receptors in that they bind MHC class I molecules and are exceptionally polymorphic. There are at least 23 members (Ly49A to Ly49W) in this family in mice. All are homodimeric type II transmembrane C-type lectins. Ly49 haplotypes also contain variable numbers of inhibitory and activating genes, some of which can recognize MHC class I molecules. Horses and donkeys have at least five highly conserved Ly49 genes. Unlike mice and horses, humans, pigs, dogs, cats and cattle possess only a single Ly49-like gene. This is a pseudogene in humans but may be functional in cattle. NK1.1 is a member of the Ly49 family that acts as an activating receptor on mouse NK cells.

NKG2 Receptors

The third family of MHC-binding receptors on NK cells are activating molecules that belong to the NKG2 receptor system. NKG2D receptors bind to nonclassical MHC class I proteins produced by stressed cells. Two of the most important of these ligands are polymorphic MHC class I-like molecules called MIC-A (*m*ajor histocompatibility complex, class *I c*hain-related *A*) and MIC-B coded for by MHC class Ic genes (Chapter 11). Unlike conventional class I molecules, these do not bind antigenic peptides. While minimally expressed on normal, healthy cells, they are expressed in large amounts by stressed cells. These stresses may include DNA damage due to ionizing radiation or alkylating agents, heat shock, and oxidative stress. MIC-A and MIC-B are overexpressed in tumor cells and virus-infected cells. When these ligands bind to NKG2D, it overrides the inhibitory effects of conventional MHC class I molecules and triggers NK cytotoxicity. NKG2D is also expressed on activated γ/δ and α/β T cells, suggesting that they too have a role in innate immunity. It may be that the combination of γ/δ T cells and NK cells kills tumors on surfaces, whereas a combination of α/β T cells and NK cells is most effective within the body.

Fc Receptors

NK cells also recognize and kill target cells using an antibody-dependent pathway employing the Fc receptor, CD16 (FcγRIII).

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CD16 is a 38-kDa transmembrane protein linked to either the γ chain of FcγRI (in macrophages) or to the zeta chain of CD3 (in NK cells). When antibodies bind to target cells, the bound antibody links to NK cell CD16, triggers cytotoxicity, and the target cells are killed. NK cells can spontaneously release their CD16 so that the NK cell can detach from an antibody-coated target after it has delivered its lethal hit.

Effector Mechanisms

NK cell activities are regulated by cytokines and pathogen-associated molecular patterns (PAMPs). For example, IL-2 and IL-4 enhance their cytotoxicity, whereas IL-3 enhances NK survival. Although NK cells are active in the nonimmune animal, virus infections or interferon inducers enhance their activity (Fig. 19.10). These activated NK cells are called lymphokine-activated killer (LAK) cells. When macrophages phagocytose invading organisms and produce tumor necrosis factor-α (TNF-α) and IL-12, these cytokines then induce interferon-γ (IFN-γ) production by NK cells. The IFN-γ enhances NK activity further by promoting the rapid differentiation of pre-NK cells.

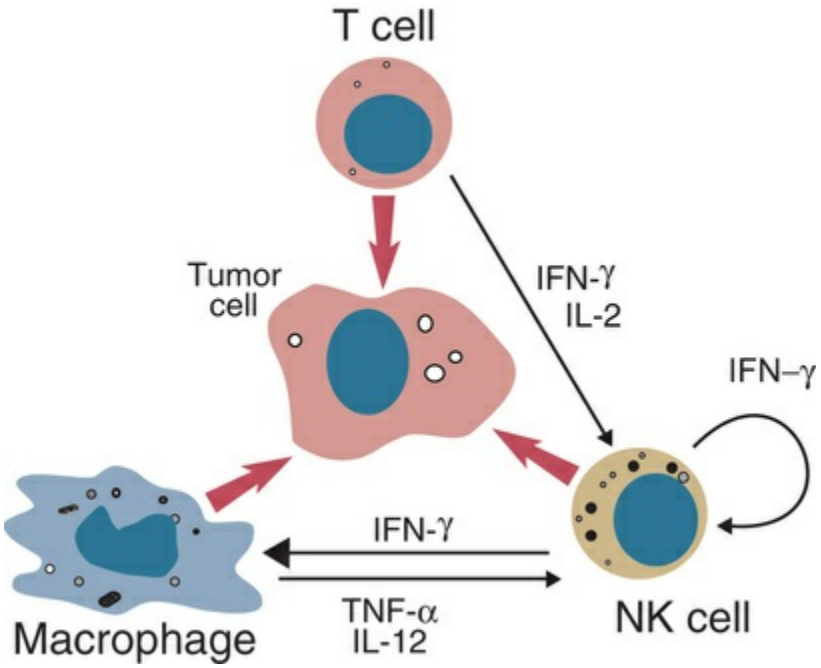


FIG. 19.10 The interactions between NK cells, macrophages, T cells, and abnormal cells. IFN-γ is a potent stimulant of both NK

cells and macrophages. Macrophages stimulate NK cell activities with TNF-α and IL-12.

Once activated, NK cells kill target cells through either a perforin/granulysin/NK-lysin pathway or through the death domain pathway involving CD95L. NK cell granules are stored preformed in resting NK cells (in contrast to cytotoxic T cells, which only produce theirs on demand). Once an NK cell encounters a target cell, a synapse forms at the contact site. Activating KIRs induces MHC molecules to form a ring around a cluster of adhesion molecules. At the center of the synapse, there is a cSMAC through which NK cell granule contents can pass. Receptors and signaling molecules also segregate in the cSMAC, whereas integrins and talin accumulate in the pSMAC. Inhibitory KIRs, in contrast, prevent the localization of lipid rafts to the immune synapse.

Perforins, granulysin, and NK-lysin are found in NK cell granules, and their expression is increased by exposure to IL-2 and IL-12. NK cell perforin is a protein of 70 to 72 kDa (slightly larger than that produced by T cells). It produces small (5 to 7 nm dia) channels in target cell surfaces. Presumably, granzymes are injected into the target cells through the perforin channels.

Functions

Unlike T and B cells that circulate as resting cells and so require several days to become fully activated, NK cells are "on call" and can be rapidly activated by IFNs released from virus-infected cells or by IL-12 from stimulated macrophages. As a result, NK cells promptly attack tumors and virus-infected cells. They participate in innate defenses long before antigen-specific primary adaptive responses can be generated.

NK cells kill some tumor cells, xenografts, and virus-infected cells (Fig. 19.11). Thus they are active against herpesviruses, influenza, and poxviruses. Some Ly49 molecules on mouse NK cells can also recognize viruses directly so that, for example, they can kill cytomegalovirus-infected cells. NK cells can also kill bacteria such as *Staphylococcus aureus, Mycobacteria,* and *Salmonella typhimurium;* protozoa such as *Neospora caninum;* and some fungi.

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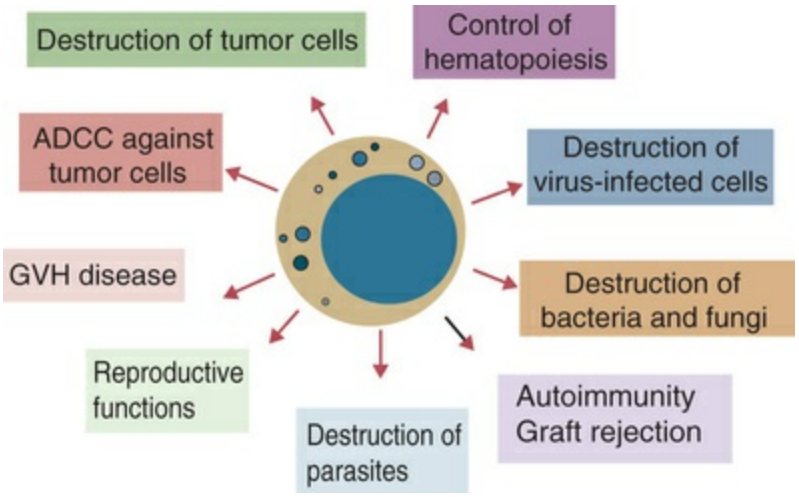


FIG. 19.11 A schematic diagram showing the many functions of NK cells. *ADCC,* antibody-dependent cellular cytotoxicity; *GVH,* graft-versus-host.

NK cells can destroy some cultured tumor cells, and there is a positive correlation between this activity *in vitro* and resistance to tumor cells *in vivo.* Experimentally, it is possible to increase resistance to tumor growth *in vivo* by passive transfer of NK cells from a resistant animal. NK cells destroy human leukemia, myeloma, and some sarcoma and carcinoma cells *in vitro,* and this activity is enhanced by IFN-γ. NK cells can also invade small primary mouse tumors. Some carcinogenic agents and low doses of radiation inhibit NK activity. Stressors such as surgery may also depress NK activity and so promote tumor growth. NK cells also play an important role in the maintenance of pregnancy (Chapter 34).

Memory NK Cells

NK cells increase in numbers in response to stimulation and are removed once invaders have been eliminated. Some may, however, develop a "memory" and mount a form of secondary response to some antigens. For example, NK cells bearing a KIR specific for cytomegalovirus can expand their numbers in response to viral antigen. Thereafter these NK "memory" cells persist in both lymphoid and nonlymphoid tissues for several months. These self-renewing memory cells are reactivated on re-exposure to the viral antigen. Adoptive transfer of these reactivated NK cells leads to a rapid expansion of their numbers and protective immunity to

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cytomegalovirus. Because NK cells employ multiple activating receptors, an NK cell activated through one receptor may well be reactivated through a different receptor. For example, NK cells initially activated through Ly49 may be reactivated through NKG2D. This NK "memory" response could be more accurately described as training rather than memory.

NK Cell Subsets

NK cell subsets have been identified in many species. Some of these subsets may simply represent cells at different stages of development. Some NK cell diversity probably reflects site-specific subpopulations such as those found in the liver or the thymus. NK phenotypes may also change with age. In mice some NK cells express Ly49, whereas others do not. In humans, some subsets differentially express CD56 and CD16. Cells that express both are primarily cytotoxic, whereas those that express CD56 in the absence of CD16 are mainly cytokine producers. This second population predominates in secondary lymphoid organs. In humans, there is also evidence of two NK subsets based on cytokine secretion. NK1 cells produce IFN-γ but almost no IL-4, IL-10, or IL-13. NK2 cells do not secrete IFN-γ but produce IL-13. Another subset of NK cells has a regulatory function, secretes IL-10, and dampens immune responses. It has been suggested that NK cells exposed to moderate levels of IL-12 secrete IFN-γ, but if exposed to very high levels of IL-12, they produce IL-10 that can then suppress T cell activities. In effect, overstimulation turns on a suppressive function. These regulatory NK cells may reduce the severity of virus-induced immunopathology.

Species Differences

Mammalian NK cell expression of KIR and Ly49 receptors is species specific and mutually exclusive. A species may have either a diverse *Ly49* or a diverse *KIR* gene family, but not both. For example, humans have multiple polymorphic *KIR* genes but only a single, nonfunctional *Ly49* gene. Likewise, cattle, dogs, cats, and pigs also possess multiple *KIR* genes and a single functional *Ly49* gene. This suggests that the use of multiple *Ly49* genes by rodents and horses is not typical of mammals in general (Fig. 19.12).

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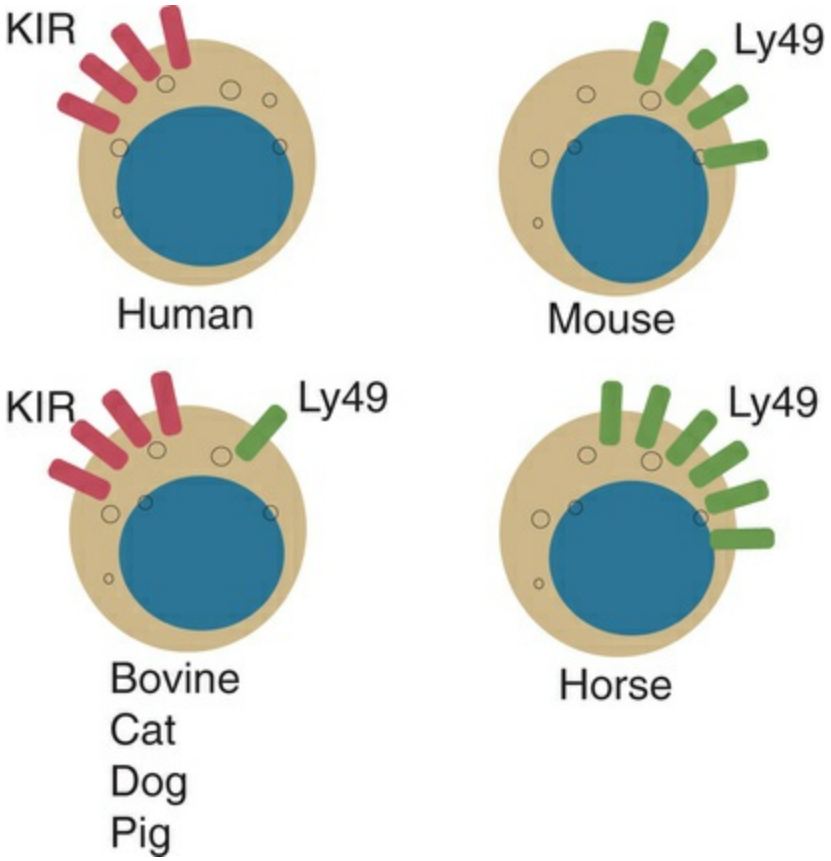


FIG. 19.12 The species difference between the MHC class I receptors on NK cells. Humans possess a single, nonfunctional *Ly49* gene. Thus they rely totally on KIR molecules, whereas mice rely totally on Ly49 molecules for recognition of their MHC ligands. Most domestic species resemble humans except for the horse that resembles the mouse in its receptor usage.

Horses

Equine NK cells are activated by recombinant human IL-2 and are active against human tumor cell targets. Horses have six transcribed *Ly49* genes but no transcribed *KIR* genes. Thus horse NK cell receptors resemble those of rodents rather than those of the other domestic mammals.

Cattle

NK cells constitute about 3.5% of blood lymphocytes in young calves and about 2% in older cattle. They are found in highest concentrations in the spleen, lymph nodes, and peripheral blood. Bovine NK cells can kill human cancer cell targets as well as bovine cells infected with parainfluenza-3, bovine leukemia virus (BLV), or

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bovine herpesvirus type 1 (BHV-1). They generate resistance to mycobacteria by preventing its replication within macrophages. They play a role in resistance to the protozoan parasite *Neospora caninum* by producing IFN-γ that kills infected cells. Three subpopulations have been described. For example, most bovine NK cells express both CD2 and NCR1, but subpopulations may be CD2 or NCR negative. Other NK cell surface molecules include CD16, perforins, CD5, CD94, WC1, MHC class II, and asialo-GM_1. (GM_1 is a ganglioside–a glycolipid molecule composed of a fatty ceramide residue buried in the lipid bilayer of a cell with at least three sugar groups projecting into the extracellular fluid. One of these is normally a sialic acid group. This is lacking in asialo-GM_1.) Cattle also have some circulating NCR1-positive T cells.

Cattle NK use KIR proteins as MHC class I receptors. Cattle have at least six *KIR* genes, some of whose products are inhibitory, whereas others are activating. They also have variable haplotype composition. Cattle NK cells also express NKG2D. Cattle probably have four MIC genes. One gene is consistently present, whereas the presence of the other three is variable. They possess one *Ly49* gene with three alleles, so cattle are the only species known to have both polymorphic *Ly49* genes and *KIR* genes. Cattle also have at least 16 LILRs, of which seven are likely inhibitory, four are activating and five are soluble. Thus overall, cattle NK cells are highly heterogeneous and differentially express many different receptors for MHC class I.

Cattle NK cells are activated by IL-2, IL-12, IL-15, IFN-α, and IFN-γ. Activation by IL-2 enables them to express CD25 and CD8 and lyse tumor cell lines. If activated by IL-12 and IL-15, they express increased amounts of granulysin, IFN-γ, and perforin, and can kill both human tumor cells and bacille Calmette-Guérin (BCG)-infected macrophages. Cattle also produce NK-lysin and, uniquely, have four functional *NK-lysin* genes (as opposed to one in other mammals). Three (*NK1, NK2A,* and *NK2B*) are predominantly expressed in the Peyer's patches, while one *(NK2C)* is found exclusively in the lungs.

Sheep

Sheep have $CD16^+$, $CD14^-$ NK cells in their blood. More than 80% of

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these cells also express perforin and NCR1 and are cytotoxic for mouse and sheep cell targets. They produce IFN-γ in response to IL-12 stimulation.

Pigs

Pig NK cells are NCR1, CD25, CD8a, CD16 positive, but subsets exist. Pigs have a single *KIR* gene and a single *Ly49* gene, and the latter may be a pseudogene. Thus it is uncertain how pig NK cells recognize their targets. Porcine NK cells are found in spleen and peripheral blood, but very few are found in lymph nodes or thymus (Fig. 19.13). They can lyse human cancer cells as well as cells infected with transmissible gastroenteritis virus or pseudorabies virus. Pigs have two *MIC* genes, one of which is a pseudogene.

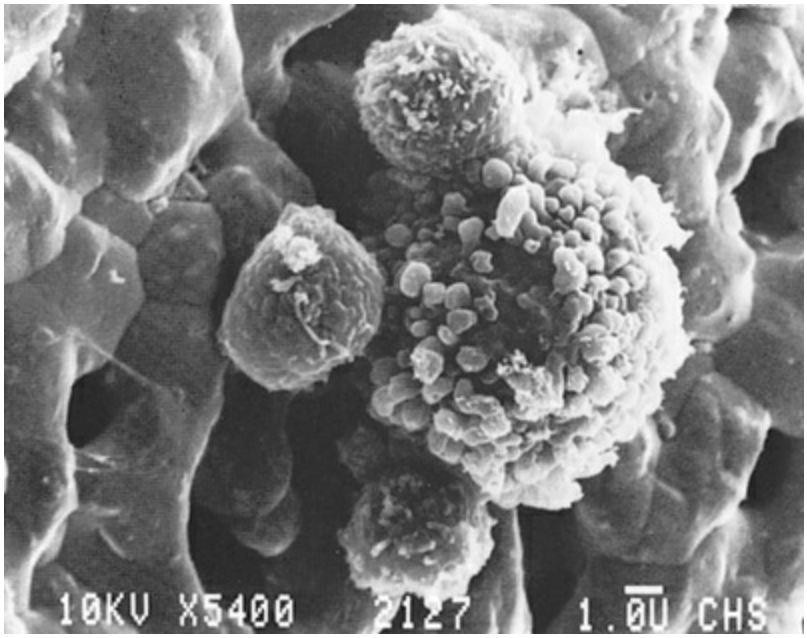


FIG. 19.13 A scanning electron micrograph of NK cells from a pig (small round cells) attached to a target cell (a human tumor cell). Original magnification ×5400. (From Yang WC, Schultz RD, Spano JS: Isolation and characterization of porcine natural killer [NK] cells, *Vet Immunol Immunopathol* 14:345-356, 1987.)

Dogs

Dog NK cells may be isolated by culturing peripheral blood mononuclear cells and removing the T cells by anti-T cell antibodies

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complexed to magnetic beads. The residual cells can be cultured in the presence of IL-2, and IL-15. The non-T, non-B large granular cells that survive are NK cells (IL-2 is necessary for survival, IL-15 is needed for expansion). These NK cells are $CD3^-$, granzyme B^+, $CD45^+$, and MHC class I^+. Both IL-15 and IL-21 stimulate their proliferation, receptor expression, and cytotoxic functions. They can lyse distemper-infected target cells as well as cancer cells from thyroid adenocarcinomas, melanomas, osteosarcomas, and mammary carcinomas.

Cats

Feline NK cells are large granular lymphocytes found in the blood and spleen. In general, cat NK cell numbers and properties are similar to those in other domestic mammals. However, unlike other species, 10% to 30% of feline NK cells are $CD11b^+$ and selectin $CD62L^+$. They are active against feline target cells infected with feline leukemia virus, herpesvirus, or vaccinia virus.

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NKT Cells

Natural killer T cells (NKT cells) are innate-like T cells that express both NK cell markers and a TCR of limited diversity. There are two functionally distinct NKT cell subsets. Type I NKT cells are T cells that express a semi-invariant TCR consisting of an invariant α chain associated with diverse β chains. This TCR recognizes lipid, lipopeptide, and glycolipid antigens presented by the MHC class I-like molecule CD1. CD1 proteins are nonpolymorphic molecules found on antigen-presenting cells (Fig. 19.14). The CD1-lipid presentation system allows the immune system to sense lipid antigens. It thus complements the MHC-peptide presentation system. Some CD1 proteins present lipid antigens to conventional T cells, while others present lipids to NKT cells. Type I NKT cells can promote immunity in response to lipid antigens from infectious agents and tumor cells. They are found in the liver, spleen, blood, and adipose tissue. Most are found in the sinusoids of the liver. They make up about 1% of the mononuclear cells in human blood.

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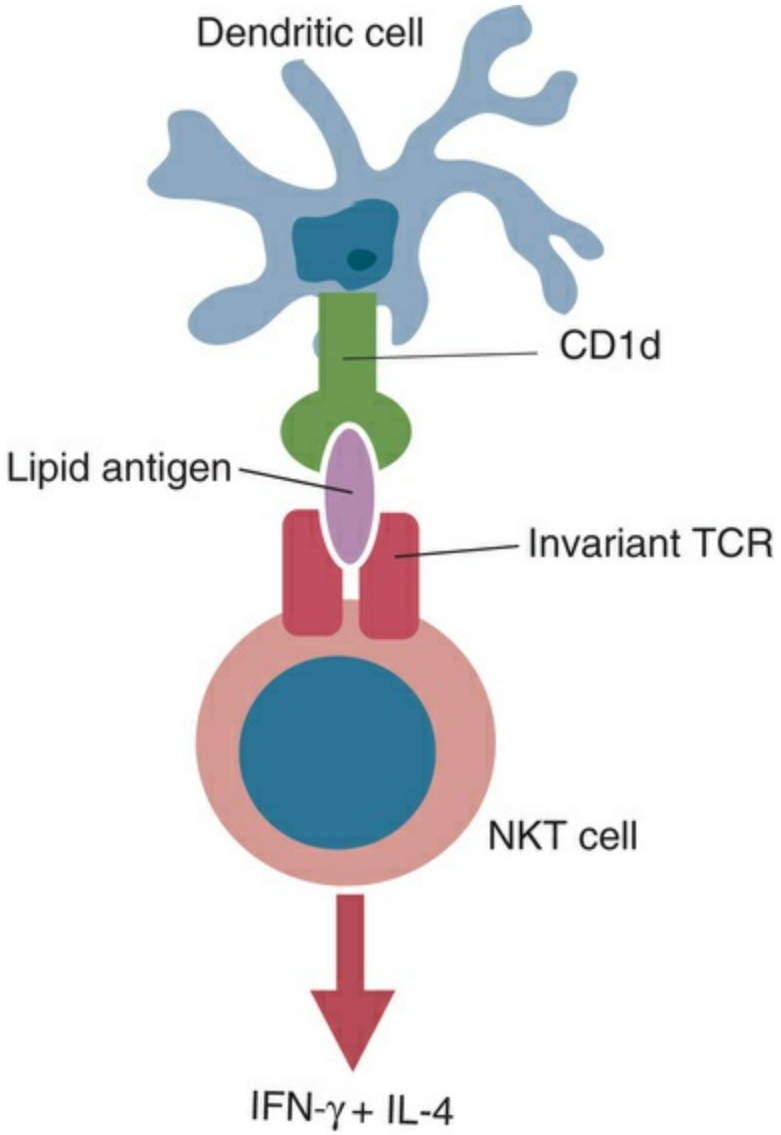


FIG. 19.14 Type I NKT cells recognize and respond to lipid antigens by using CD1d as an antigen-presenting molecule.

In the absence of prior antigenic stimulation, NKT cells respond more rapidly than conventional T cells. They produce proinflammatory cytokines such as IFN-γ and TNF-α as well as antiinflammatory cytokines such as IL-4 and IL-10. As a result, they can modulate immunity in a broad spectrum of diseases. NKT cells trigger chemokine and cytokine release, enhance NK function, and promote dendritic cell maturation and B cell responses. NKT cells inhibit the development of Th17 cells and regulate IL-17 production. NKT cells secrete IL-12 that acts on neutrophils to decrease their production of IL-10. They play a role in allergies, antitumor immunity, autoimmunity, and antimicrobial immunity, especially to mycobacteria. Thus they link the T cell system with the

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innate NK cell system. Type I NKT cells also express receptors for inflammatory cytokines produced by antigen-presenting cells. They can be activated either in combination with TCR-mediated signals or even without such signals.

Type II NKT cells, in contrast, use an oligoclonal TCR where both α and β chains contribute equally to lipid recognition. Their functions are predominantly antiinflammatory and they recognize diverse hydrophobic antigens. The type II cells also suppress the type I cells, suggesting that they have an immunoregulatory role.

Species Differences

Equine NKT cells recognize the cell wall lipids of *Rhodococcus equi* when they are bound to CD1. The equine genome contains 13 expressed CD1 genes, which makes it the largest CD1 family yet recognized in mammals. All but one of these molecules are expressed on equine antigen-presenting cells. The structural differences between these proteins are located in their antigen-binding sites suggesting that horse NKT cells can recognize many different lipid antigens. It is possible that this unusually large number of CD1 molecules reflects their key role in protection against *R. equi.* Other cells that express CD1 include dendritic cells, Kupffer cells, endothelial cells, and hepatocytes. Pigs and horses possess functional *CD1* genes that form receptors similar to those found in humans. In pigs, type I NKT cells recognize α-galactosylceramide. Their production is enhanced by IL-2, -15 and -33. The *CD1* genes in cattle and other ruminants are pseudogenes, so functional NKT cells may not be present. Cattle do, however, possess a novel T cell subset that has features of both T and NK cells. Thus these cells express both NCR1 and CD3. They can be activated by either the NKR or TCR pathways and can kill *Theileria parva*-infected cells. Presumably these are the functional equivalents of NKT cells in other species.

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20

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Regulation of Adaptive Immunity

CHAPTER OUTLINE

Tolerance, 208
T Cell Tolerance, 208
Central T Cell Tolerance, 208
Negative Selection, 208
Receptor Editing, 210
Peripheral T Cell Tolerance, 210
Clonal Anergy, 210
B Cell Tolerance, 210
Peripheral B Cell Tolerance, 211
Duration of Tolerance, 211
Control of Immune Responses, 211
Antigen Regulation of Immune Responses, 212
Antigen Processing and Immune Regulation, 212
Antibody Regulation of Immune Responses, 212
Inhibitory Receptors, 212
Regulatory Cells, 213
Regulatory T Cells, 213
Interleukin-10, 215

Transforming Growth Factor-β, 215
Regulation by the Innate Immune System, 216
Th17 Cells, 216
Interleukin-17, 216
Regulatory Macrophages, 218
Indoleamine 2,3-Dioxygenase and Tolerance, 218
Tolerogenic Dendritic Cells, 218
Regulatory B Cells, 218
Natural Suppressor Cells, 218
When Do Regulatory Cells Work?, 218
Regulation of Apoptosis, 218
Neural Regulation of Immunity, 219
Stress, 219
Autonomic Nervous System, 220
Hypothalamic-Pituitary-Adrenal Cortical Axis, 220
Neuropeptides and Lymphocytes, 220

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Explain how and why T cells do not respond to normal body components.
- Define negative selection, positive selection, clonal anergy, and immunological tolerance and differentiate between central and peripheral tolerance.
- Understand why B cells are much harder to tolerize than T cells.

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- Discuss how antigens regulate immune responses, although very low or very high doses of antigen may cause tolerance.
- Explain how antibodies regulate antibody production through negative feedback mechanisms.
- Describe the origins and properties of regulatory T cells (Treg cells).
- Discuss the properties and significance of interleukin-10.
- Explain how Th17 cells enhance inflammatory responses by secreting interleukin-17 (IL-17).
- Describe the properties and significance of IL-17.
- Discuss the role of indoleamine 2,3-dioxygenase in inducing tolerance.
- Discuss briefly how the immune system and the central nervous system are closely interconnected and influence each other.

The adaptive immune system can recognize and respond to foreign invaders and can learn from the experience so that the body responds faster and more effectively when exposed to the invader a second time. There is, however, a risk associated with this—the risk of collateral damage. One of the reasons why the adaptive immune system is so complex is that much effort must be put into ensuring that lymphocytes will only attack invaders or abnormal cells and will ignore normal healthy tissues. As might be anticipated, many different regulatory circuits balance lymphocyte activities and minimize the chances of inappropriate or damaging responses. In addition, immune responses are regulated to ensure that they are appropriate with respect to both quality and quantity.

Since both T and B cells randomly generate antigen-binding receptors, it is clear that the initial production of self-reactive cells cannot be prevented. An animal cannot control the amino acid sequences and hence the binding specificity of these receptors. As a result, when first generated, as many as 50% of the T cell antigen receptors (TCRs) and B cell antigen receptors (BCRs) may bind self-antigens. If autoimmunity is to be avoided, lymphocytes with these inappropriate receptors must either be destroyed or at least turned off.

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Tolerance

Tolerance is the name given to the situation in which the immune system will not respond to a specific antigen. Tolerance is primarily directed against self-antigens from normal tissues. In 1948 two Australian immunologists, Burnet and Fenner, recognized this need for self-tolerance and suggested that immature lymphocytes would become tolerant to an antigen if they first met it early in fetal life.

Support for this suggestion came from observations on chimeric calves. In 1945 Owen noted that when cows are carrying twin calves, blood vessels in the two placentas commonly fuse. As a result, the blood of the twins intermingles freely, and bone marrow stem cells from one animal colonize the other. Each calf is born with a mixture of blood cells, some of its own and some originating from its twin. In dizygotic (nonidentical) twins, this is called a chimera. These "foreign" blood cells persist indefinitely because each chimeric calf is fully tolerant to the presence of its twin's cells (Fig. 20.1). Burnet and Fenner suggested that this could only happen because each calf was exposed to the foreign cells early in fetal life at a time when lymphocytes become tolerant upon encountering antigens. Cells from an unrelated calf would be rejected normally if administered after birth. Thus immune tolerance is not innate. It is established during fetal and postnatal development and relies on mechanisms that control lymphocyte development.

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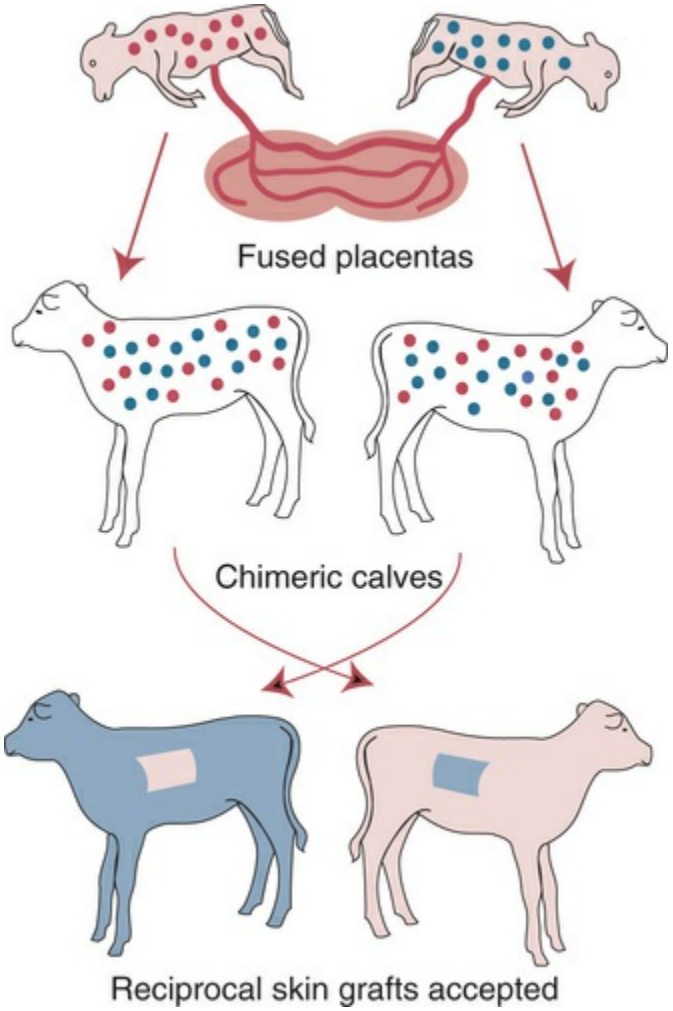


FIG. 20.1 Fusion of the placentas of dizygotic twin calves results in the development of calf chimeras. Hematopoietic stem cells from each animal colonize the bone marrow of the other. Each chimera is tolerant to its twin's cells and will accept a skin graft from its twin despite the genetic differences.

Subsequent studies have shown that self-tolerance is of two types, central and peripheral. In central tolerance, immature self-reactive lymphocytes within the thymus, bursa, or bone marrow either die or alter their receptor specificity. In peripheral tolerance, mature lymphocytes that recognize self-antigens either die, are turned off, or are suppressed by regulatory T cells (Treg cells). By reconstituting lethally irradiated mice with T or B cells derived from normal or tolerant donors, tolerance can be shown to occur in both cell populations. However, their susceptibility to peripheral tolerance differs. T cells can be made tolerant rapidly and easily within 24 hours and remain in that state for more than 100 days (Fig. 20.2). In contrast, B cells develop tolerance in about 10 days and return to normal within 50 days.

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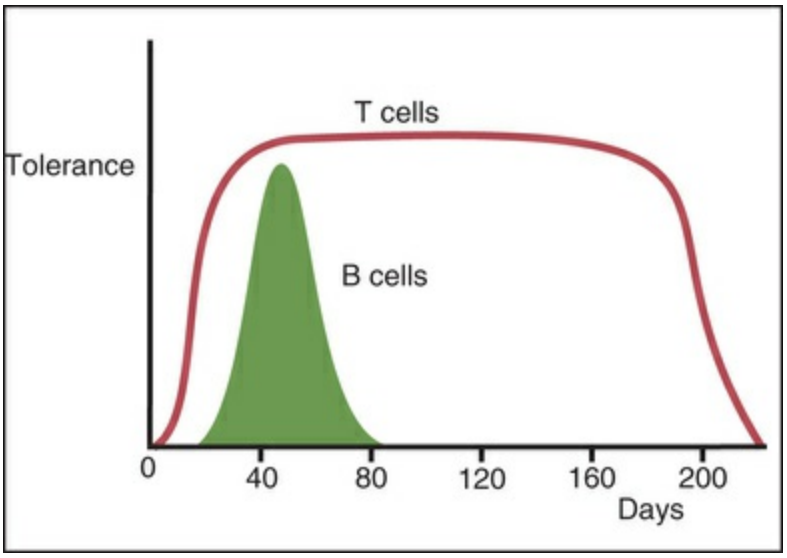


FIG. 20.2 The duration of tolerance in T cells and B cells. T cells are much more easily rendered tolerant than B cells. Once tolerant, they remain that way for much longer.

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T Cell Tolerance

Central T Cell Tolerance

Negative Selection

Developing T cells must undergo education in the thymus where they learn to discriminate between self and non–self-antigens. Tolerance to self-antigens results when there are no functional T cells that can bind self-antigens (Fig. 20.3). (It should be pointed out that an exception must be made for MHC molecules. T cells must be able to recognize them if they are to respond to foreign peptides.) Although lymphocytes generate an enormous diversity of TCRs, far fewer receptors are actually used by mature T cells than might be anticipated. Several processes limit receptor diversity. First, the mechanisms used to generate TCRs inevitably result in the production of non-functional receptors. For example, two-thirds of possible gene arrangements will be out of frame. Cells with these non-functional TCRs die. As T cells mature within the thymus, positive selection ensures that the cells that can bind self-antigens survive. At this point, however, the cells whose receptors bind too strongly to self-antigens also die (Fig. 20.4). The timing and extent of this apoptosis depend on the affinity of the TCR for a self-antigen. T cells that bind self-antigens strongly die earlier and more completely than weakly binding cells. Thus the T cells that eventually leave the thymus have been purged of dangerous, self-reactive cells.

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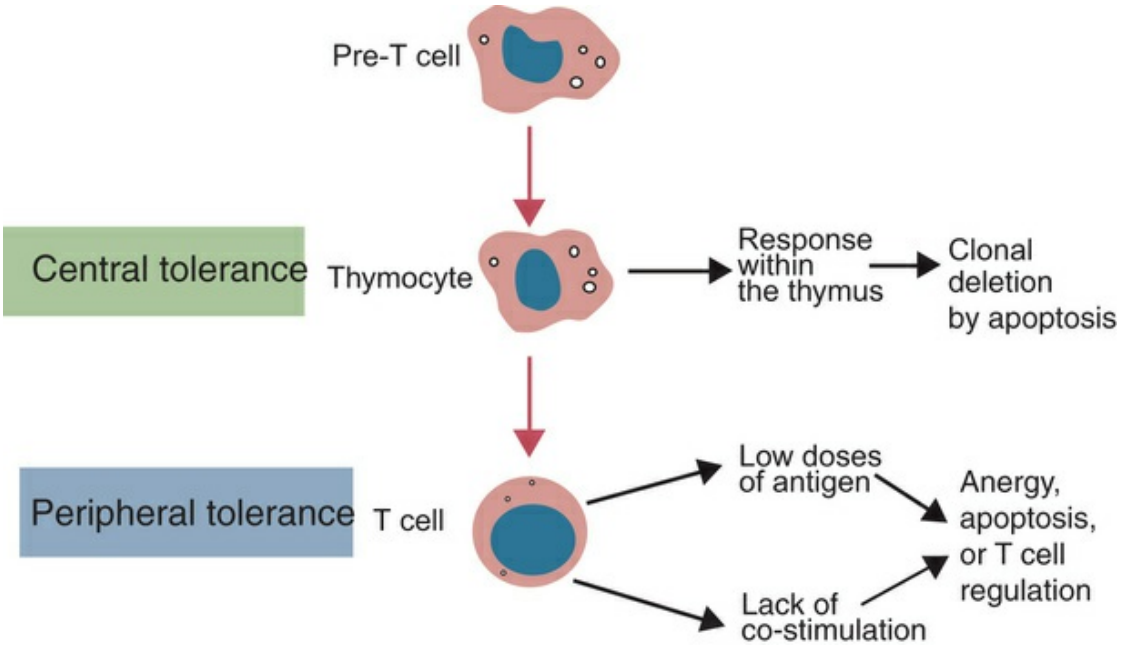
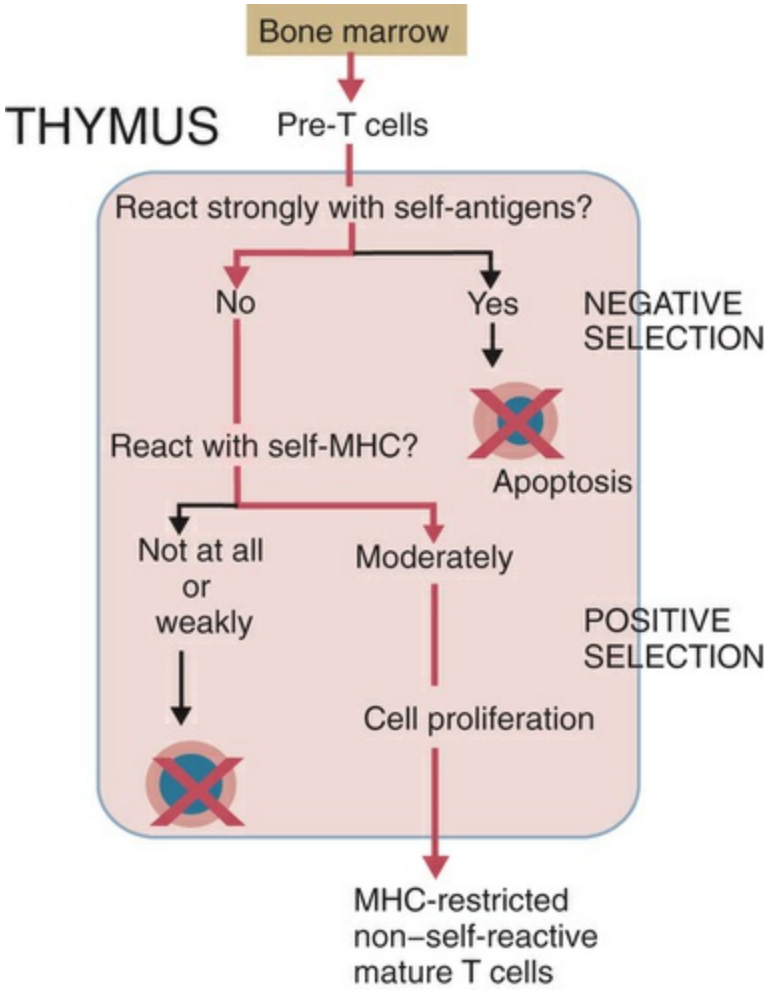


FIG. 20.3 The principal ways by which T cells are made tolerant.



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FIG. 20.4 How the thymus induces central T cell tolerance by negative selection. Surviving T cells are unreactive to autoantigens yet can still respond to foreign antigenic peptides in association with MHC molecules as a result of positive selection.

The negative selection process is assisted by the presence of many different self-antigens in the thymus. Normally, each tissue possesses its own tissue-specific antigens. Thus "skin antigens" are usually restricted to the skin, whereas "liver antigens" are restricted to the liver, and so forth. However, the epithelial cells in the thymic medulla show uniquely "promiscuous" gene expression. Thymic epithelial cells use a transcription factor called the autoimmune regulator (AIRE) that promotes the expression of thousands of non-thymic protein antigens in the thymic medullary epithelium. Examples include insulin, thyroglobulin, and myelin basic protein. In this way, the thymic epithelial cells ensure that self-reactive T cells encounter most normal tissue antigens and are eliminated if they respond too strongly. In addition, some normal tissue antigens may be taken up by macrophages and carried to the thymus. Self-reactive T cells that respond to these antigens are also eliminated. However, this raises another question: What about self-antigens that are not expressed in, or do not enter, the thymus? Antigens from the eye, testis, or brain are not processed in this way, and as a result central tolerance to these antigens does not develop.

These selection processes together ensure that cells that can bind self-antigens are positively selected and those that bind these antigens with very low or very high affinity are subsequently deleted. As a result, the moderate-affinity clones survive and are available to recognize foreign antigens. An additional factor that probably determines thymocyte survival is the dose of antigen presented to the cells. If the amount of a specific antigen is high (as one might anticipate for a self-antigen), multiple TCRs will be occupied on each thymocyte and trigger apoptosis. In contrast, if the amount is low, this will occupy only a few TCRs, and the weak signal may result in positive selection and thymocyte proliferation.

Receptor Editing

When the antigen receptors of a developing T cell bind to self-antigens, another strategy that prevents autoimmunity is receptor

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editing (Chapter 36). Although cell maturation stops when a T cell leaves the thymus, its *RAG* genes remain active, and as a result, V(D)J recombination continues. Consequently, the *TCR* genes continue to diversify, and altered receptors are expressed on the cell surface. This process is called receptor editing. If a cell successfully edits its receptors, its maturation can proceed. Failure to do so will result in its death. This is a potentially hazardous process since it may permit the development of self-reactive T cells that have not undergone selection within the thymus.

Peripheral T Cell Tolerance

Clonal Anergy

Low-affinity self-reactive T cells may survive the selection process and leave the thymus. These must then be regulated by peripheral tolerance mechanisms. One form of peripheral tolerance is clonal anergy, the prolonged, antigen-specific suppression of T cell function. Clonal anergy is triggered when T cells are exposed to antigens in the absence of effective co-stimulation. T cells normally require multiple co-stimulatory signals from several sources in order to respond to antigen. If these signals are insufficient or inappropriate, T cell responses will be suppressed and tolerance results.

As pointed out in Chapter 14, binding of an antigen to a TCR is by itself insufficient to trigger a T cell response. Indeed, occupation of the TCR in the absence of co-stimulation causes tolerance. For example, foreign protein solutions normally contain some aggregated molecules. These aggregated molecules are readily taken up and processed by dendritic cells and thus are highly immunogenic. If a solution of such a protein, such as bovine γ-globulin, is ultracentrifuged so that all the aggregates are removed, then the aggregate-free solution will induce tolerance in mice due to the absence of co-stimulation from antigen-presenting cells (Fig. 20.5).

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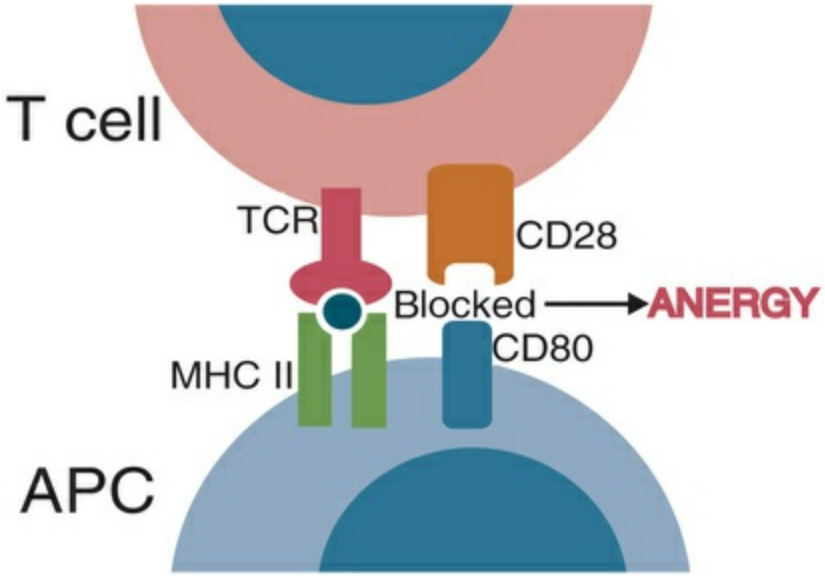


FIG. 20.5 Peripheral tolerance through clonal anergy will develop if a TCR is stimulated by antigen in the absence of simultaneous co-stimulation through the CD28/CD80 or CD28/CD86 pathway.

Binding to the TCR by an antigen in the absence of co-stimulation activates the tyrosine kinases and phospholipase of the T cell and raises its intracellular Ca^{2+}. This results in enhanced production of IκB that inhibits NF-κB. This prevents the cell from making cytokines, especially IL-2. Tolerant Th1 cells produce less than 3% of normal IL-2 levels and much less IFN-γ and TNF-α. Once induced, this "anergy" can last for several weeks.

Triggering of T cell responses normally requires prolonged interactions with antigen-presenting cells (APCs). Tolerance induction, on the other hand, is characterized by relatively short interactive episodes. Thus a key difference between T cell activation and anergy may simply be the duration of their encounter with APCs.

Very high doses of an antigen can induce a form of clonal anergy called immune paralysis (Fig. 20.6). The high doses of the antigen probably bypass APCs, reach the Th cell receptors directly, and in the absence of co-stimulation, trigger paralysis.

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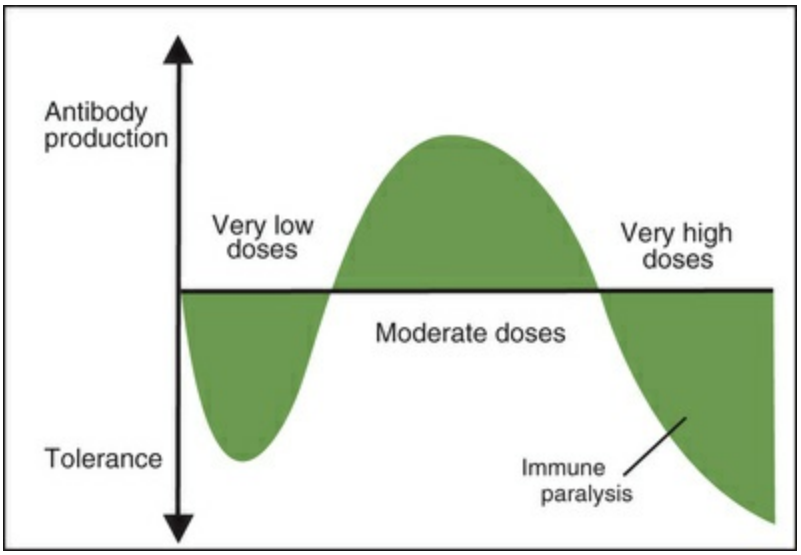


FIG. 20.6 The ability of different doses of antigen to induce peripheral tolerance. Both very low and very high doses can induce tolerance. Moderate doses, in contrast, induce an immune response.

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B Cell Tolerance

Unlike the TCR repertoire, B cell antibody diversity is generated in two phases. The first phase involves VDJ rearrangement or gene conversion in the primary lymphoid organs; the second phase involves random somatic mutation in secondary lymphoid organs. B cells therefore have several opportunities to generate receptors that can bind self-antigens. It has been estimated that 55% to 75% of early immature B cells have self-reactive receptors, so suppression of these B cells must begin at an early stage in an animal's development.

Immature B cells in the bone marrow can be made tolerant once they have rearranged their V-region genes and are committed to express complete immunoglobulin M (IgM) molecules. When these immature cells encounter and bind antigen, the BCR transmits a signal that arrests cell development, blocks synapse formation, and triggers apoptosis. An immature B cell population can be rendered tolerant by one millionth of the dose of an antigen required to make mature B cells tolerant. Immature B cells may also undergo receptor editing as described previously. If receptor editing fails to generate a non–self-reactive B cell, it will die.

Peripheral B Cell Tolerance

Peripheral B cell tolerance is induced by multiple mechanisms including apoptosis, clonal anergy, clonal exhaustion, and blockage of BCRs.

Because BCRs undergo random somatic mutation within germinal centers, self-reactive B cells will develop in secondary lymphoid organs. These cells will not, however, make autoantibodies if APCs and helper T cells are absent or if Treg cells are active (Fig. 20.7). This is not, however, a foolproof method of preventing self-reactivity. In the absence of T cell help, B cells may be activated by PAMPs such as LPS, flagellins, or unmethylated CpG DNA acting through TLRs. B cells may also be activated by either cross-reacting epitopes or by foreign carrier molecules stimulating non-tolerant helper T cells (see Fig. 36.2).

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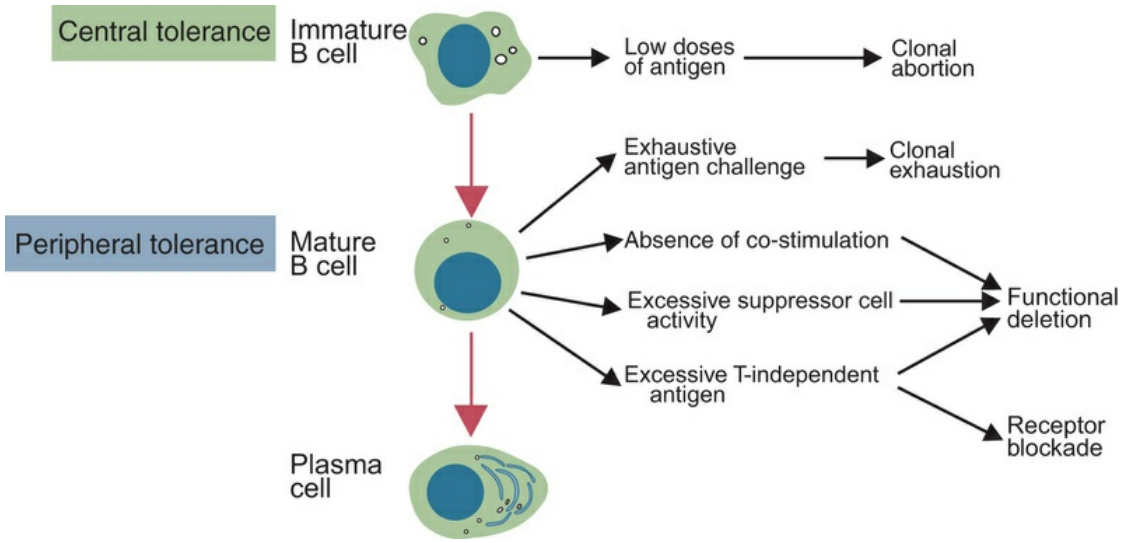


FIG. 20.7 Central and peripheral tolerance mechanisms in B cells.

As with T cells, B cell anergy occurs when they encounter antigens in the absence of co-stimulation. B cells are difficult to maintain in a tolerant state, however, and will reactivate rapidly unless steps are taken to maintain tolerance. Self-reactive B cells must also bind to a critical threshold of self-antigen to be made tolerant. This results in selective silencing of high-affinity B cells. Presumably the failure of low-affinity anti-self B cells to become tolerant poses little threat of autoimmune disease because the low-affinity antibodies will not cause tissue destruction.

B cells subjected to repeated exhaustive antigenic stimulation may differentiate into short-lived plasma cells. If all B cells develop into such plasma cells, no memory B cells will remain to respond to antigen, and tolerance will result.

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Duration of Tolerance

The duration of tolerance depends on antigen persistence and on the ability of the bone marrow to generate fresh T or B cells. Once an antigen is completely eliminated, tolerance fades. If, however, the antigen is persistent, such as occurs with an animal's own self-antigens, tolerance also persists. In the continued presence of an antigen, newly formed antigen-sensitive cells will undergo apoptosis as soon as their receptors bind self-antigen. Treatment that promotes bone marrow activity, such as low-dose X-irradiation, hastens the fading of tolerance, whereas immunosuppressive drug treatment has the opposite effect.

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Control of Immune Responses

Tolerance is not the only mechanism of immune regulation employed by the body. The magnitude of immune responses must also be regulated. An inadequate immune response may lead to immunodeficiency and increased susceptibility to infection. An excessive immune response may result in the development of allergies or autoimmunity (Chapters 29 and 36). Failure to control the lymphocyte proliferation that occurs during immune responses may permit development of lymphoid cell tumors. Failure to control the immune response to the fetus may lead to abortion (Chapter 34). The immune responses must therefore be carefully regulated to ensure that they are appropriate in both quality and quantity. As might be anticipated, many different control mechanisms exist.

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Antigen Regulation of Immune Responses

Adaptive immune responses are antigen driven. They commence only on exposure to an antigen, and when it goes, they stop. If an antigen persists, the stimulus persists and the immune response is prolonged. Prolonged responses occur after immunization with slowly degraded antigens, such as the bacterial polysaccharides, or with antigens incorporated in oil or insoluble adjuvants. Antigens that do not reach organized lymphoid tissues, irrespective of their origin, may fail to induce either immunity or tolerance. Thus self-antigens restricted to sites such as the brain, or viruses such as papillomaviruses that never enter lymphoid organs, are usually ignored by the immune system.

Antibody responses are also regulated by antigen. Rigid polymeric antigens such as those on a bacterial surface or antigens linked to TCR activators such as LPS can induce B cell responses in the absence of T cell help. On the other hand, non-polymeric, flexible antigens such as soluble proteins induce B cell responses only in the presence of $CD4^+$ T cells.

Antigen Processing and Immune Regulation

The nature of the immune response may vary in different parts of the body depending on dendritic cell populations. Langerhans cells in the skin seem especially suited for promoting T cell responses, whereas follicular dendritic cells prime B cells. cDC1 cells are optimized to present antigens to Th1 cells, whereas cDC2 cells present antigens to Th2 cells. Adjuvants also influence the type of immune response through their effects on APCs (Chapter 24).

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Antibody Regulation of Immune Responses

As a result of negative feedback, antibodies generally suppress B cell responses. IgG antibodies tend to suppress the production of both IgM and IgG, whereas IgM antibodies suppress only the synthesis of IgM. Specific antibodies tend to suppress a specific immune response better than non-specific immunoglobulins.

This negative effect of antibodies on B cell functions is mediated through the inhibitory B cell receptor CD32b (FcγRIIb). In diseases where immunoglobulin levels are abnormally high, as in patients with myelomas (Chapter 15), this feedback depresses normal antibody synthesis, and patients become susceptible to infections. A similar phenomenon occurs in newborn animals that acquire antibodies from their mother. The presence of maternal antibodies, while conferring protection, inhibits immunoglobulin synthesis and so prevents the successful vaccination of newborn animals (Fig. 20.8).

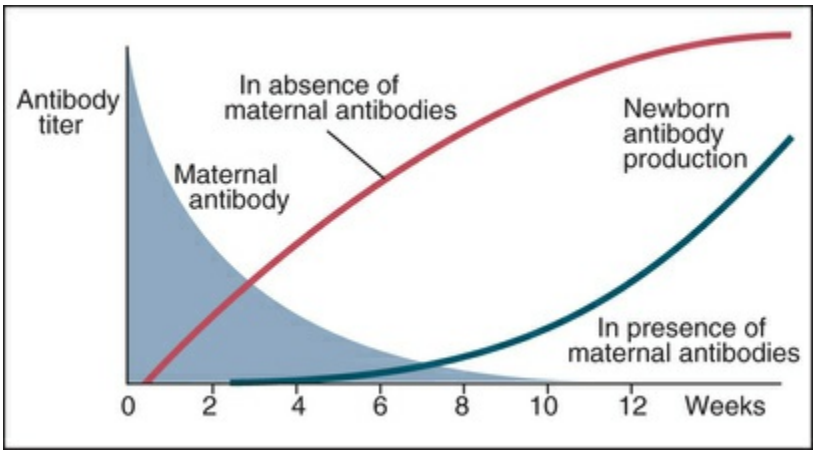


FIG. 20.8 The presence of maternal antibody in a newborn animal effectively delays the onset of immunoglobulin synthesis through a negative feedback process.

Serum IgG levels are also regulated through the neonatal immunoglobulin receptor (FcRn) (see Fig. 23.6). Despite its name, it is present throughout life and is widely distributed on endothelial

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cells, in muscle, vasculature, and hepatic sinusoids. Because it binds IgG and albumin with high affinity, it regulates the serum half-lives of these proteins. When IgG binds to FcRn it is protected from degradation and so has a longer half-life. If FcRn expression remains constant, IgG levels remain stable. If IgG levels rise, the surplus will fail to bind FcRn and be degraded. Conversely, if IgG levels drop, a greater proportion will bind to FcRn and be protected. This receptor also plays a key role in the transfer of maternal immunoglobulins to newborn mammals (Chapter 23).

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Inhibitory Receptors

A key feature of the adaptive immune system is that, while poised to launch a potent array of destructive mechanisms against invaders, the body maintains control of the process. It is critically important to limit and eventually terminate a response by inactivating or eliminating pathways that are no longer required. This regulation involves the extensive use of inhibitory receptors. These suppress the activity of lymphocytes once they have completed their task and so provide a crucial safeguard against inappropriate immune responses. Thus activation and inhibition must be paired to initiate and terminate immune responses. In some cases, activating and inhibitory receptors recognize similar ligands, so the net outcome is a product of the relative strength of these signals. Loss of inhibitory signals is often associated with autoimmunity or hypersensitivity.

An excellent example of an inhibitory receptor is CD32b (FcγRIIb) expressed on B cells. Any antibodies present will occupy these receptors. If these receptor-bound antibodies are cross-linked to a BCR through an antigen, the BCR and CD32 come together (Fig. 20.9). As a result, their signal transduction pathways interact, and BCR signaling is blocked. This prevents B cell activation and triggers its apoptosis. The CD32 pathway is a feedback mechanism whereby B-cell activation is suppressed by antibody and uncontrolled B-cell responses are prevented. Since another receptor, FcγRIII, stimulates B cells, B cell responses can be regulated by altering the ratio of FcγRIIb to FcγRIII. Macrophage activation is regulated in a similar manner, and activated macrophages have a high FcγRIII-to-II ratio.

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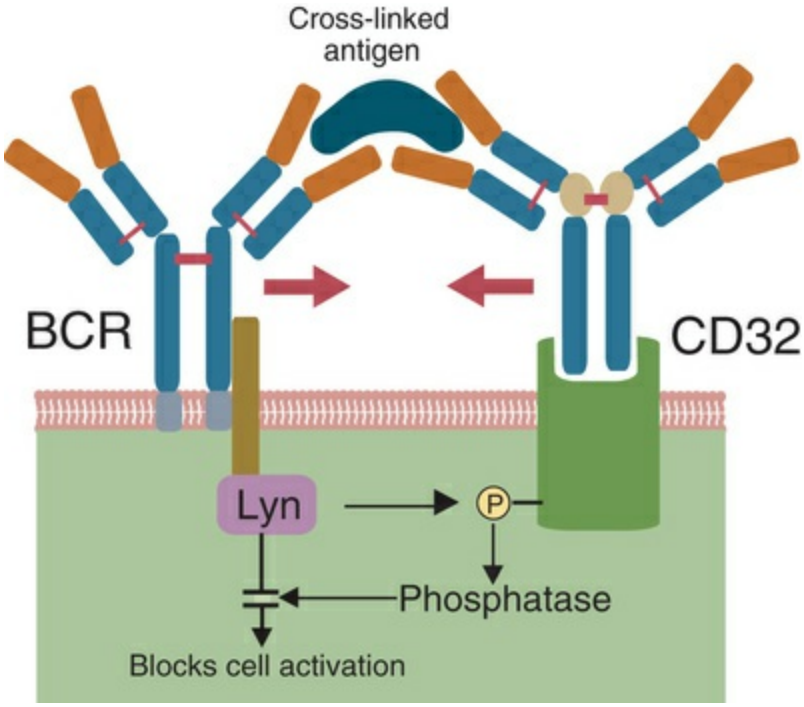


FIG. 20.9 Cross-linkage between a BCR and CD32 (an Fc receptor) by antibody and antigen can turn off a B cell by activating a phosphatase that in turn blocks signaling by tyrosine kinase.

CD28 and CTLA4 on T cells both bind the same ligand (CD80) but deliver antagonistic signals. CD28 is an activator, whereas CTLA4 is an inhibitor. A deficiency of CTLA4 leads to uncontrolled T cell proliferation and autoimmunity.

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Regulatory Cells

Although much immune regulation is "passive" in that self-reactive lymphocytes are eliminated by central tolerance, cells in peripheral tissues also "actively" regulate the immune system. Cells with regulatory functions include T cells, B cells, macrophages, dendritic cells, and natural suppressor cells.

Regulatory T Cells

Regulatory T cells (called Tregs) play a master role in regulating the immune system and maintaining the balance between peripheral tolerance and immunity (Fig. 20.10). In their absence, multiorgan autoimmune disease or uncontrolled inflammation results. Some of these Treg cells develop naturally, whereas others are induced by cytokine exposure.

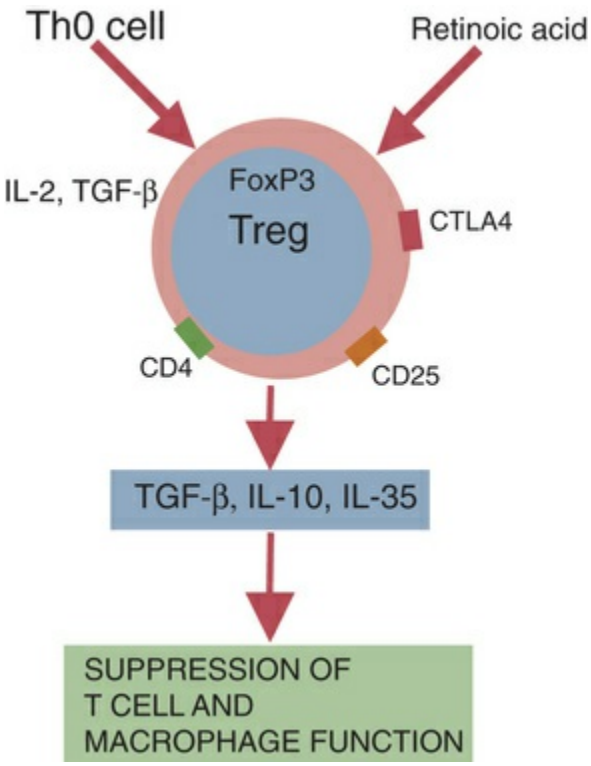


FIG. 20.10 The production and functions of regulatory T cells. They are generated by the combined actions of IL-2 and TGF-β as well as the presence of retinoic acid. They characteristically produce the suppressive cytokines, TGF-β, IL-10, and IL-35.

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Treg cells are typical lymphocytes that express CD4 and CD25 (the α chain of the IL-2 receptor). All activated T cells express CD25, but Treg cells are the only ones that express it when naïve. Their most characteristic feature, however, is their use of a specialized transcription factor called FoxP3. This is yet another example of a situation in which cattle are different from mice and humans. FoxP3$^+$, CD4$^+$, CD25$^+$ cells are found in cattle, but these are not Tregs. The Treg cells in cattle are WC1.1$^+$, WC1.2$^+$, γ/δ $^+$T cells.

Natural Treg cells originate in the thymus (tTregs), whereas peripheral Treg cells (pTregs) are produced in secondary lymphoid organs, especially the intestine. The intestine is a major site of pTreg development, and specialized intestinal dendritic cells promote this through pathways that use a combination of transforming growth factor-β (TGF-β), IL-2, and retinoic acid, a metabolite of vitamin A. The retinoic acid is generated by the bacterial microbiota in the gut and is required for normal T cell function. Intestinal pTreg cells develop from naïve T cells in response to antigen and co-stimulation. These signals induce the transcription of FoxP3. FoxP3 in turn induces transcription of the genes for CTLA-4, TGF-β, and IL-10. pTregs are scattered throughout the body. They account for roughly 5% of circulating T cells and 10% of lymph node T cells in the dog.

Tregs suppress immune responses through multiple pathways (Fig. 20.11). Thus tTreg cells inhibit immune responses through direct cell-to-cell contact. This may be mediated by delivery of suppressive molecules through gap junctions, by binding of membrane-bound suppressive cytokines such as TGF-β, by producing cytotoxic granzymes and perforins, or by CTLA-4 reverse signaling through CD80. CD25 on Treg cells may bind and hence reduce the availability of IL-2. Galectin 1 on Tregs can bind receptors on effector T cells resulting in cell cycle arrest. CTLA4 cells can induce apoptosis of effector T cells by releasing granzymes or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL).

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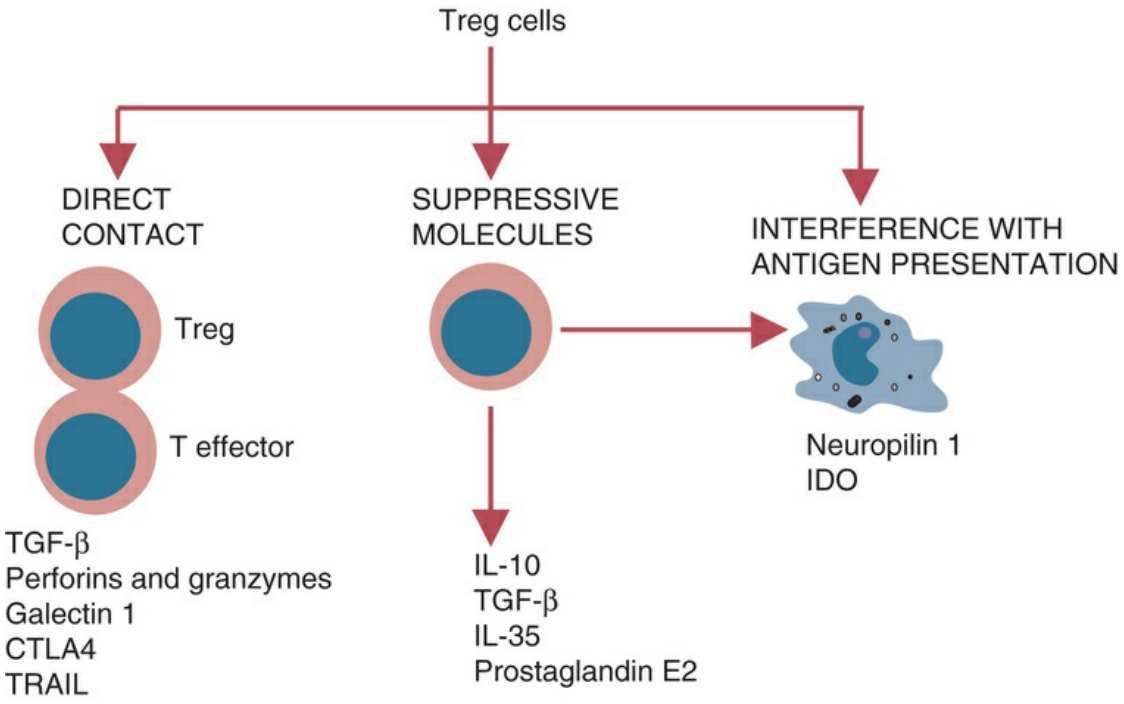


FIG. 20.11 The mechanisms by which Treg cells can suppress other immune responses.

A second set of pathways, inhibition by suppressive cytokines, is used predominantly by pTreg cells. These cytokines include IL-10, TGF-β, and IL-35 as well as competitors for cytokine receptors. IL-10 is the most important of these (Fig. 20.12). As a result of all these mechanisms, Treg cells suppress the response of helper T cells to antigens and prevent inappropriate T cell activation in the absence of an antigen. Treg cells can also suppress CD4 and CD8 T cell responses by pathways independent of IL-10 and TGF-β. For example, they appear to shorten the interaction time between T cells and APCs and thus prevent activation.

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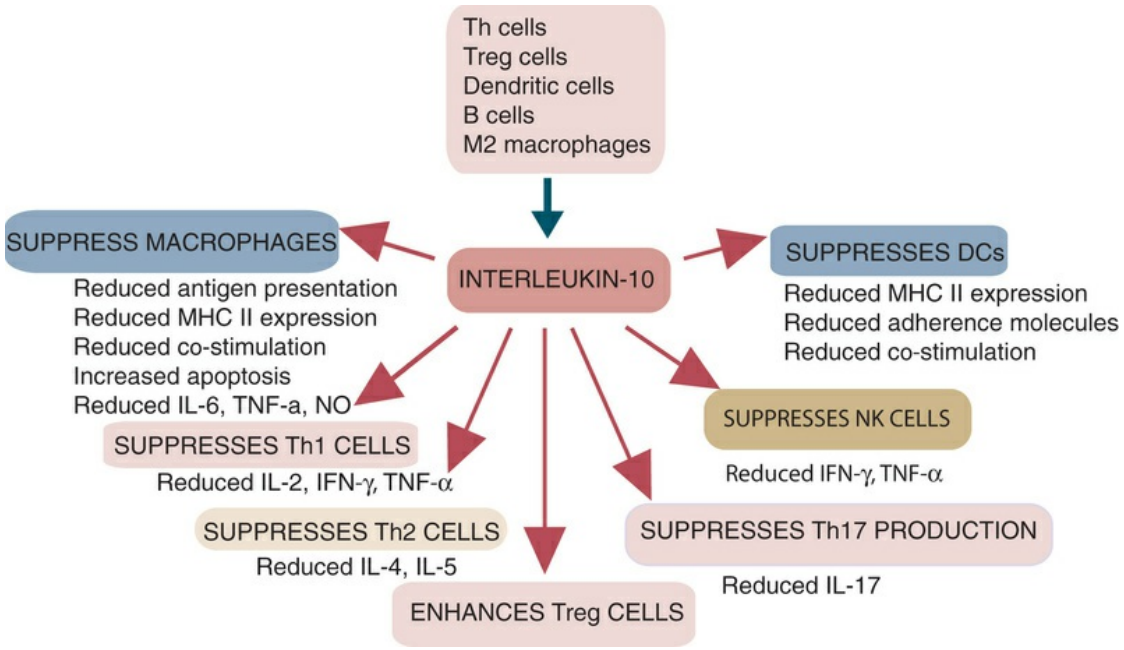


FIG. 20.12 The origins and properties of interleukin-10.

Oral administration of an antigen may induce pTreg cells. Treg cells from the mesenteric lymph nodes of orally tolerant animals secrete TGF-β, IL-4, and IL-10. This may account, in large part, for tolerance to food antigens.

Treg cells are not the only way in which cellular control of immune responses is exercised. Many of the regulatory activities of T cells reflect the antagonistic functions of Th1 and Th2 cells. For example, IFN-γ from Th1 cells can suppress IgE production, whereas IL-10 from Th2 cells is suppressive for dendritic cell IL-12 production and thus for the production of Th1 cytokines.

In cattle, γ/δ T cells are a major regulatory T cell subset in peripheral blood. They spontaneously secrete IL-10 and proliferate in response to IL-10 and TGF-β. They can inhibit both antigen-specific and nonspecific proliferation of $CD4^+$ and $CD8^+$ T cells *in vitro*.

In horses, Tregs are $FoxP3^+$, $CD4^+$, and $CD25^+$. They act in a manner similar to other species by cell-cell contact and by IL-10 and TGF-β production. As in humans the circulating $CD4^+$ $CD25^{hi}$ population contains tTreg cells, while pTreg cells can be induced *in vitro* by appropriate stimulation.

Pigs possess $CD4^+$ $CD25^{hi}$ $FoxP3^+$ Tregs that produce immunosuppressive IL-10. They require IL-2 for activation, but excessive IL-2 may reduce their suppressive activity.

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Interleukin-10

IL-10 is a cytokine that inhibits both innate and adaptive immune responses (see Fig. 20.12). It is produced by macrophages (especially M2 macrophages) and classical dendritic cells in response to microbial products. It is also produced by multiple T cell subsets, including some populations of helper T cells in response to high antigen doses and IL-12. It is produced in especially large amounts by Treg cells in response to TGF-β. Small amounts of IL-10 may also be produced by B cells, mast cells, neutrophils, and natural killer (NK) cells.

IL-10 down-regulates MHC class II and co-stimulatory molecule expression on dendritic cells and macrophages and hence impairs antigen presentation. IL-10 or IL-10-treated dendritic cells can induce a long-lasting, antigen-specific, anergy in T cells. IL-10 inhibits the synthesis of the Th1 cytokines, IL-1, IFN-γ, and TNF-α, and the Th2 cytokines IL-4 and IL-5. Thus it can regulate both Th1 and Th2 responses. IL-10 also inhibits the production of IL-5, CXCL8, IL-12, granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF). It down-regulates the production of IFN-γ and TNF-α by NK cells.

Transforming Growth Factor-β

TGF-β comprises a family of five glycoproteins; three (TGF-β1, TGF-β2, and TGF-β3) are found in mammals, and two others (TGF-β4 and TGF-β5) have been described in chickens and *Xenopus* toads. They are secreted as inactive precursors that are activated on the cell surface by proteases after binding to integrins. TGFs are produced by platelets, activated macrophages, neutrophils, B cells, and T cells and act on T and B cells, dendritic cells, macrophages, neutrophils, and fibroblasts (Fig. 20.13).

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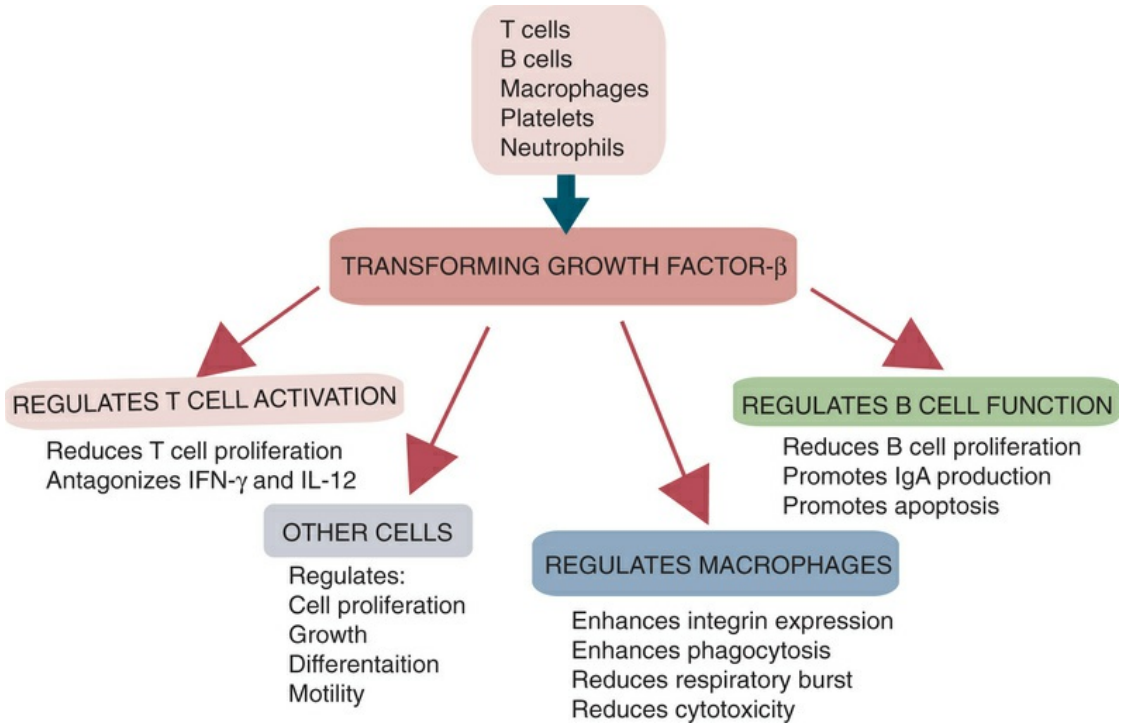


FIG. 20.13 The origins and properties of transforming growth factor-β.

TGF-β regulates macrophage activities. It may be either inhibitory or stimulatory, depending on the presence of other cytokines. Thus it can enhance integrin expression as well as phagocytosis by blood monocytes. On the other hand, it suppresses the respiratory burst and NO production and blocks monocyte differentiation and the cytotoxic effects of activated macrophages. TGF-β is required for optimal dendritic cell development and regulates the interaction between follicular dendritic cells and B cells. TGF-β inhibits T and B cell proliferation and stimulates their apoptosis. Apoptotic T cells release TGF-β, contributing to the suppressive environment. TGF-β influences the differentiation of Th subsets. It tends to promote Th1 responses and the production of IL-2 in naïve T cells, but it also antagonizes the effects of IFN-γ and IL-12 in memory cells. It also controls the development and differentiation of B cells, inhibiting their proliferation, inducing apoptosis, and regulating IgA production.

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Regulation by the Innate Immune System

The triggering of innate immune responses through pattern-recognition receptors (PRRs) activates many pathways that affect the adaptive immune responses. For example, signaling by most PRRs activates the transcription factors, NF-κB and NF-AT. These can then activate T and B cells. Stimulation of TLR4 can also induce Th1, Th2, and Th17 responses. The cytosolic PRRs such as the RLRs and NLRs can also activate Th1 responses and trigger $CD8^+$ cytotoxicity (Chapter 18). Thus if a virus infects dendritic cells and is recognized by cytosolic PRRs, not only will it trigger a type I interferon response but it may also trigger the production of cytokines and other co-stimulators needed to activate T cells.

Th17 Cells

Naïve $CD4^+$ T cells differentiate into Th17 cells when exposed to IL-23 supported by IL-21, IL-6 and TGF-β. Th17 cells play an important role in host defense and are potent inducers of acute inflammation (Fig. 20.14). Th17 cells are conventional small lymphocytes that are abundant in mucosal surfaces. Their presence in these surface tissues is regulated by the gut microbiota.

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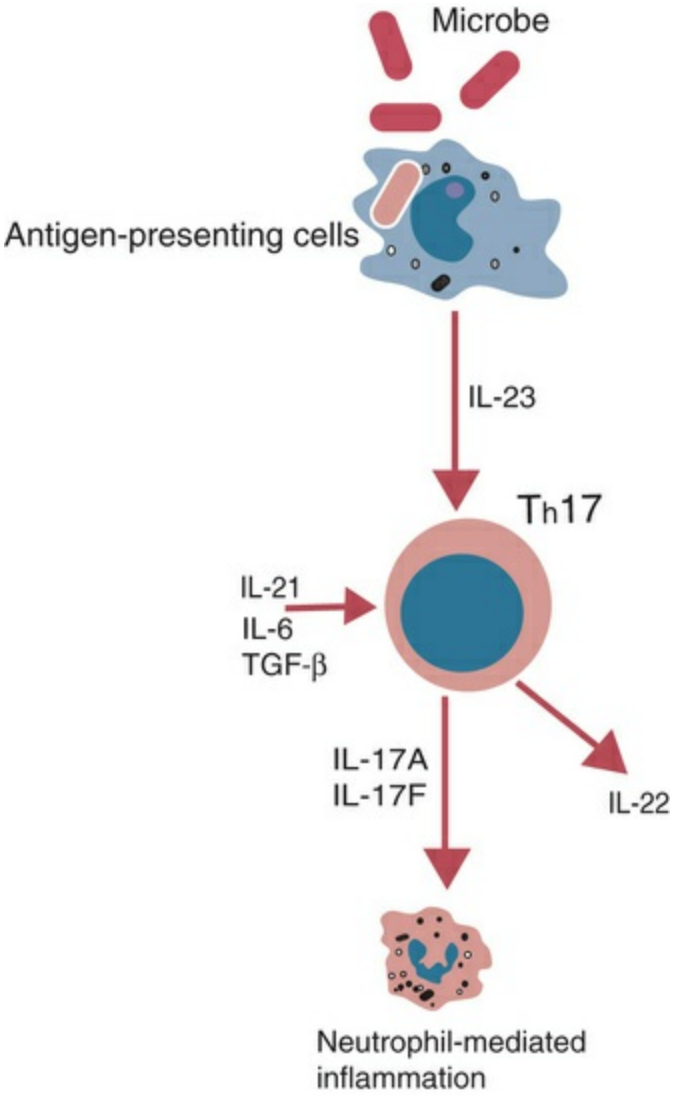


FIG. 20.14 The production of Th17 cells. The original differentiation signal comes from IL-23. This signal is amplified by IL-21, IL-6 and TGF-β .

Dendritic cells and macrophages activated by microbial PAMPs through TLR2 secrete IL-23 (Box 20.1). IL-23 promotes the activation of Th17 cells. Th17 cells secrete IL-17A, IL-17F, IL-21, and IL-22 (Fig. 20.15). IFN-γ suppresses the development of Th17 cells and inhibits IL-17-mediated inflammation.

Box 20.1

Interleukin-23

IL-23 is closely related to IL-12. Both are heterodimers consisting of an identical p40 subunit and a second smaller subunit, IL-23p19 or

IL-12p35, respectively. Both cytokines are produced by macrophages and dendritic cells in response to LPS, and both enhance T cell proliferation and the production of IFN-γ. Whereas IL-12 drives a pathway leading to Th1 cell production, IL-23 drives a pathway leading to the stabilization of IL-17-producing $CD4^+$ T cells. IL-23 recruits diverse inflammatory cells in addition to Th17 cells and is thus involved in the pathogenesis of several immune-mediated diseases. The production of IL-23 is increased in some tumors, where it promotes inflammation and enhances angiogenesis (Chapter 35).

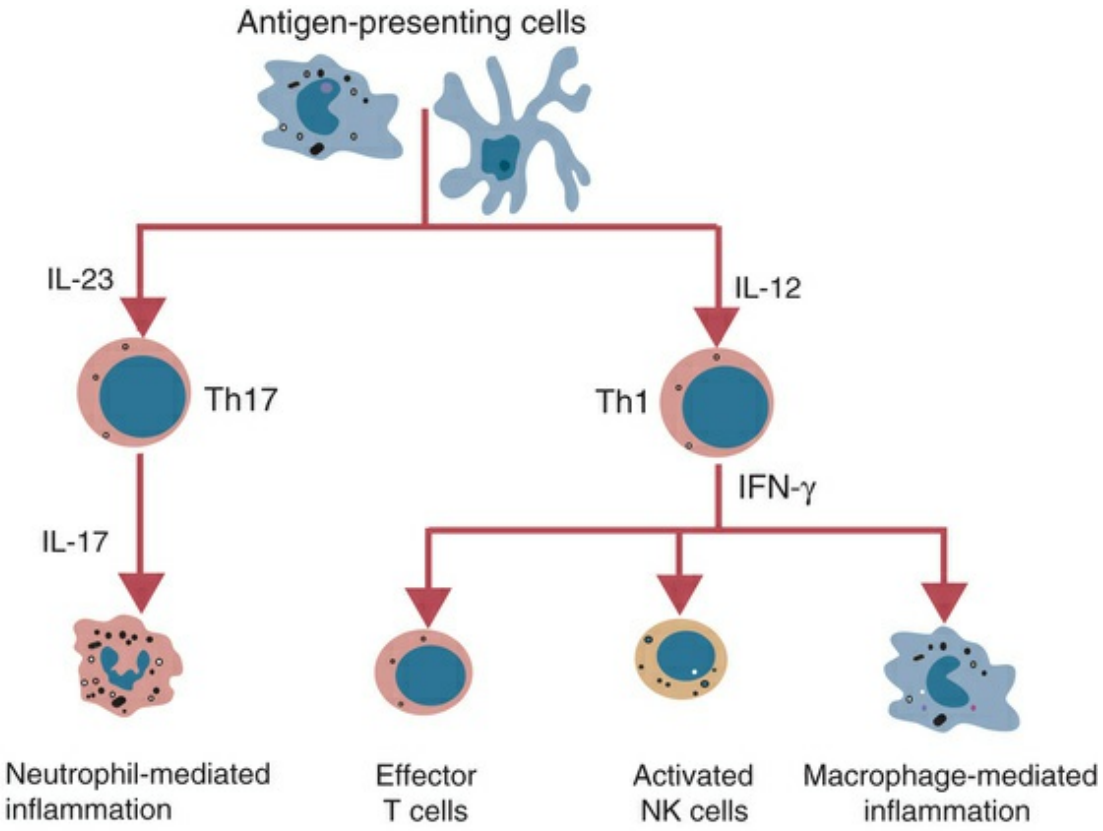


FIG. 20.15 The generation of Th1 and Th17 cells depends upon the production of IL-12 or IL-23 by antigen-presenting cells. Th1 cells promote cell-mediated responses. Th17 cells promote innate responses.

Interleukin-17

Six cytokines belong to the IL-17 family (IL-17A through IL-17F), but the two most important members are IL-17A and IL-17F. IL-17A

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is involved in the development of autoimmunity, inflammation, and some tumors. IL-17F is mainly involved in mucosal defense. IL-17E (also known as IL-25) promotes Th2 responses. The functions of the others are unclear. IL-17A is a homodimer of 35 kDa produced by Th17 cells. Endothelial cells and macrophages are the main targets of these cytokines since IL-17 plus TNF-α can stimulate them to produce proinflammatory molecules such as chemokines, G-CSF, GM-CSF, IL-1, and IL-6, inflammatory mediators such as acute-phase and complement proteins, and antibacterial defensins (Fig. 20.16). IL-17 is especially potent in triggering inflammation in autoimmune diseases such as rheumatoid arthritis.

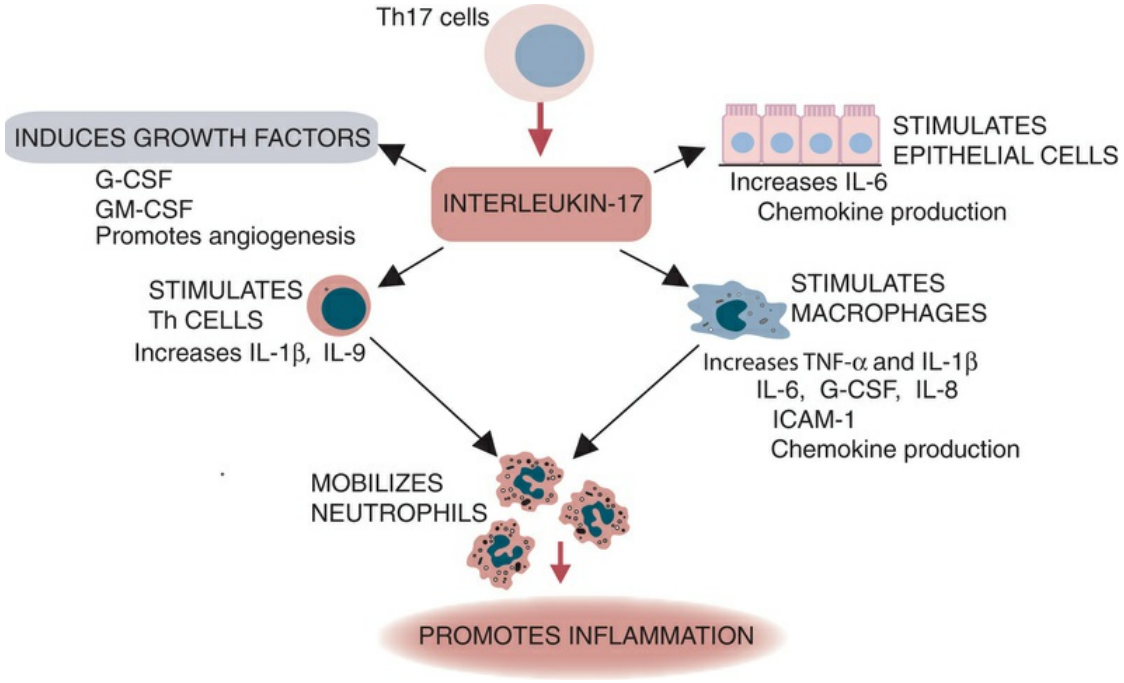


FIG. 20.16 The origins and properties of interleukin-17.

IL-17 regulates neutrophil accumulation in acute inflammation and is crucial for coordinating host defenses against many bacteria and fungi. For example, Th17 cells play a key role in defense against pathogens such as *Klebsiella pneumoniae, Citrobacter rodentium, Salmonella enterica, Mycobacterium tuberculosis,* and *Candida albicans.* It is believed that they do so by triggering inflammation and recruitment of inflammatory cells leading to rapid pathogen eradication.

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Regulatory Macrophages

Cytokines cause macrophage polarization. Thus exposure to IFN-γ generates classically activated M1 cells. Exposure of macrophages to IL-4 or IL-13, in contrast, results in the development of M2 cells (Chapter 18). M2 cells participate in tolerance induction, suppress inflammation, and participate in tissue repair. M2 cells increase expression of the macrophage mannose receptor, the β-glucan receptor, and CD163; enhance endocytosis and antigen processing; and increase MHC class II expression. M2 macrophages produce large amounts of the Th2 and regulatory cytokines such as IL-4, IL-13, IL-10, TGF-β, and IL-1RA. They are not killer cells because they produce arginase, which generates ornithine rather than nitric oxide (see Fig. 6.7).

In healthy animals, M2 polarized cells may be found in the placenta and lung, where they inhibit dendritic cell antigen presentation and mitogen responses in lymphocytes. M2 macrophages are responsible for control of granuloma formation as well as for skin immune tolerance induced by ultraviolet B radiation. They can also be found in healing tissues, where they are associated with angiogenesis.

Indoleamine 2,3-Dioxygenase and Tolerance

Many regulatory cells, including Tregs, dendritic cells, some macrophages, fibroblasts, trophoblast giant cells, endothelial cells, and some tumor cell lines, produce indoleamine 2,3-dioxygenase (IDO). This enzyme catalyzes the oxidative degradation of the amino acid tryptophan, resulting in its local depletion. Tryptophan is required for the mTOR pathway in T cells so that they undergo cell cycle arrest and apoptosis when deprived of tryptophan. IDO therefore inhibits T cell activation, proliferation, and survival and promotes peripheral tolerance. Th1 cells appear to be more sensitive to tryptophan depletion than are Th2 cells. Treg cells may also induce IDO expression in some dendritic cells. IDO activity has also been documented in T cell tolerance to tumors, as a negative regulator in autoimmune diseases, and in some allergies. IDO plays a key role in preventing immunological rejection of the fetus and of liver and corneal allografts (Chapter 34). IDO may also act as a

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defensive enzyme since by removing tryptophan it prevents the growth of *Toxoplasma gondii, Chlamydia pneumoniae,* streptococci, and mycobacteria.

Tolerogenic Dendritic Cells

The function of dendritic cells is to capture and process foreign antigens for presentation to T cells. However, the precise signals generated by dendritic cells depend on their state of maturity, on their co-stimulating molecules, and on the presence or absence of inflammatory cytokines. Thus proteins from dead and dying cells that are captured by immature dendritic cells in the absence of inflammation may cause the dendritic cells to kill responding T cells or cause the T cells to differentiate into Treg cells. Treatment of dendritic cells with IL-10 can block their ability to activate Th1 cells while preserving their ability to promote Th2 responses.

Regulatory B Cells

A subpopulation of B cells may secrete IL-10, IL-35 and TGF-β. As a result, they suppress the functions of Th17, Th1, and effector T cells and induce the differentiation of Treg cells.

Natural Suppressor Cells

Natural suppressor (NS) cells are large granular innate lymphocytes that produce cytokines with Treg-inducing activity. They suppress B and T cell proliferation as well as immunoglobulin production. NS cells occur normally in the adult bone marrow and neonatal spleen and possibly regulate innate immune responses.

When Do Regulatory Cells Work?

Regulatory cells control almost all aspects of immunity. Treg cells, for example, work constantly throughout an animal's life to prevent self-reactivity. They are responsible for lack of immune responses in the newborn; immunosuppression following trauma, burns, or surgery; prevention of autoimmunity; and some cases of hypogammaglobulinemia. Regulatory cells are found in some

tumor-bearing animals, where they may block tumor rejection, and in pregnant animals, where they block rejection of the fetus.

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Regulation of Apoptosis

The thymus of the mouse releases about 1 million new T cells into the circulation every day. (Presumably a cow would produce many more.) To keep the number of mouse lymphocytes relatively constant, a million must also die. Likewise, the mouse bone marrow releases about 10^7 B cells daily, and a similar number must die. In addition, lymphocytes divide in response to antigens. All this proliferation must be balanced by the removal of cells by apoptosis. Apoptosis also removes autoreactive lymphocytes and limits the clonal expansion of lymphocytes during an immune response. This homeostatic system is carefully regulated because if it fails, excess lymphocytes may cause lymphoid tumors or autoimmunity. The regulatory process depends on providing cells with survival signals. If these are inadequate, cells will die. These regulatory signals are provided by IL-2, IL-4, IL-9, and IL-21.

Apoptosis is mediated by intracellular caspases. Caspases are expressed as inactive precursors in lymphocytes. Proteins of the bcl-2 family modulate their activity. Thus, in a quiescent cell, survival depends on the ongoing presence of bcl-2. Cytokines also regulate apoptosis. Survival is signaled through IL-2, IL-4, IL-7, and IL-15, whereas cell death is signaled through CD95 and TGF-β.

Activated lymphocytes become more susceptible to killing through TNF and CD95. Thus, activation of T cells enhances expression of CD95L. However, the CD95 pathway is normally blocked by stimulatory signals such as those transmitted through CD28 on T cells and CD40 on B cells. Once these co-stimulatory signals are lost, activated cells will undergo CD95-induced apoptosis. This is why unwanted lymphocytes are eliminated at the end of the immune response.

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Neural Regulation of Immunity

The nervous and immune systems communicate by multiple pathways. They communicate through parasympathetic and sympathetic nerves and by soluble neurotransmitters. Neuroendocrine hormones such as corticotrophin-releasing factor and α-melanocyte-stimulating hormone, as well as some neurotransmitters, act on cells of the immune system to regulate cytokine balances. Conversely, cytokines and chemokines modulate nervous system activities such as appetite, body temperature, and sleep behaviors. There are transport systems that carry cytokines across the blood-brain barrier. In addition, inflammation in tissues can lead to activation of sensory nerves that then relay messages to the brain. Many of the cytokines produced in the brain are produced by brain and meningeal macrophages—microglia. For example, IL-1 is produced in the brain in response to endotoxin.

Memory T cells may secrete acetylcholine in response to vagal nerve stimulation. Electrical stimulation of the vagus inhibits cytokine release and reduces inflammation-mediated injury in endotoxemia and sepsis. This is called the inflammatory reflex and requires acetylcholine production.

Stress

Stress influences immunity (Fig. 20.17). Small bouts of stress are believed to enhance immune responses, but prolonged stress is detrimental. One obvious example is shipping fever. This is a complex pneumonia of cattle caused by several viral respiratory pathogens with secondary infection by *Mannheimia hemolytica.* It develops in cattle that have been transported in confined spaces for long distances (and hence, many hours) with minimal feed and water and usually after rapid weaning and castration. The stress involved in the shipping process is sufficient to make these cattle highly susceptible to pneumonia. Stress depresses T cell responses, NK cell activity, IL-2 production, and expression of IL-2R on lymphocytes. Reduction in stress can have a reverse effect. In studies investigating innate responses among calm and

temperamental Brahman bulls, it was found that calm bulls had elevated neutrophil L-selectin expression and increased neutrophil phagocytic and oxidative burst activity when compared to temperamental bulls, 48 hours posttransportation. By 96 hours, neutrophil phagocytosis, oxidative burst, and cell adhesion molecule expression were enhanced in all bulls, but the effects were most pronounced in calm bulls.

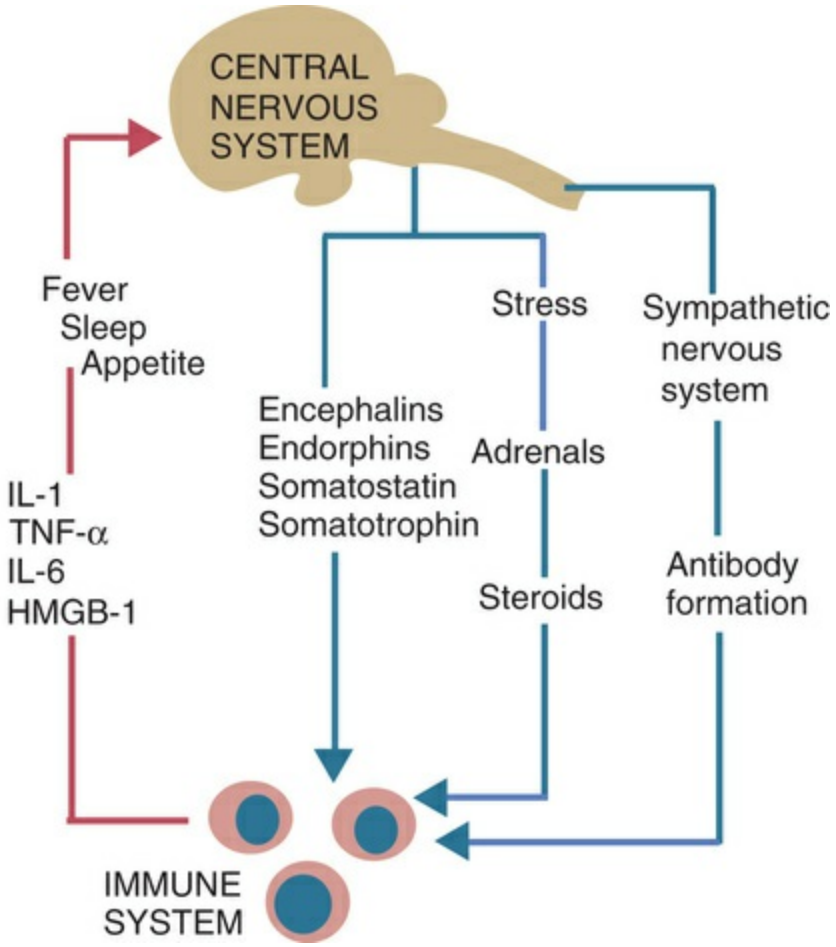


FIG. 20.17 Some of the ways in which the central nervous system and the immune system interact.

Stress can be due to something as simple as early weaning, which reduces IL-2 production in piglets. Stress in pregnant sows results in immunosuppression of their offspring. Thus confinement stress late in pregnancy results in the birth of piglets with T and B cells that have a reduced ability to respond to mitogens. Both morbidity and mortality are increased in piglets from stressed sows. A different form of stress may result from mammalian social

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structures. A dominance hierarchy regulates many mammalian populations. Depending on the way the hierarchy is established, some members may be very highly stressed. Animals of high rank will be stressed if it requires constant fighting to maintain dominance. This occurs, for example, in wild dogs, lemurs, and mongooses. In hierarchies in which dominant members intimidate through psychological intimidation, such as in mice, rats, and many monkeys, low ranking individuals may be stressed and immunosuppressed. If new individuals are introduced into a group, or a dominant animal loses its position, stresses occur as a result of the reorganization. In pigs, it has been shown that there is a relationship between social status and disease susceptibility. Thus morbidity and mortality among pigs challenged with pseudorabies virus were highest among subordinate animals. Dominant pigs had lymphocytes that were more responsive to virus antigens. This of course makes sense from an evolutionary point of view in that the least reproductively fit animals were more likely to die of disease, but it is difficult to separate cause and effect in this phenomenon. Were subordinate animals immunosuppressed because they were under stress as a result of their lowly status? Alternatively, could it be that those animals with a highly effective immune system were healthier and thus better able to reach high social status within the population? Certainly high levels of social stress are found in confined, crowded animal populations. Social subordination in rhesus monkeys results in changes in gene expression that are biased toward inflammation including genes associated with lymphocyte proliferation, innate immunity, and cytokine responsiveness. Thus in stressed subordinate monkeys, TLR4 signaling predominantly used the MyD88- NF-κB pathway, while in dominant monkeys they used the TRIF-IFN pathway (Chapter 2).

When the behavior of pigs is examined, they can be divided into two groups: aggressive animals that tend to fight other animals and then may flee rapidly, and passive animals that tend to cope with stress by withdrawing gradually from stressful situations. Aggressive pigs had higher *in vitro* and *in vivo* cell-mediated immune responses but lower humoral responses than passive animals. This suggests that there were differences in their relative Th1 and Th2 responses. However, when these animals were

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stressed, the aggressive ones showed a much greater drop in these responses than the passive animals. Differences in the way animals cope with stress are thus reflected in differences in immune reactivity.

The stress effect is mediated by two major pathways. One involves the autonomic nervous system producing its neurotransmitters, epinephrine, norepinephrine, and acetylcholine, and the other is the hypothalamic-pituitary-adrenal cortical axis producing glucocorticoids. Stress signals the brain to activate these pathways.

Autonomic Nervous System

Almost all primary and secondary lymphoid organs are supplied by nerves through the autonomic nervous system, and many cells of the immune system express receptors for the neurotransmitters released by both arms of the autonomic nervous system. This may occur either through adrenergic signals from the sympathetic nervous system or cholinergic signals from the parasympathetic nervous system.

The sympathetic nerves act through the neurotransmitter norepinephrine. They innervate the thymus, the splenic white pulp, and the lymph nodes. They influence blood flow, vascular permeability, and lymphocyte migration and differentiation. Surgical or chemical sympathectomy of the spleen enhances antibody production and can induce changes in the distribution of lymphocyte subpopulations. NK cell activity appears to be modulated directly by the hypothalamus through the splenic nerve. Autonomic nerves innervate Langerhans cells in the skin. By releasing neuropeptides, these nerves can depress the antigen-presenting ability of these cells. This might explain why "hot spots" in dogs worsen with anxiety. Denervated skin shows reduced inflammation after tissue damage and heals more slowly. Most important, sympathetic nerves innervate the adrenal medulla.

Immune cells have a complete set of cholinergic receptors. Efferent activity in the vagus nerve activates a cholinergic antiinflammatory pathway. Vagal stimulation suppresses the systemic response to endotoxin by downregulating hepatic TNF-α

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synthesis. Activation of acetylcholine receptors on macrophages inhibits production of IL-1 and TNF-α.

The production of antimicrobial proteins such as the defensins is regulated by the autonomic nervous system, and stress reduces cutaneous epithelial antimicrobial activity. This appears be a result of increased glucocorticoid and acetylcholine production.

Both adrenergic and cholinergic stimulation increase norepinephrine signaling, leading to NF-κB activation in mononuclear cells. The sympathetic nervous system can alter the Th1/Th2 balance through the β-adrenergic receptor. Stimulation of sympathetic nerves enhances production of Th2 cytokines while inhibiting production of Th1 cytokines. Norepinephrine suppresses production of IL-6 and TNF-α.

Hypothalamic-Pituitary-Adrenal Cortical Axis

The adrenal cortex is stimulated by adrenocorticotropic hormone (ACTH) from the pituitary under the influence of corticotrophin-releasing hormone from the hypothalamus. As a result, glucocorticoids are secreted and suppress T cell function by blocking the NF-κB pathway. IL-1 and IL-6 act on both the hypothalamus and the pituitary to increase ACTH production and subsequent cortisol release.

Neuropeptides and Lymphocytes

Cells of the immune system have receptors for neuropeptides such as the enkephalins and endorphins that thus can influence lymphocyte activity. The generation of cytotoxic T cells is enhanced by metenkephalin and β-endorphin, whereas α-endorphin suppresses antibody formation and β-endorphin reverses this suppressive effect. Other neuropeptides that influence the immune system include ACTH, oxytocin, vasoactive intestinal peptide, somatostatin, prolactin, and substance P.

Many neuropeptides such as vasoactive intestinal peptide and neurokinin-1 (NK-1) have a similar structure to the antimicrobial peptides so that they also have antimicrobial properties and may be involved in host defense. For example NK-1, also known as

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substance P, not only mediates pain and inflammation but also has significant antibacterial activity. Other neuropeptides have similar effects. As a result, appropriate nervous stimulation can promote neuropeptide release and enhance local antibacterial activity. The pain associated with acute inflammation may well reflect this local resistance to infection. Some neuropeptides can promote Th17 activity by triggering monocyte production of IL-23.

Immune responses are also modulated by environmental factors. Changes in day length (photoperiod) influences immune responses. These effects can be complex, but in general reduced day length appears to promote immune reactivity. The effect appears to be mediated through the hormone melatonin. Circadian rhythms also regulate some immune functions such as the activities of hematopoietic stem cells and lymphocyte recruitment from the blood. These appear to be a result of oscillations in chemokine levels and the expression of adhesion molecules on vascular endothelial cells.

Finally, the innate immune system can influence nervous function. For example, cytokines such as IL-1, IL-6, and TNF-α as well as other inflammatory mediators induce "sickness behavior," including fever, fatigue, depressed activity, and excessive sleep. All these are closely associated with the systemic response to infectious agents and chronic inflammation (Chapter 7).

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21

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The Microbiota and the Immune System

CHAPTER OUTLINE

The Locations of the Microbiota, 222
- The Skin, 222
- The Respiratory Tract, 223
- The Genitourinary System, 224
- The Gastrointestinal Tract, 224
  - *The Rumen, 224*
  - *The Cecum and Large Intestine, 225*

The Functions of the Microbiota, 225
- Nutritional Efficiency, 225
- Intestinal Protection, 225
- Development of Lymphoid Organs, 226
- Microbiota Signals to the Body, 227

Immune Responses to the Microbiota, 228
- Enterocytes, 228
- ILC3 Cells, 229
- B Cell Functions, 229
- T Cell Functions, 229

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Dysbiosis, 230
Odors, 232
The Virome, 232
Inflammatory Bowel Diseases, 232

LEARNING OBJECTIVES

After you read this chapter, you should be able to:

- Explain how the presence of a dense microbial population on surfaces ensures that the immune systems operate optimally.
- Understand how the skin microbiota plays a role in regulating skin defenses.
- Explain how the microbiota in the respiratory system influences its defenses as well as the development of allergies.
- Recognize the importance of the rumen as a source of microbial metabolites.
- Explain how the intestinal microbiota influences the development of lymphoid organs and the immune system.
- Understand the role of ILC3 cells in regulating immune responses to the microbiota.
- Describe why the microbiota does not trigger an excessive inflammatory response.
- Explain how the responses of proinflammatory Th17 cells are counterbalanced by antiinflammatory Treg cells.
- Explain the adverse effects of dysbiosis on the immune system.
- Explain how the microbiota influences inflammatory and autoimmune disease development.
- Describe the hygiene hypothesis and explain its significance.
- Understand the role of the microbiota in the development of inflammatory bowel disease.

As animals evolved over millions of years they were obliged to develop a relationship with the microbes in their environment. As a result, the immune systems depend upon the microbiota for their

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development and function while they regulate the composition and behavior of the microbes. The surfaces of the animal body consist of many stable, nutrient-rich ecosystems where microbes thrive. As a result, each surface is populated by enormous numbers of bacteria, archaea, fungi, and viruses collectively termed the microbiota. The bacteria are the best studied of these. Thus bacteria live on the skin, in the respiratory tract, in parts of the genitourinary tract, and sometimes within the body but mainly within the gastrointestinal tract. It has been estimated that in an animal body, at least half of all the cells are microbial. As a result of their life-long, intimate association with body surfaces, the microbiota can be considered to be an integral part of the body—a "virtual organ." As such, they influence both innate and adaptive immunity and conversely, they are influenced by signals generated by its host. This has given rise to the concept that animals and their microbiota together form "superorganisms" that share nutrition and exchange energy and metabolites and whose complex interactions are regulated in large part by immune mechanisms.

By harnessing the immensely diverse genomes present in the microbiota, animals enhance their metabolic potential and obtain new ways to utilize food. (Mammals possess about 20,000 protein-encoding genes, while our microbiota may collectively possess about 10 million.) Thus they increase an animal's ability to extract energy from plant structural carbohydrates and to obtain essential vitamins. Because of the microbiota, animals can utilize food sources that would otherwise be unavailable. Microbial metabolism permits animals to adapt to otherwise noncompetitive lifestyles. For example, mice with a conventional microflora need to eat 30% fewer calories than germ-free mice to maintain their body weight. This is due to the ability of the microbiota to extract more energy from food.

Domestic mammals do, however, have some additional complexities associated with their diet and lifestyle. The large domestic herbivores contain massive amounts of microbial material in their rumen and large intestine. These reflect a major role of the microbiota—providing nutrition by extracting energy from complex, plant-derived polysaccharides such as the celluloses (Fig. 21.1). Carbohydrate digestion is the primary function of the

gastrointestinal microbiota. Many members of the microbiota are optimized to ferment oligosaccharides and thus generate energy-rich short-chain fatty acids (SCFAs). The fatty acids in turn can be used as energy sources by other, more specialized bacteria. It is estimated that in an omnivore such as a human, as much as 10% of daily energy needs are provided by colonic fermentation. This figure is much higher for herbivores such as ruminants and horses.

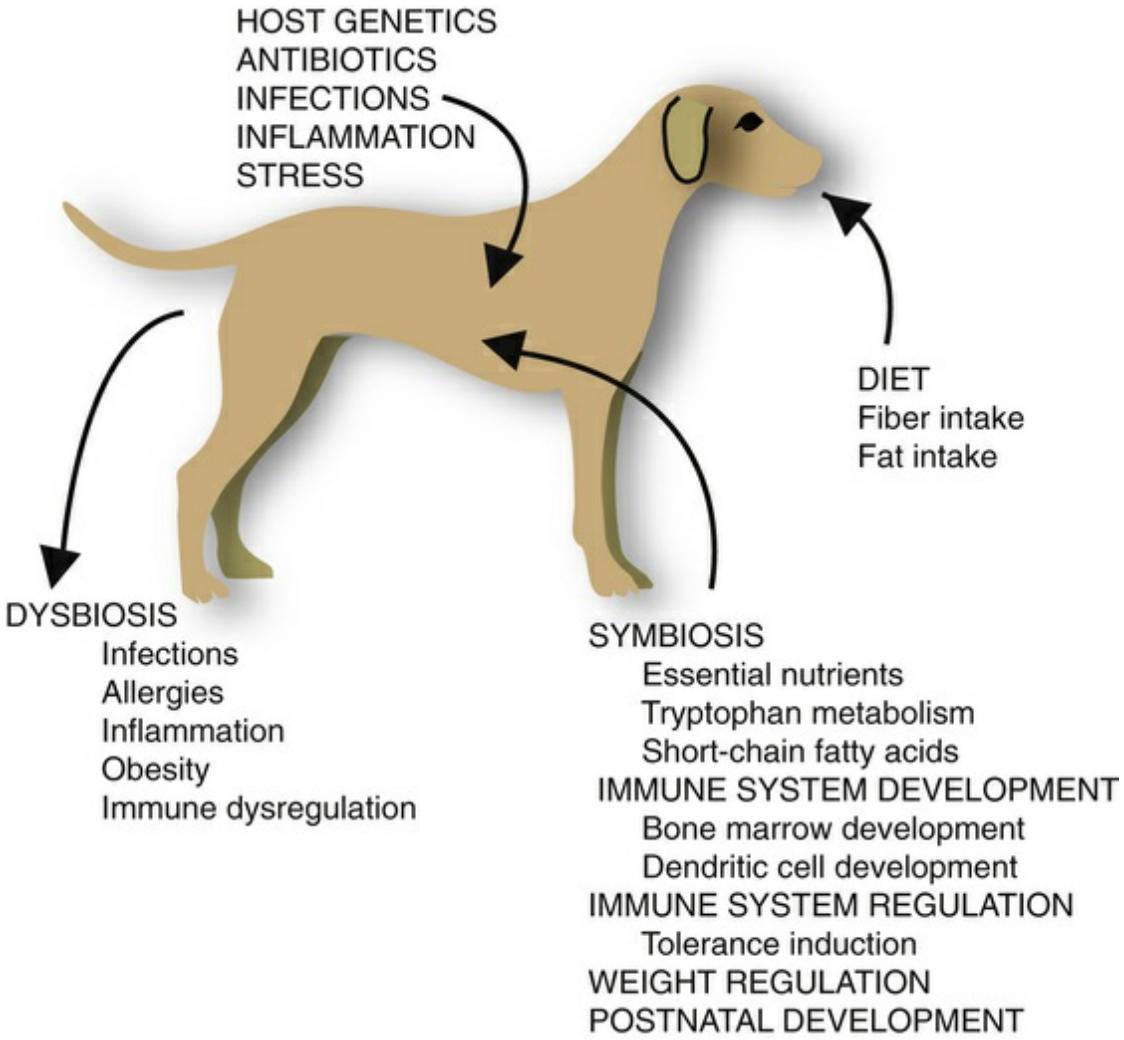


FIG. 21.1 The importance of the microbiota to the proper functioning of the animal body, some of the factors that modify its composition, and the consequences of dysregulation.

It is now well accepted that the intestinal microbiota both contribute to local host defenses and also have effects on the immune system and metabolism that extend throughout the body. Nutrients and microbial metabolites are continually released into

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the body where they influence immune cell and inflammatory functions.

The immunological defenses on body surfaces are therefore faced with the difficult task of co-existing with the microbiota while at the same time controlling any inadvertent invasion through breaks in the epithelial barriers.

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The Locations of the Microbiota

The Skin

Normal skin harbors trillions of microorganisms. These are found on the keratinocyte surface and extend into sebaceous glands and hair follicles. The skin is not, however, a hospitable surface. The outer cell layers are constantly shed and replaced by new cells from below. It is cool in some areas and hot in others. Haired skin may be very different from mucocutaneous junctions. Some areas of skin may be very dry, with a high salt content, hydrophobic, and acidic, as well as being nutrient poor. Other areas may be moist but bathed in a complex mixture of proteases, lysozyme, and antimicrobial peptides such as β-defensins and cathelicidins. Nevertheless, by using modern molecular techniques, it has been estimated that up to a billion bacteria may live on a square centimeter of human skin. Given the sheltering effects of hairs or feathers, it is likely that the skin microbiota in domestic animals may be even more complex. For example, the skin microbiota of dogs varies greatly between different dogs and different skin sites. For example, there is higher microbial diversity in haired skin compared to mucocutaneous junctions. The highest microbial diversity was found in the axilla and the dorsum of the nose. On average, about 300 different bacterial species were identified on the dorsal canine nose.

The skin microbiota can be divided into a resident population that is relatively stable and consistent—a true commensal population, and a changing population of transient bacteria that may only persist on the skin for hours or days. Both populations can contain a mixture of commensals and potential pathogens, yet invasion and disease are relatively uncommon. Large populations of Proteobacteria and Oxalobacteriaceae predominate (Fig. 21.2). The precise composition of the skin microbiota thus depends on location (hairy, wooly or bald skin; back versus skin in the axilla or groin or ear) and the presence of disease such as seborrhea or atopic dermatitis. There is also great variation between individuals. Grooming activities will have some impact on these microbial populations but their significance is unclear.

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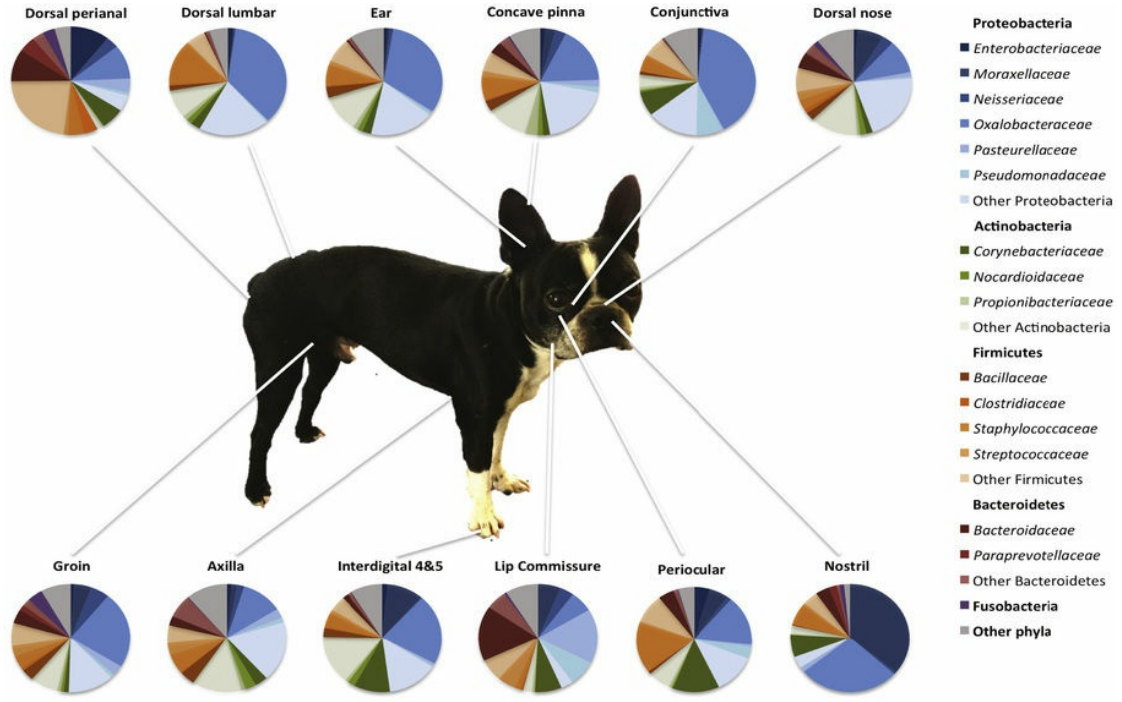


FIG. 21.2 The composition of the microbiota on canine skin. (From Hoffmann AR, Patterson AP, Diesel A, et al: The skin microbiome in healthy and allergic dogs. *PLoS One* 9(1):e83197, 2014.)

In mice, it has been shown that the skin microbiota influences local inflammatory and T cell responses (Fig. 21.3). Epidermal Th17 and $CD8^+$ T cells are especially affected. The microbiota control the balance between effector and regulatory T cells within skin tissue. They influence keratinocyte production of IL-1 and its effects on epidermal dendritic cells and thus control local T cell responses. Skin bacteria can activate antigen-specific T cells across the intact epithelium. However, the presence of Treg cells in neonatal skin mediates tolerance to skin commensal bacteria at a time when the skin is establishing its microbiota. Wound healing in germ-free mice is significantly accelerated and scarring is much reduced. Skin bacteria certainly contribute to the failure of some wounds to heal promptly.

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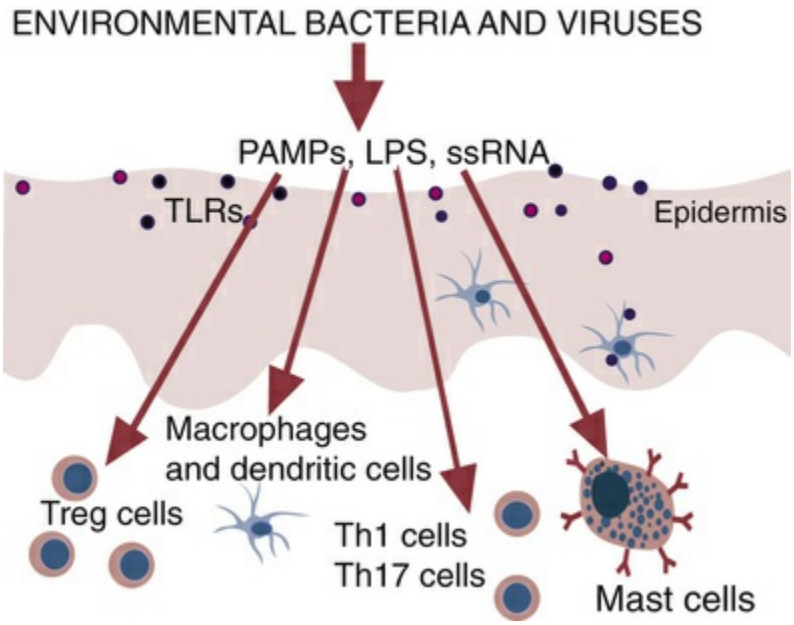


FIG. 21.3 The role of the skin microbiota in regulating immunity. Environmental bacteria trigger innate responses with PAMPs such as bacterial lipopolysaccharides (LPS) and single-stranded RNA (ssRNA) acting through toll-like receptors (TLRs).

The Respiratory Tract

Like all body surfaces exposed to the external environment, the upper respiratory tract houses a dense and complex microbiota. It has been calculated that a human inhales 10^5 organisms/day just breathing normally. Many nasal bacteria are also found on the skin while others are common environmental bacteria. Deeper in the airways, in the lower respiratory tract, many diverse bacteria are also present. Neisseria and Gram-negative cocci are common.

Contrary to previous belief, the lung is not sterile. Healthy lungs harbor a complex microbiota, closely related to but much less dense than that found in the upper respiratory tract. The bronchi contain about 2000 bacterial genomes per cm^2. Lung tissues contain between 10 and 100 bacterial cells per 1000 lung cells. These include both aerobes and anaerobes, and like other surfaces, the populations differ greatly between individuals. The predominant phyla are Firmicutes with lesser numbers of Proteobacteria and Actinobacteria. The organisms generally live within the mucus layer and include bacteria, fungi (yeasts), and viruses including bacteriophages. Pathobionts are also present and may induce disease in immunodeficient individuals. Perhaps the best example

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of these are the fungi of the genus *Pneumocystis* (Chapter 39). As might be expected, the microbiota of individuals with chronic respiratory disease differs from healthy individuals. The airway microbiota plays a role in resistance to respiratory infections as well as the development of asthma and probably chronic obstructive pulmonary disease. Thus in the absence of the microbiota, the airways are prone to mount exaggerated Th2 responses. The presence of a microbiota induces Treg activity that suppresses this. This probably explains the protective effects of inhaled microbial antigens (as in a farming environment) on the development of allergies. Dietary fiber also has a protective effect on allergic airway inflammation in mice and this results from increased levels of circulating short-chain fatty acids. The intestinal microbiota also regulate pulmonary adaptive responses. Thus segmented filamentous bacteria (SFBs) in the intestine regulate pulmonary immunity to bacteria and fungi. Conversely, influenza infection in the lungs generates type I interferons. These in turn induce changes in the gut microbiota such as depletion of obligate anaerobic bacteria and an increase in Proteobacteria resulting in intestinal dysbiosis—a possible cause of "stomach flu."

The Genitourinary System

In adult females, the healthy cervicovaginal microbiota is usually dominated by lactobacilli and other lactic acid-producing bacteria. The vagina is also lined by a squamous epithelium composed of cells rich in glycogen. When these cells desquamate, the glycogen provides a substrate for the lactobacilli that, in turn, generate large quantities of lactic acid. This reduces the pH to a level that protects the vagina against invasion by many pathogenic bacteria and yeasts. Glycogen storage in the vaginal epithelial cells is stimulated by estrogens and thus occurs only in sexually mature animals.

The Gastrointestinal Tract

The gut microbiota is a complex community containing bacteria, archaea, fungi, and viruses. The most obvious of these are trillions of bacteria belonging to hundreds of different species. They are

dominated by members of two phyla, the Firmicutes and the Bacteroidetes, with lesser numbers of Actinobacteria and Proteobacteria and many minor phyla such as the Fusobacteria and the Verrucomicrobia. It has been estimated that the canine small intestine harbors more than 200 different bacterial species, while the canine colon may house up to a thousand (Fig. 21.4).

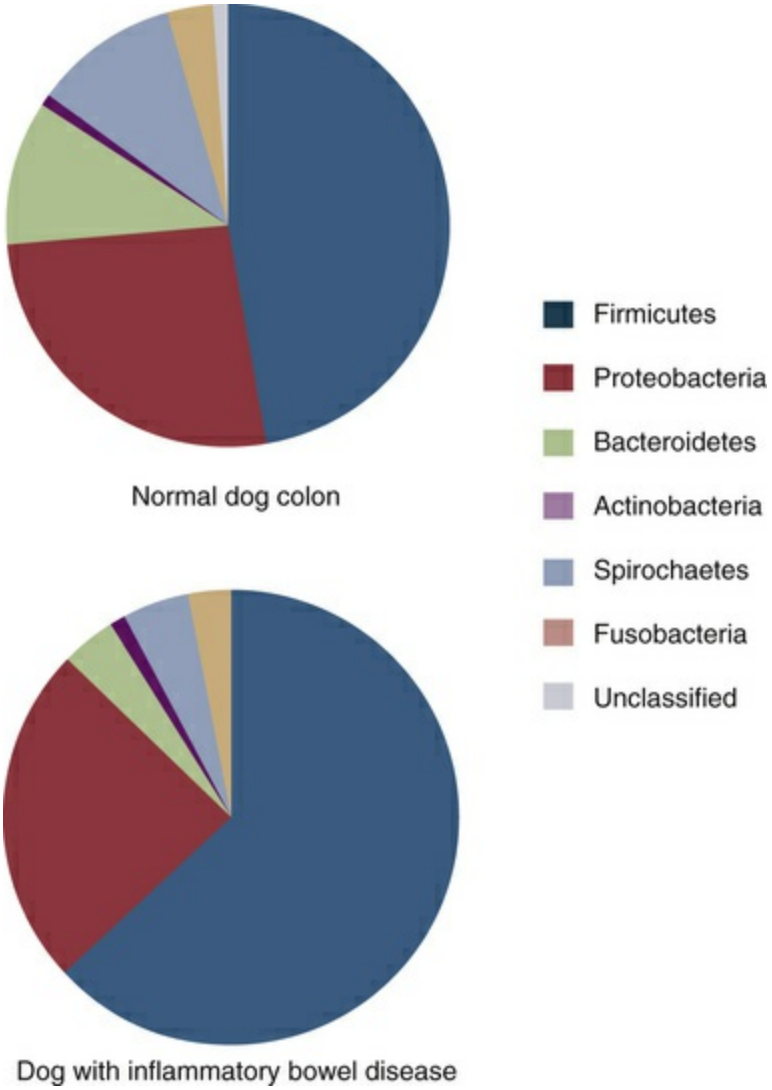


FIG. 21.4 The enormous diversity of the gut microbiota in the dog is well seen in this comparison of the composition of the colon microbiota in normal dogs and dogs with inflammatory bowel disease. (Courtesy Dr. P. Xenoulis.)

Each individual animal's microbiota is unique and its composition is determined by management, diet, genetics, antibiotic exposure, and environmental factors. The composition of

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the microbiota also changes along the gastrointestinal tract under the influence of nutrient availability and the local microenvironment. The Firmicutes consist of mainly Gram-positive bacteria. Many are spore-forming. Important members include the Clostridia that may be beneficial or pathogenic. They also include potentially pathogenic streptococci and staphylococci. The Actinobacteria are also Gram-positive bacteria with a different G+C content than the Firmicutes. The Bacteroidetes are Gram-negative bacteria that ferment indigestible plant carbohydrates to produce short-chain fatty acids. The Proteobacteria contain Gram-negative enterobacteria such as *E. coli* and *Klebsiella*.

The canine stomach has a microbiome dominated by *Helicobacter* spp. Bacterial counts in the canine and feline duodenum are in a range from 10^2 to 10^9 per gram of content. In the colon, the count ranges from 10^9 to 10^{11} colony forming units/g.

The Rumen

The surface of the rumen is covered by stratified squamous epithelium. Thus its defenses have more in common with skin rather that with the rest of the gastrointestinal tract. Although this epithelium is largely leak-proof, the presence of such a large source of microbial antigens suggests that some provision must be made for defense against leakage and that it may have a profound influence on the innate and adaptive immune systems of animals.

Disturbances in rumen metabolism, often caused by feeding very high-energy diets, result in changes in the ruminal microbiota, leading to an increase in fatty acid and ethanol production, a drop in rumen pH, and the development of subacute rumen acidosis. This in turn results in local inflammation, opening of intercellular junctions, and a disruption in the barrier function of the ruminal squamous epithelium. This can permit bacterial PAMPs such as endotoxins, flagella, and other microbial products to cross the ruminal wall and enter the bloodstream. This may lead to endotoxemia, a systemic innate immune response, an acute-phase response, and prolonged systemic inflammation.

While much is known about the relationships between the intestinal immune system and lymphoid tissues in simple-stomached animals, much less is known about the interactions

between the rumen and the immune system. The ruminal lymphatics drain to many lymph nodes (Fig. 21.5). Toll-like receptors (TLRs) such as TLR4, cytokines such as IL-1β and IL-10, and caspase-1 can be found in the ruminal walls suggesting that inflammation can readily occur here. Interferon-γ is found in ruminal contents as are T and B cells. It appears that the ruminal microbiota may communicate with its associated lymphoid tissues and so promote regulatory responses. There is little evidence that the ruminal microbiota directly shapes the development of the immune system but it has been suggested that the complex innate immune systems of ruminants, especially their high diversity of antimicrobial peptides, may have evolved in response to this source of microbial invasion.

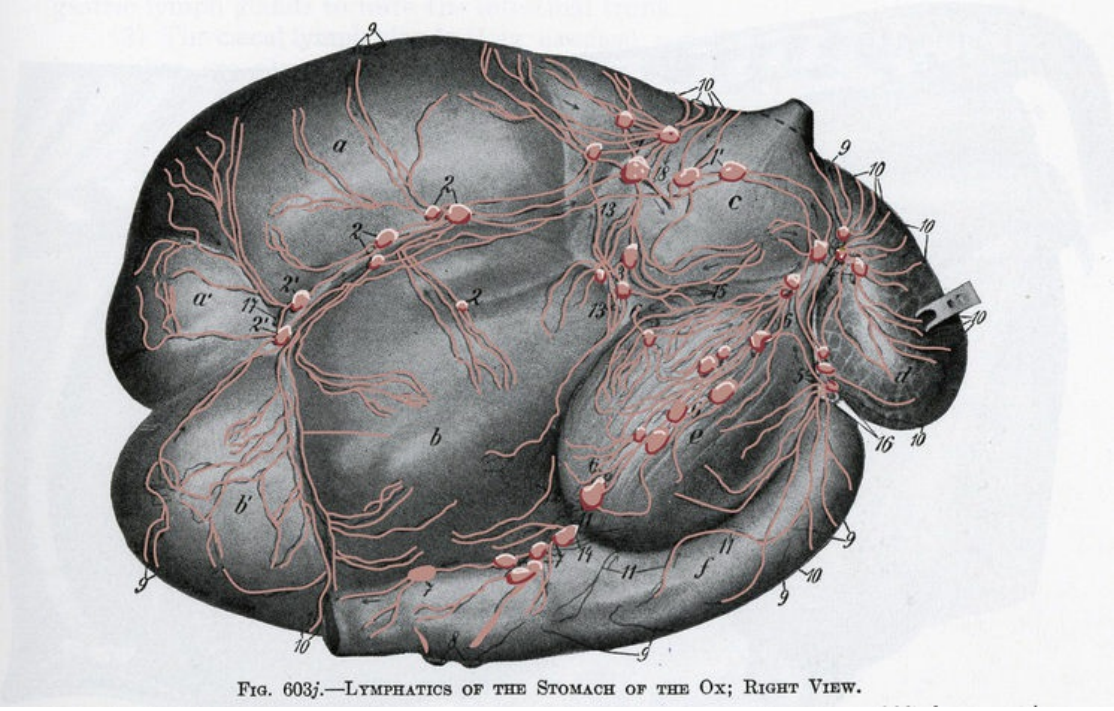


Fig. 603*j*.—Lymphatics of the Stomach of the Ox; Right View.

FIG. 21.5 The lymphatic drainage of the rumen. The rumen is an organ specialized for the fermentation and digestion of complex plant polysaccharides. It is lined by stratified squamous epithelium and is thus generally impermeable to most invading microorganisms. However, leakage is inevitable, and as a result, ruminants have large numbers of lymph nodes located where they can trap any ruminal escapees. (From Sisson S [Revised by Grossman JD]: *Anatomy of the domestic animals,* ed 4, Philadelphia, 1953, Saunders.)

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The Cecum and Large Intestine

Hindgut fermenters (such as the horse) are monogastric herbivores that digest fibrous plant material by anaerobic fermentation in the cecum and colon. The short-chain fatty acids produced are absorbed through the mucosa. Firmicutes are the main bacterial phylum (especially Clostridia), but the predominant organism is a member of the Verrucomicrobia. Other phyla found in the equine large intestine include Spirochaetes, Fibrobacteres, Ruminococcus, and Bacteroidetes. Under certain conditions such as carbohydrate overload, the microbiota of the large gut change drastically, as a result of which their pH drops, and bacterial PAMPs escape into the bloodstream. This can result in the development of acute laminitis (Chapter 7).

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The Functions of the Microbiota

Nutritional Efficiency

The composition and metabolism of the microbiota is critically dependent on diet. Thus the microbiota in animals fed a low-fat, high-plant polysaccharide diet is very different from that in animals on a high-fat, high-sugar and low-plant polysaccharide diet. There are great differences in the microbiota of African children compared to European children or in Indian cattle compared to American cattle. Environmentally induced fluctuations in the intestinal microbiota can permit the host to adjust its metabolic and immunologic performance in response to nutritional and environmental changes.

The intestinal microbiota changes during pregnancy. In mothers during the third trimester, there is a reduction in species richness. When transferred to germ-free mice, late pregnancy microbiota induced greater adiposity and reduced insulin sensitivity. This effect is beneficial in a normal pregnancy since it supports fetal growth and the onset of lactation. The maternal microbiota also drives early postnatal immune development including the potential for young animals to develop type 2 responses and hence allergies.

Intestinal Protection

The microbiota protects the body against colonization by pathogens and prevents the overgrowth of pathobionts. They do this by competing for essential metabolites and nutrients, and by inducing intestinal immune responses (Fig. 21.6). By fully occupying and exploiting the intestinal environment, commensal bacteria block subsequent colonization by pathogenic bacteria. (It is, for example, possible to prevent or reduce *Salmonella* colonization of the chicken intestine by feeding an appropriate mixture of commensal bacteria to birds.) The microbiota also modify local environmental conditions by keeping the pH and oxygen tension low. This is also influenced by the diet; for example, the intestine of milk-fed animals contains many lactobacilli that produce bacteriostatic lactic

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and butyric acids. These acids inhibit colonization by *E. coli,* so young animals suckled naturally tend to have fewer digestive disturbances than animals weaned early in life. There is a strong negative correlation between the level of lactobacilli and the level of pathogens in the intestine.

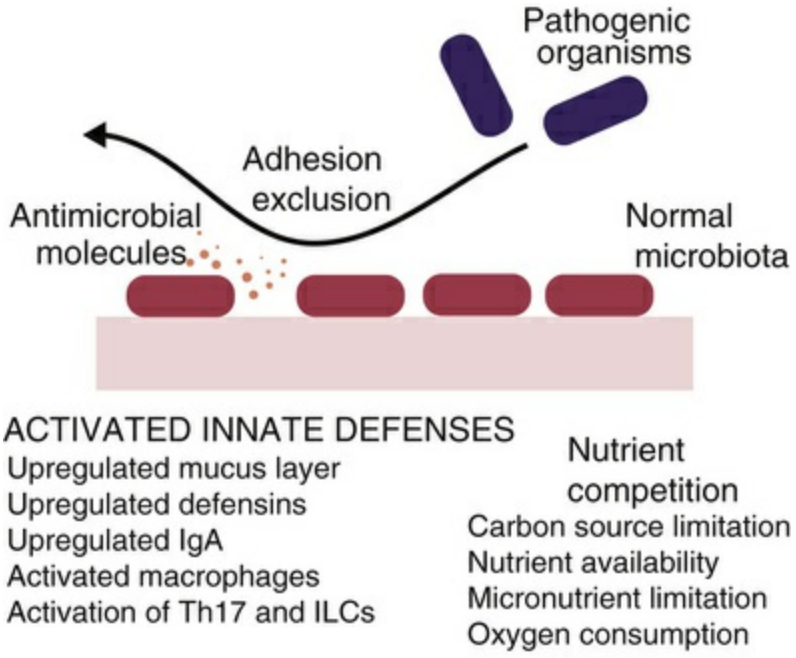


FIG. 21.6 The mechanisms by which the microbiota protect body surfaces against colonization and invasion by pathobionts.

Development of Lymphoid Organs

The development of the lymphoid tissues in the gastrointestinal tract begins well before birth. However, their complete maturation and the recruitment of IgA secreting B cells and T cells only occurs after birth. The microbiota recruit immune cells to surfaces, and drive the development and organization of all the major lymphoid tissues.

It has long been possible to derive animals by cesarean surgery and raise them within sealed chambers in such a way that they are free of microbes. Compared to conventionally raised animals, these "germ-free" animals have fewer and smaller Peyer's patches, smaller mesenteric lymph nodes, and fewer $CD4^+$ T cells in the

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lamina propria of the gut wall. They have fewer intraepithelial T lymphocytes (IELs) within their intestinal epithelium. These IELs have reduced expression of TLR and MHC class II, as well as reduced cytotoxicity. Systemic immune defects are also apparent. Germ-free mice have fewer $CD4^+$ T cells in the spleen, and fewer and smaller germinal centers as a result of reduced B cell numbers. Their production of macrophages and neutrophils by bone marrow stem cells is impaired. Their immunoglobulin levels are only about 2% of normal so that if exposed abruptly to the external environment, they are vulnerable to bacterial diseases. The presence of the microbiota is also necessary for the production of tertiary lymphoid structures such as cryptopatches and isolated lymphoid follicles.

Mammals have evolved two strategies to generate B cell populations with a diverse antibody repertoire (Chapter 17). Thus mice and humans rely mainly on random rearrangements of the VD and J genes during B cell development within the bone marrow. Other mammals such as cattle, sheep, pigs, and rabbits use an alternative strategy. These species undertake an initial burst of B cell proliferation with limited diversification *in utero.* These newly produced cells then migrate to the gut-associated lymphoid tissues where they expand both their numbers and their diversity. As described in Chapter 12, in sheep the ileal Peyer's patch is the site of B cell repertoire expansion, while the jejunal Peyer's patch is the source of antigen-specific IgA responses.

The process of microbial-driven B cell diversification and IgA production appears to depend upon the presence of a select subgroup of bacteria within the microbiota. For example, a combination of *Bacteroides fragilis* and *Bacillus subtilis* can induce B cell development and VDJ diversification in germ-free rabbits. Neither species alone has this effect, suggesting that two signals are needed. Once B cell proliferation and diversification are triggered, the microbiota continue to regulate any additional diversification. It is believed that microbial molecules trigger these B cell responses by binding to their TLRs and activating NF-κB pathways. Alternatively, soluble bacterial superantigens might trigger a polyclonal B cell response and drive the process by preferentially stimulating the production of B cells expressing certain Vh regions.

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It may be relevant to note here that the distribution of TLRs on cells differs significantly between conventional and germ-free pigs.

Microbiota Signals to the Body

Bacteria, be they on the skin, respiratory tract, genital tract, or intestine, communicate directly and effectively with their host's immune system. Indeed, this interaction is essential to the proper functioning of the innate and adaptive immune responses. Alterations or imbalances in the microbiota therefore have profound effects on the functions of the immune system. The proper interaction between the immune system and the microbiota is required for optimal health.

Dietary plant fibers contain complex carbohydrates. When digested by Clostridia in the cecum and colon, these complex carbohydrates generate large amounts of short-chain fatty acids (SCFAs) such as butyrate, propionate, and acetate that suppress macrophages and promote production of intestinal $Foxp3^+$ Treg cells (Fig. 21.7). Butyric acid has antiinflammatory effects since it acts on macrophages and prevents epigenetic changes by inhibiting histone deacetylases. Butyrate also increases barrier functions by stimulating enterocytes and increasing transcription of mucin genes, goblet cell differentiation, and mucus production. It can also stimulate some bovine neutrophil functions. As a result, high-fiber diets play a key role in regulating intestinal inflammation.

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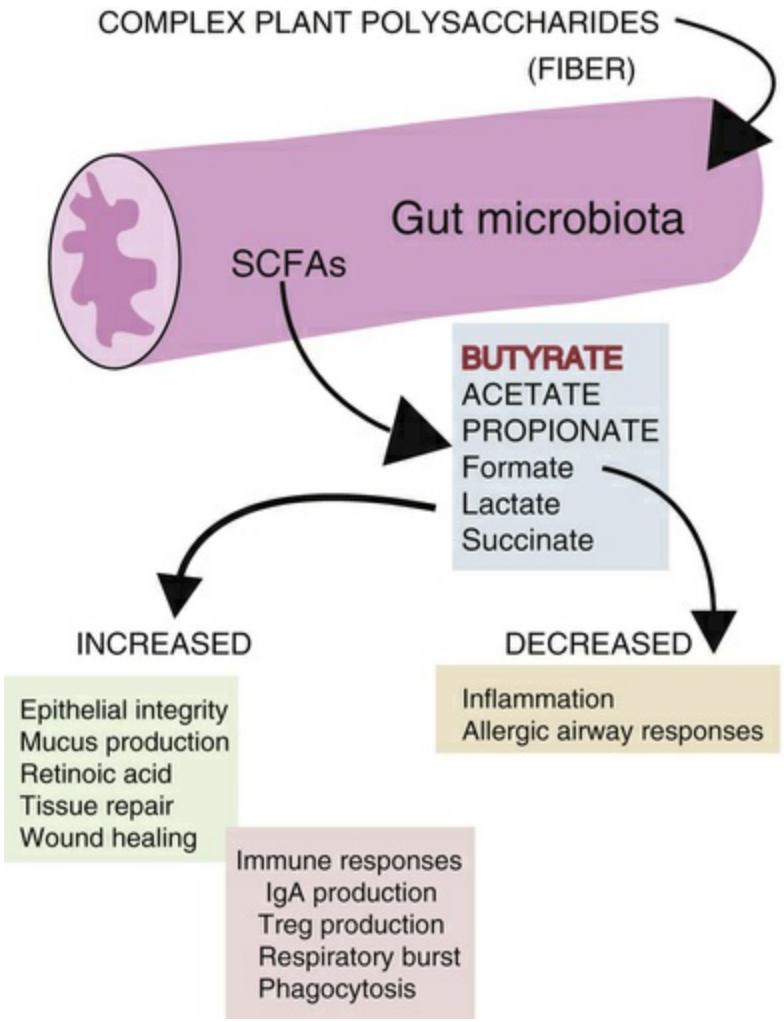


FIG. 21.7 The importance of short-chain fatty acids (SFCAs) in promoting immunity.

If the intestinal microbiota is altered in such a way that there is increased production of acetate, then this will activate the parasympathetic nervous system and promote glucose-stimulated insulin production. This stimulates production of a hunger hormone called ghrelin that enhances appetite, leading to increased food intake and obesity.

Among the intestinal microbiota, some species play a key role in regulating immune responses. One such group are classified as Clostridial clusters. Several of these bacteria (Clostridia clusters IV, XIVa, and XVIII) specifically induce Treg cells and IL-10 production in the gut. These Clostridia form a thick layer over the epithelium and enhance the release of transforming growth factor-β (TGF-β) and indoleamine 2,3-dioxygenase (IDO) from enterocytes (Fig. 21.8). They also promote mucus production by goblet cells.

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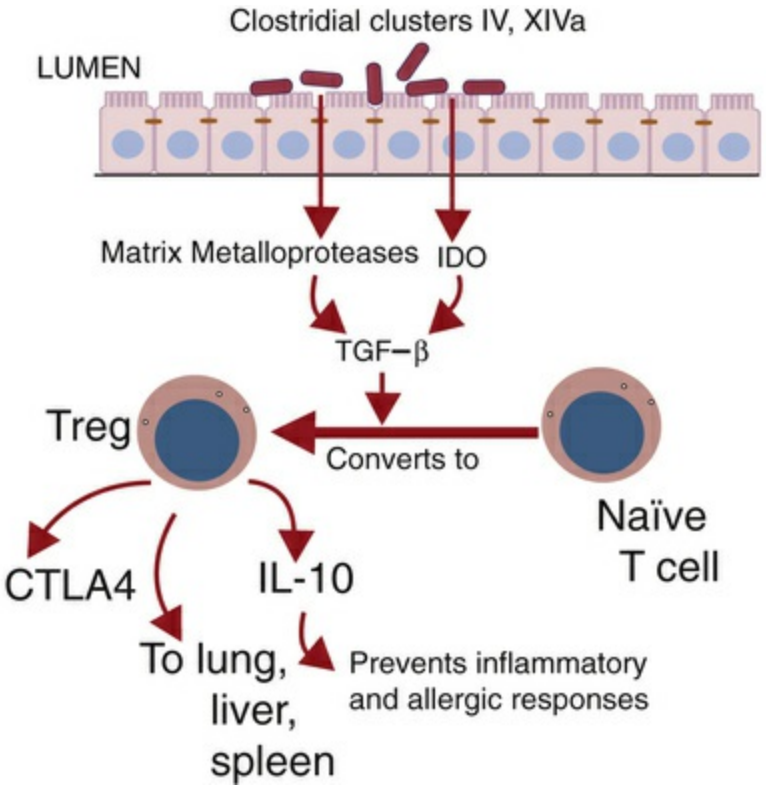


FIG. 21.8 The role of clostridial clusters in promoting both local and systemic Treg activity.

In addition to the Clostridia, the capsular polysaccharide-A of *Bacteroides fragilis* triggers IL-10 production through the TLR2-MyD88 pathway. Numerous Clostridia species can trigger IL-10 production by a non-MyD88 pathway involving TGF-β production by intestinal epithelial cells. Other mechanisms such as desensitization of TLRs to bacterial PAMPs and bacterial stimulation of IL-10 and IL-2 production by Treg cells also help minimize inflammation.

Additionally, some commensal bacteria actively suppress intestinal inflammation. For example, *Lactobacilli* and *Bacteroides* inhibit the innate signaling pathways triggered by TLRs and NLRs. A common commensal, *Bacteroides thetaiotaomicron* inhibits NF-κB signaling and intestinal lactobacilli prevent degradation of the NF-κB inhibitor IκB.

The presence of Clostridial clusters in the colon also results in an increase in the numbers of IL-10 producing Treg cells in distant tissues such as the spleen and lung, and they play a role in inhibiting allergic responses. Thus T cells educated by commensal bacteria may emigrate from the gut to remote tissues and determine

the body's T cell balance.

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Immune Responses to the Microbiota

For many years, it was believed that the role of the immune system was simply to ensure the complete exclusion of all invading microbes by distinguishing between self and not-self and eliminating foreign antigens. We now know, however, that that decision alone is insufficient to ensure health. The immune system must also determine the degree of threat posed by the microbes it encounters and adjust its response accordingly. It must maintain tolerance to microbiota or food antigens while, at the same time, be highly responsive to invading pathogens. This discrimination is determined in part by the way in which enteric antigens are processed and the behavior of enterocytes. It is also determined by intestinal T and B cells as well as by the gut microenvironment.

The presence of the intestinal microbiota must either be tolerated or ignored if an animal is to remain healthy. An animal cannot afford to act aggressively toward its own microbiota. The presence of all these bacterial products has the potential to trigger massive acute inflammation, but this inflammation must not happen unless necessary for the defense of the body.

Enterocytes

The intestinal epithelium is not simply a barrier. It is a highly responsive tissue that employs innate and adaptive immune cells to restrain the microbiota without triggering unnecessary inflammation, but is always ready to activate more potent defensive responses should the need arise. Enterocytes interact with the intestinal microbiota. They produce many peptides that kill or inactivate bacteria and as a result shape its composition. Enterocytes block access of intact antigens to the lamina propria. They ensure that a balance exists between inflammation and tolerance. They secrete and respond to regulatory cytokines. They display antigens to dendritic cells. Within the epithelium and the underlying lamina propria are IELs that upon appropriate stimulation by microbiota-induced IL-1 or IL-23 can regulate their differentiation into effector or regulatory cells.

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Enterocytes express receptors for many microbial-associated molecular patterns (MAMPs) including TLRs-1, -2, -3, -5, and -9 as well as NOD-2. When exposed to MAMPs, recruitment of MyD88 and TRIF result in NF-κB and MAP kinase activation and cytokine synthesis. In practice, bacterial signals trigger the production of some antimicrobial peptides and cytokines but not inflammation. This is because the PRRs are not expressed on the luminal side of enterocytes, where they would normally come into contact with commensals. They are located at the base of the cells and at intracellular locations. Thus they are activated only after bacteria penetrate the epithelial barrier. By preventing microbial invasion, enterocytes also prevent the development of inflammation within the intestinal wall. Some of the cytokines produced by enterocytes influence the regulatory activities of antigen-processing cells such as macrophages and dendritic cells. IL-10 inhibits the TLR-MyD88 pathway, whereas IL-2 inhibits TLR-independent pathways.

The antimicrobial peptides within the inner mucus layer keep most of the microbiota from contacting the enterocytes and thus ensure that the microbiota remain within the intestinal lumen. They not only protect the host from microbial invasion but also from the potentially harmful inflammatory response that would occur when MAMPs are absorbed into the body (Chapter 22).

ILC3 Cells

Group 3 innate lymphoid cells also regulate the interactions between the microbiota and its host (Fig. 19.4). They respond to IL-23, IL-1β, and TSLP from dendritic cells by producing IL-17 and IL-22. These attract neutrophils and promote the production of antimicrobial peptides, especially REGIIIγ in the small intestine. REGIIIγ interacts with the mucus layer to maintain a relatively bacteria-free zone adjacent to the mucosal surface. ILC3 cells also activate B cells and induce IgA production. They can promote tolerance to food antigens by producing GM-CSF that in turn promotes Treg production. Their production is regulated by the aryl hydrocarbon receptor (Box 21.1).

Box 21.1

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The Aryl Hydrocarbon Receptor

The aryl hydrocarbon receptor (AhR) was originally identified as a transcription factor in the liver where it controls cellular responses to aromatic hydrocarbons such as dioxanes and bisphenols. It also regulates the enzymes that metabolize xenobiotics such as cytochrome p450. Its endogenous ligands include derivatives of tryptophan, indoles, and lipoxins. Dietary tryptophan is metabolized by lactobacilli to generate indole-3-aldehyde, tryptamine, and indole, all of which are AhR ligands.

AhR plays an important role in regulating immune responses. Thus it is expressed at high levels in antigen-presenting cells, intraepithelial lymphocytes, Th17, and Treg cells. Ligand binding to the AhR stimulates IL-23 production and Th17 differentiation. IL-22 from these cells enhances production of anti-microbial peptides. The development of ILC3 cells depends upon AhR expression and activation. AhRs are essential for the formation of intraepithelial lymphocytes, cryptopatches, and isolated lymphoid follicles. Defective AhR signaling results in severe intestinal inflammation and the development of allergies. Thus AhR knockout mice mount enhanced Th2 responses and higher levels of IgE and IgG1. Their dendritic cells express higher levels of CD86 and MHC class II molecules. AhR signaling negatively affects the type I interferon response.

AhR ligands such as indoles and flavonoids are naturally found in cruciferous vegetables (cabbage, cauliflower, lettuce). These act through AhRs to maintain IELs and promote normal intestinal lymphoid function. AhRs also play an important role in resistance to *Pseudomonas aeruginosa, Mycobacterium tuberculosis, Listeria, Toxoplasma, Leishmania* and *Trypanosoma cruzi.*

B Cell Functions

There is more immune activity in the intestine than in all other lymphoid tissues combined. It has been estimated that more than 80% of the body's activated B cells are found in the intestine. Their function is to defend against possible invasion by the microbiota.

Although the microbiota are separated by the inner mucus layer and glycocalyx from direct contact with enterocytes, intestinal

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dendritic cells can extend their processes into the intestinal lumen and sample the microbiota. These bacteria can persist within the dendritic cells for several days while the cells carry them into the mucosa and mesenteric lymph nodes and present them to B cells. In addition, some bacteria are taken up by specialized antigen-capturing M cells, penetrate the Peyer's patches, and become resident within the tissues. Although most of these invading bacteria are killed by macrophages, some are also presented to B cells. The B cells produce IgA, which may modify the composition of the microbiota and block further mucosal penetration. Bacteria are thus prevented from breaching the mucosal barrier by the ongoing IgA response, and the mesenteric lymph nodes form an additional barrier that prevents the commensals from reaching the systemic immune system. It is likely that a similar local IgA response occurs against food antigens. (The role of IgA is discussed in detail in Chapter 22.)

T Cell Functions

The intestinal immune system must be relatively unresponsive to the microbiota while still being able to respond to potential pathogens. Thus it must be highly regulated. The key to successful accommodation with the intestinal microbiota depends on the body's ability to control inflammation in the gut wall. This is achieved by a maintaining a balance between proinflammatory Th17 cells and antiinflammatory Treg cells (Fig. 21.9).

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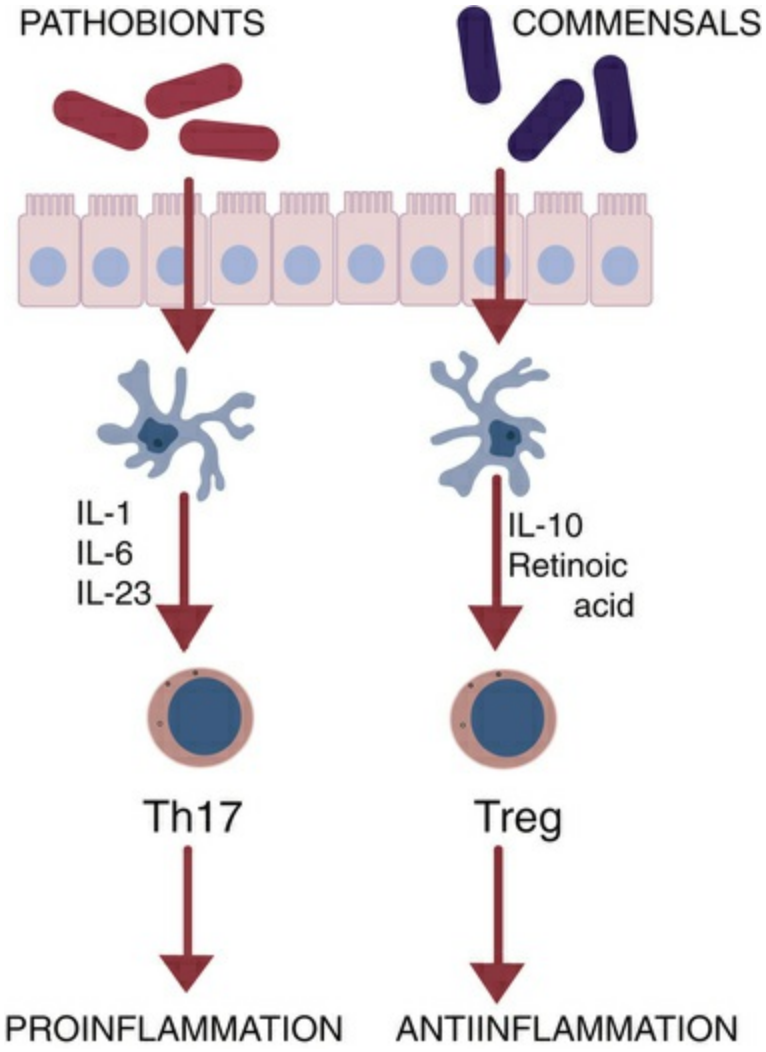


FIG. 21.9 The countervailing activities of Tregs and Th17 cells require a balanced microbiota to maintain health.

Intestinal helper T cell phenotypes are "plastic," and precursor cells can differentiate into either Treg or Th17 cells, while Th17 cells may further differentiate into Th1 cells. This differentiation is regulated by signals from the microbiota. In effect the microbiota program the T cell system to optimize its function. Additionally, helper T cell differentiation is determined by cytokines. For example, the development of both Tregs and Th17 cells are promoted by TGF-β. Treg cells require TGF-β plus retinoic acid and IL-2, whereas Th17 cells require TGF-β plus IL-6 and IL-23. The major transcription factors used by Tregs and Th17 are FoxP3 and RORγt respectively, and these are co-expressed in naïve and effector $CD4^+$ cells.

Treg cells.

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Intestinal Treg cells are a subset of $CD4^+$ Th cells required to maintain the body's commensal relationship with its microbiota. Treg cells produce IL-10 as well as expressing high levels of CTLA-4 (cytotoxic T lymphocyte antigen 4), the inhibitory ligand for CD80. Treg production occurs in response to signals from both the microbiota and enterocytes. These Treg cells, when acted on by proinflammatory cytokines, can convert to IL-17 or IFN-γ -expressing effectors and so "break tolerance." Under these circumstances, Th17 cells can give rise to IFN-γ producers that functionally resemble Th1 cells, and it is likely that many intestinal Th1 cells develop through this pathway. Under other circumstances the Treg cells may convert to helper cells and promote the switch to IgA production. Indeed, about 75% of the IgA reactive to the microbiota is produced through a T-dependent pathway controlled by Treg cells.

Mucosal inflammation is therefore actively suppressed by the production of large numbers of IL-10-producing $FoxP3^+$ Tregs. Under stable conditions, the production of Treg cells is favored while that of Th17 cells is suppressed. In the absence of Treg cells, uncontrolled effector T cells will respond to microbial antigens and trigger inflammatory bowel disease. Likewise, IL-10 deficient mice develop chronic unremitting colitis driven by IL-23 and the Th17 pathway.

It is also clear, however, that this tolerance can only go so far. Should a potential pathogen seek to invade the body from the intestine, then the immune system must be prepared to act aggressively to prevent this. This is mediated by proinflammatory Th17 cells.

Th17 cells.

As described in Chapter 20, Th17 cells are a subset of $CD4^+$ T cells that regulate inflammation and maintain the intestinal epithelial barrier. Under the influence of IL-23, they produce the proinflammatory cytokines IL-17A, IL-17F, and IL-22. They also produce the antibacterial cytokine IL-26 (Fig. 21.10). Like Treg cells, the development of Th17 cells is regulated by signals from the microbiota and from enterocytes. Th17 cell development is specifically stimulated by the attachment of SFBs to enterocytes.

Enterocytes can sense this tight attachment (Fig. 21.11). The mechanism by which this affects enterocytes is unclear, but SFBs cause enterocytes to produce serum amyloid A (SAA) (Chapter 7). The SAA acts as a cytokine and stimulates IL-23 production by macrophages leading to ILC3 secretion of IL-22 and -17. This in turn triggers development of Th17 cells. Lamina propria macrophages and dendritic cells can also detect SFB-derived molecules through their TLRs and produce IL-23 and TGF-β, promoting further Th17 cell differentiation. As a result the T cells differentiate into RORγt-expressing Th17 cells. Th17 cells in turn regulate the abundance of SFBs by promoting the production of antibacterial peptides such as β-defensins, lipocalins, and calprotectin by enterocytes.

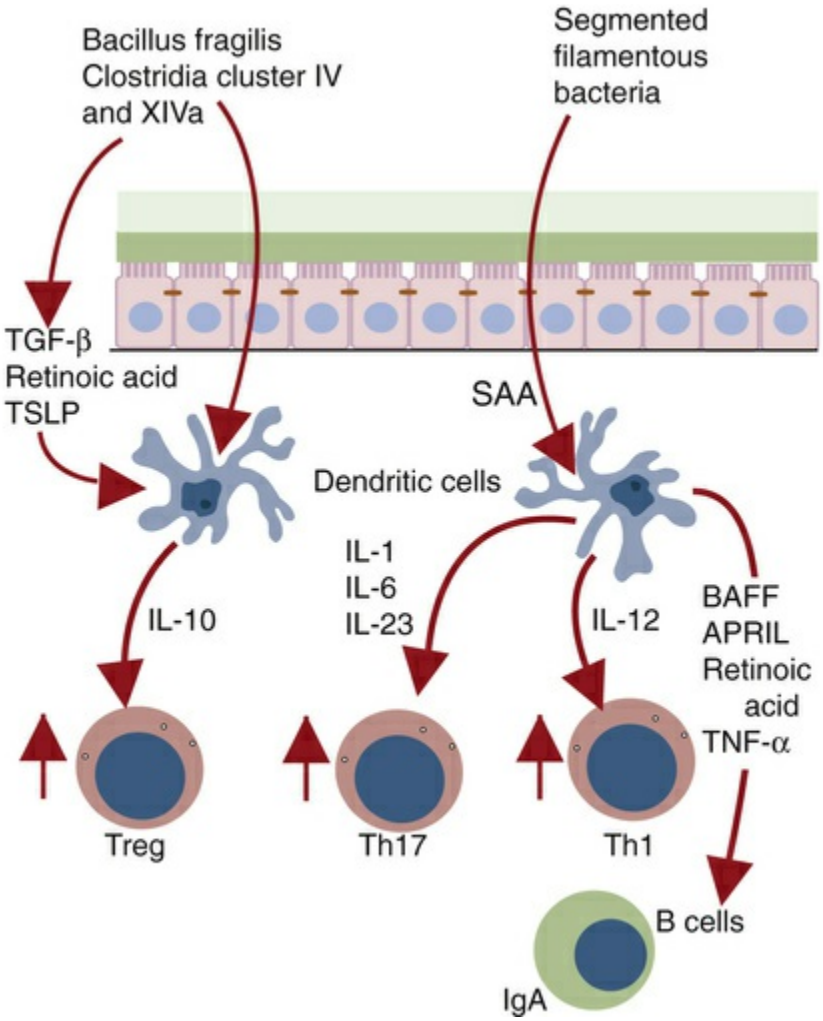


FIG. 21.10 The role of segmented filamentous bacteria and Clostridia in regulating T cell subset formation and in controlling intestinal inflammation. Bacillus fragilis and the clostridia activate dendritic cells by stimulating production of TGF-β retinoic acid and

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thymic stromal lymphopoietin (TSLP). These promote the activation of Treg cells. Segmented filamentous bacteria stimulate enterocytes to produce serum amyloid A (SAA). This stimulates dendritic cells to produce cytokines that generate Th17, Th1, and B cells.

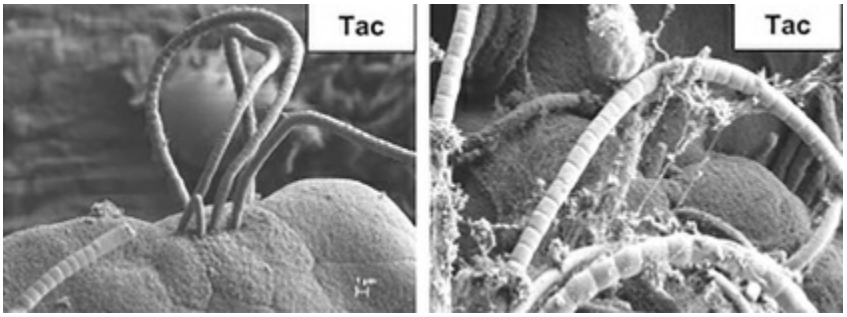


FIG. 21.11 Scanning electron micrographs of segmented filamentous bacteria in the terminal ileum of mice. (From Ivanov II, Atarashi K, Manel N et al: Induction of intestinal Th17 cells by segmented filamentous bacteria, *Cell* 139:485-498, 2009. Elsevier. With Permission.)

SFB are unique spore-forming, long filamentous Gram-positive anaerobic commensals found in the small intestine of mammals and birds. They do not yet have a formal scientific name. Their interim name is "*Candidatus* Savagella." (*Candidatus* is a term used for the provisional name of unculturable bacteria.) They have a unique ability to stimulate the maturation of T and B cells and especially stimulate Th17 responses and IgA production. They can also stimulate the upregulation of host innate defense genes, inflammatory cytokines, and chemokines. They attach very strongly to the enterocytes of the terminal ileum and the cells overlying Peyer's patches, where they are in a good position to be sampled by dendritic cells. (Most other bacteria remain within the mucus.) SFB induce the development of germinal centers in Peyer's patches and other intestinal lymphoid organs and increase production of IgA and Th17 cells. In the absence of SFB, mice mount weaker IgA responses and poorer intestinal T cell responses and recruitment of intraepithelial lymphocytes. It is not believed that the Th17 cells are directed against specific antigens in the SFBs; they appear to develop polyclonally through bystander activation.

Retinoic acid.

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Retinoic acid, the active metabolite of vitamin A, is a central regulator of mucosal immunity. In association with TGF-β, it enhances T cell proliferation and cytotoxicity and is especially important in promoting Th2 and Treg differentiation and in the homing of IgA^+ B cells to mucosal surfaces (Chapter 22). It is essential for maintaining the stability of Th1 cells and prevents their transition to Th17 cells. Retinoic acid normally suppresses Th17 responses and favors tolerance to food antigens.

Interleukin-22.

IL-22 is produced by Th17 cells, activated T cells, and ILC3s in response to IL-23. IL-22 reduces the absorption of food allergens. IL-22-producing ILC3s are present in normal intestinal tissues, especially in cryptopatches (Chapter 12). The IL-22 acts on nearby Paneth cells to produce antimicrobial peptides (lipocalin-2 and calprotectin). IL-22 acts on cells of the skin and the digestive and respiratory systems to increase expression of several β-defensins and promotes innate immunity and repair in these tissues. It promotes enterocyte growth and survival.

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Dysbiosis

It is clear from the above discussion that the gut microbiota exerts a significant influence on the systemic immune response. If this microbiota becomes unstable or imbalanced, dysbiosis occurs. Dysbiosis is a cause of equine laminitis and ruminal acidosis, and has been implicated in the development of several immune-mediated diseases such as canine chronic enteropathy and inflammatory bowel diseases. Antibiotic treatment is an important cause of dysbiosis. This can radically alter the composition of the intestinal microbiota and increase the risk of developing infections with organisms such as *Clostridium difficile* or overgrowth with other unwanted pathogens. Antibiotics alter the composition of the microbiota resulting in an increased risk of obesity. (Obese individuals have more Firmicutes and fewer *Bacteroides* than lean ones.) However, much remains to be learned about this very complex subject. Perhaps the most significant dysbiosis is that which leads to the development of allergies.

The role of the intestinal microbiota also extends to inflammatory diseases. For example, antibiotic-induced intestinal dysbiosis in mice affected circulating cytokine levels as well as the severity of ischemia/reperfusion injury on the heart. Oral vancomycin decreased circulating leptin levels, resulted in smaller myocardial infarcts, and improved functional recovery as compared to untreated controls. Modification of the gut microbiota by the probiotic *Lactobacillus plantarum* had a similar effect. Even oral antifungal drugs can disrupt the intestinal fungal microbiota in mice and increase the severity of airway allergic disease.

In critically ill animals, breakdown of the intestinal epithelium and mucosal barriers may permit leakage of bacterial components into the body. Conversely, depletion of the gut microbiota, especially as a result of antibiotic treatment, can make the mucosal defenses vulnerable and perhaps reduce the priming of the systemic immune responses. It may therefore be advantageous to modulate the composition of the gut microbiota in some critically ill animals by the judicious use of probiotics (Box 21.2).

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Box 21.2

Probiotics

Probiotics are cultures of live bacteria that when given orally may improve health by minimizing dysbiosis and its effects on the animal body. Provided the dose of these bacteria is large enough, the probiotics may change the composition of the microbiota at least for a time and thus influence the functions of the immune system. The results obtained depend not only on the dose of microbes administered but also the specific strain or mixture of organism(s). Lactobacilli and bifidobacteria are usually favored for probiotic use and appear to enhance immunity to diarrhea and respiratory tract infections. They possibly act by outcompeting pathogens. They may also reduce the severity of atopic disease. No single microbe is likely to solve all gastrointestinal problems, so it may be important to select the mixture of bacteria used with care. A widely employed technique involves oral administration of diluted feces or complex bacterial mixtures to outcompete *Salmonella* in poultry and pigs. Fecal transplants have proven successful in treating *C. difficile* infections in humans.

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Odors

Another way the microbiota affect their host is by increasing the diversity of the communication signals available to the host. Thus it is postulated that bacteria in mammalian scent glands as well as those in the mouth and the intestine generate odorous metabolites such as short-chain fatty acids. Some of these odors are used for host communication. Variations in these host chemical signals may be a result of underlying variations in the microbiota of the scent glands. Thus surveys of the microbiota of hyena scent glands suggest that variations in their microbiota correlate with the volume and fatty acid profiles of scent secretions. The bacterial populations also vary with species, gender, pregnancy, and reproductive state. In other mammals, odors are used for kin identification and mate choice. Given that genes for many odor receptors are linked to the MHC, it is not difficult to imagine how animals recognize others by the odors generated by their microbiota.

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The Virome

The viral component of the microbiota is called the virome—the collection of all viruses found within an animal. They include not only the viruses of eukaryotic cells that cause infection and disease but also the endogenous viruses that are integrated into and form a significant component of mammalian genomes. This virome differs greatly between individuals and may be even more diverse than the bacterial microflora. It has been estimated that the number of distinct viruses in human stool samples can range from 50 to almost 3000. In addition to mammalian viruses, the virome contains huge numbers of bacteriophages, perhaps as many as 100 phages for every bacterium. These phages prey on the bacteria and may play a role in transmitting genes between bacteria. When alterations in diet change the composition of the bacterial microflora, they also alter the composition of the phage population.

Certain enteric viruses such as the noroviruses can influence the development of the enteric immune system. Thus they can restore intestinal morphology and lymphocyte function in germ-free mice. Conversely, the ability of noroviruses to persistently infect animals depends on the microbiota. Antibiotics prevent persistent norovirus infections by changing the composition of the microbiota. Removal of the virome with antiviral drugs can result in enteritis.

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Inflammatory Bowel Diseases

Chronic enteropathies with persistent clinical signs in domestic species are a diverse group of diseases that result from a combination of genetic, microbial, nutritional, allergic, and environmental factors. Some resemble inflammatory bowel diseases in humans (Crohn's disease and ulcerative colitis) but most cases probably have a very different pathogenesis. Some result from dysbiosis and dysregulation of the immune responses to the intestinal microbiota. As pointed out, commensal bacteria within the intestine are prevented from invading the intestinal wall by a glycocalyx, by high concentrations of defensins, and by an ongoing IgA response. They also suppress inflammation by blocking NF-κB activation and generating IL-10–secreting Treg cells. If these control mechanisms fail and the animal responds aggressively to its commensals, perhaps by increasing Th1 responses, then severe inflammation may result, making the intestine much more susceptible to bacteria-induced injury.

Canine chronic enteropathies are characterized by persistent or recurrent gastrointestinal inflammation of undetermined cause. These diseases present with a history of chronic vomiting, diarrhea, and weight loss. There is a breed predisposition in Weimaraners, Rottweilers, German Shepherds, Border Collies, and Boxers. The most common form is a lymphocytic-plasmacytic enteritis.

Affected dogs may show an increase in Proteobacteria, especially *E. coli* or *Pseudomonas,* and a decrease in Firmicutes and Bacteroidetes. Other changes, such as increased bacterial adherence to the mucosa, reduced bacterial diversity, changes in the bacterial mixture, and overgrowth of other bacteria, may all contribute to the inflammation (see Fig. 21.4).

Many cases are associated with an increase in T cell and IgA^+ plasma cells in the small intestine. The T cells are primarily α/β $CD4^+$ cells, although there is an increase in intraepithelial γ/δ T cells as well. There may also be an increase in the numbers of intestinal mast cells. Some dogs with chronic enteropathy have reduced IgA levels in feces, duodenum, and blood. Hypoalbuminemia may reflect severe protein loss and carries a poor prognosis.

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The affected small intestine shows increased messenger RNA for IL-12, IFN-γ, TNF-α, and TGF-β. In dogs, cases have been associated with altered expression or dysregulation of TLR-2, -4, and -5. Polymorphisms in TLR-4 and -5 have also been associated with disease susceptibility. Thus it has been suggested that excessive TLR activation might increase IL-1β levels. If this is accompanied by decreased production of its receptor antagonist, IL-1RA then acute inflammation may result. The IL-1RA:IL-1 ratio in affected dogs is negatively correlated with disease severity. There is no evidence for changes in the IL-17 group of cytokines in these diseases in dogs. Likewise, there is no evidence of a Th1/Th2 imbalance in either dogs or cats. The expression of IL-12 does appear to be consistently increased.

It is convenient to divide these diseases into subgroups based on their response to treatment.

Food-responsive enteropathy: In about 50% of cases, feeding a hypoallergenic or a novel antigen diet may result in rapid clinical improvement within a few days and suggests that some forms of enteropathy result from food hypersensitivities.

Antibiotic-responsive enteropathy: Other forms may result from enteric infections and some animals may respond well to antibiotic therapy. Drugs commonly used include oxytetracycline, metronidazole, and tylosin. These responsive dogs include young large-breed animals and German Shepherds. The effects of antibiotic therapy may be short lived.

Immunosuppression-responsive enteropathy: Other cases are immunologically mediated and dogs may respond well to glucocorticoids such as prednisolone and the immunosuppressive drugs such as cyclosporine or azathioprine. Unfortunately the results of many clinical trials have been mixed and confusing, and long-term control remains difficult.

Histiocytic ulcerative colitis in Boxers is a severe form of inflammatory bowel disease. The lesions are characterized by the presence of large macrophages that stain intensely with periodic acid-Schiff stain. It is possible that this disease is triggered by an unidentified infectious agent since it somewhat resembles Johne's disease. The lesions also show accumulations of IgG^+ plasma cells, MHC class II-positive cells, macrophages, and granulocytes.

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Immunoproliferative enteropathy of Basenji dogs is an inherited autosomal disease that presents as gastric mucosal hypertrophy with lymphoid cell infiltration and ulceration. The whole small intestine may show villous blunting, crypt elongation, and infiltration of the mucosa with lymphocytes, plasma cells, and some neutrophils. Dogs show a polyclonal increase in serum IgA. The disease may be controlled by high doses of corticosteroids.

Protein-losing enteropathy of soft-coated Wheaten Terriers is also an inherited disease. Histological examination shows an inflammatory bowel disease. The cellular infiltrates are mainly lymphocytes and plasma cells, but neutrophils and eosinophils are often present. This disease may result from food hypersensitivity, possibly to wheat gluten.

Gluten sensitive enteropathy of Irish Setters is an autosomal recessive small intestinal disease also caused by exposure to wheat gluten. As with the diseases described previously, affected small intestine is infiltrated with lymphocytes and other inflammatory cells. The mucosa shows increased numbers of $CD4^+$ cells and decreased $CD8^+$ T cell numbers. Affected dogs may also have elevated serum IgA levels.

Lymphocytic-plasmacytic enteritis has also been described in cats, horses, and a cow. Equine inflammatory bowel disease also encompasses several different diseases. However, rectal biopsies of affected horses suggest that increase numbers of IL-17 cells may be involved in the active disease. There is also a reduction in antiinflammatory Treg activity.

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22

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Immunity at Body Surfaces

CHAPTER OUTLINE

Immunity on Body Surfaces, 234
- The Defenses of the Skin, 234
- Immunity in the Mammary Gland, 236
- Immunity in the Respiratory Tract, 236
- Immunity in the Urogenital Tract, 238
- Immunity in the Gastrointestinal Tract, 239

Mucosal Lymphoid Tissues, 241
- Inductive Sites, 241
- Effector Sites, 242
- *B Cells, 242*
- *T Cells, 242*

Adaptive Protective Mechanisms, 243
- Immune Exclusion, 243
- *Immunoglobulin A, 243*
- *Immunoglobulin M, 245*
- Immune Elimination, 245
- *Immunoglobulin E, 245*
- *Immunoglobulin G, 246*

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Immunity to Food, 246
Vaccination on Body Surfaces, 246

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Describe the immune defenses of the skin.
- Describe the immune defenses of the mammary gland and explain its vulnerabilities.
- Understand that the intestine is the largest immune organ in the animal body.
- Describe briefly the major defense mechanisms of the respiratory tract.
- Describe the defenses of the genitourinary system.
- Describe the basic features and properties of immunoglobulin A (IgA).
- Understand how surface defenses such as IgA exclude invaders by preventing them from binding to surface epithelia.
- Explain why γ/δ T cells are specialized for epithelial defense.
- Explain the role of IgA in preventing microbial invasion.
- Discuss the concept that IgE is a backup surface defense molecule.
- Describe briefly the phenomenon of oral tolerance.
- Define M cells, Paneth cells, intraepithelial lymphocytes, γ/δ T cells, inductive sites, and immune exclusion.

Although mammals possess an extensive array of innate and adaptive defense mechanisms within tissues, it is at their surfaces that invading microorganisms are first encountered and largely repelled or destroyed (Fig. 22.1). Although the skin is the most obvious of these surfaces, it in fact represents only a small fraction of the area of the body exposed to the exterior. The surface areas of the mucous membranes of the intestine and respiratory tracts are at least 200 times larger. The majority of pathogens enter the body through mucosal surfaces when ingested or inhaled, and respiratory and enteric infections are the most significant causes of death in young animals. While the immune systems ensure that the

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interior of the body remains largely free of microbial invaders, it is not possible to keep the surfaces of the body sterile. Indeed a vast number of microorganisms live in a commensal relationship on body surfaces. As a result, by far the greatest numbers of immune cells are found associated with the gastrointestinal tract or other body surfaces. The immune system functions on the basis that microorganisms that invade the body must be eliminated. Organisms that penetrate the epithelial barriers are promptly detected, attacked, and destroyed by innate and adaptive mechanisms.

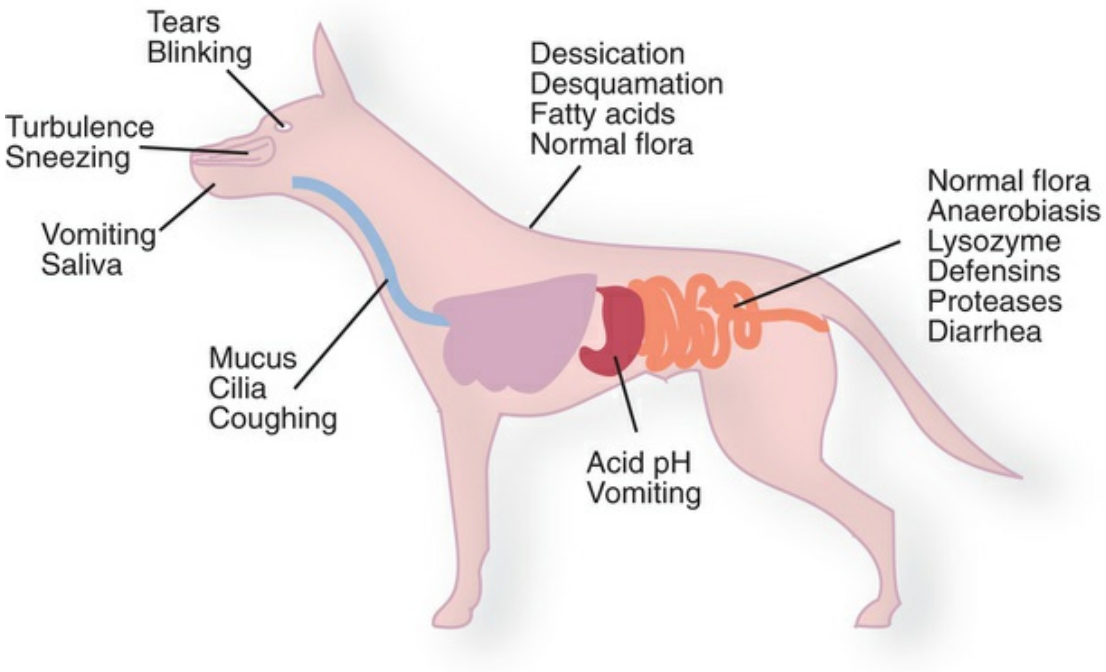


FIG. 22.1 The wide diversity of innate surface protection mechanisms.

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Immunity on Body Surfaces

The Defenses of the Skin

The skin is the first line of defense against many microbial invaders. It carries out its barrier function effectively, and few bacteria can penetrate intact skin unaided. Skin forms a tough physical barrier supplemented by continuous desquamation, desiccation, and a low pH because of fatty acids in sebum. In addition, the skin carries a resident microbiota that excludes pathogenic bacteria and fungi. If the skin microbiota is disturbed, its protective properties are reduced, and microbial invasion may result. Thus skin infections tend to occur in areas such as the axilla or groin, where both pH and humidity are high. Similarly, animals forced to stand in water or mud show an increased frequency of foot infections; as the skin becomes sodden, its structure breaks down and its resident microbiota changes in response to alterations in the environment. Major anatomical and immunological differences exist between the thick, hairless skin of humans and the haired skin of most mammals. Hair itself prevents desiccation and may protect against some fungal infections as well as being important in waterproofing and insulation.

Each layer of the skin has its own defensive mechanisms. For example, keratinocytes express multiple PRRs such as the toll-like receptors (TLRs), mannose receptors, and C-type lectins, so they are well able to recognize PAMPs associated with microbial invasion. Upon stimulation, keratinocytes produce a complex mixture of interleukins, interferons, chemokines, and other cytokines, growth factors, and antimicrobial proteins, all of which assist in excluding microbes seeking to penetrate the skin. Keratinocytes are the main source of cathelicidins and β-defensins. Pigs, for example, have 11 different skin cathelicidins. Calprotectin is also produced in the skin. It is a metal chelator and restricts the availability of the essential trace elements Zn and Mn to bacteria. Mast cells and skin secretory cells such as sweat, apocrine, and sebaceous glands also add antimicrobial peptides and lipids. Under normal conditions, keratinocyte precursor cells divide and continually renew the

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epidermis in a coordinated manner. If the skin is wounded or inflamed, alterations in adhesion molecules, surface receptors, and the cytokine environment change the behavior of the keratinocytes. Keratinocytes also express MHC class II and can act as antigen-presenting cells.

Once they invade the skin, microbes encounter dendritic cells. Both the epidermis and dermis contain large numbers of antigen-trapping dendritic cells. The best known of these are Langerhans cells. Langerhans cells bind exogenous antigen and present it to nearby helper T cells. They account for 50% to 70% of the dendritic cells in pig epithelium. The dermis also contains resident dendritic cells, as does the subcutaneous adipose tissue layer.

Healthy skin resident T cells are mainly located in the basal layer associated with Langerhans cells. $CD4^+$ and $CD8^+$ cells are present in equal numbers. In humans and mice they are predominantly α/β T cells. In some domestic mammals, many are γ/δ T cells. In cattle, for example, 44% of dermal T cells are γ/δ positive. The three major Th cell subsets are present. The microbiota regulate the development of immunity in the skin and intestine. For example, *Staphylococcus epidermidis* induces Th17 cells in the skin, and these skin Th17 cells are regulated by the skin microbiota independently of the intestinal microbiota. A subset of circulating T cells that home to the skin and produce IL-22 (Th22 cells) have also been identified. IL-22 plays an important role in maintaining barrier function on exposed body surfaces. It promotes antimicrobial immunity, inflammation, and tissue repair. Human skin also contains resident memory T cells that can provide global skin immunity and thus protect noninvolved skin. Treg cells facilitate cutaneous wound healing by enhancing local production of the epithelial growth factor receptor.

Ruminant γ/δ T cells recirculate continuously between epithelial surfaces such as the skin or intestinal epithelium and the bloodstream. In sheep, they are located in skin near the basal layer of the epidermis and in the dermis close to hair follicles and sebaceous glands. They are uncommon in wool-covered skin but are present in large numbers in bare and hairy skin. They are also found in the epithelium of the tongue, esophagus, trachea, and bladder. γ/δ T cells are a major population in skin-draining afferent

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lymph. They can produce both interferon-γ (IFN-γ) and IL-17 and they express high levels of the skin-seeking molecule, E-selectin. They survey the skin and inflammatory sites when attracted by CCR6 and E-selectin ligands. B cells also circulate in the skin and skin washings contain immunoglobulins. For example, in cattle, serum IgM, IgG1, and IgG2 cross the skin by transudation, but the IgA is locally synthesized.

Immunity in the Mammary Gland

The protective mechanisms of the udder are presumably not at their most effective in that biological anomaly, the modern dairy cow. Most infections result from invasion through the teat canal and subsequent bacterial growth in the teat cistern and mammary tissue. In a nonlactating animal, a keratin plug blocks the teat orifice and excludes bacteria. In a lactating animal, the flushing action of the milk helps to prevent invasion by some potential pathogens, whereas milk itself contains many innate antibacterial molecules. These include complement, lysozyme, lactoferrin, and lactoperoxidase. Lactoferrin competes with bacteria for iron and makes it unavailable for bacterial growth. It also enhances the neutrophil respiratory burst. Milk contains lactoperoxidase and thiocyanate (SCN^-) ions. In the presence of exogenous hydrogen peroxide, lactoperoxidase can oxidize the SCN^- to bacteriostatic products such as $OSCN^-$.

$$H_2O_2 + SCN^- \rightarrow OSCN^- + H_2O$$

The hydrogen peroxide may be produced by bacteria such as streptococci or by the oxidation of ascorbic acid. Bacterial lipopolysaccharides will also trigger local production of lipopolysaccharide-binding protein (LBP) and soluble CD14 in the mammary gland. As described in Chapter 2, both these proteins enhance lipopolysaccharide-induced cell activation and facilitate LPS elimination by promoting its binding to TLR-4.

Phagocytic cells that enter the gland in response to inflammation also contribute to antimicrobial resistance. They express PRRs,

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especially TLRs. Their activation by invading bacteria triggers the production of TNF-α, IL-1β, and IL-8 and results in clinical mastitis. Thus these cells are not only actively phagocytic (see Fig. 5.13) but they also release lactoferrin, hydrogen peroxide, and lysosomal peroxidases. The binding of bovine lactoferrin to *Streptococcus agalactiae* can activate C1q. Experimental mastitis induced by inoculation of *Escherichia coli* into dairy cattle results in increased concentrations of IL-6, IL-22, TNF-α, and IL-10 in milk. There is also a significant increase in Th17 cells in infected glands. Infusion of IL-17A at the onset of infection is associated with decreased bacterial numbers, decreased IL-10 production, and increased neutrophil recruitment.

Milk also contains IgA, secretory component, and IgG1. The IgA and secretory component are closely associated with the milk fat globules. In simple-stomach animals, IgA predominates, whereas in ruminants, IgG1 does. IgA is synthesized in the mammary tissue, although many of the IgA-producing cells in the gland are derived from precursors originating in the intestine. These cells are a source of antibodies against intestinal pathogens. Colostral IgG1, in contrast, is selectively transferred by active transport from serum using the FcRn receptor on mammary gland epithelial cells (see Fig. 23.6).

If antigen is infused into a lactating mammary gland, it tends to be promptly flushed out again in the milk. If it is infused into a nonlactating gland, a local antibody response develops. Because of the continuous removal of milk, antibody concentrations remain low (<100 mg/dL), even though, over a period of time, the total amount of immunoglobulin produced in the udder may be considerable. In acute mastitis, the inflammatory response leads to the influx of actively phagocytic cells, especially neutrophils, and to the exudation of serum proteins. As a result, immunoglobulin levels in mastitic milk may rise to levels at which they can exert a protective influence (~8000 mg/dL).

Because the local immune response in the udder is relatively ineffective in preventing infection, attempts to vaccinate against mastitis-causing organisms have been generally unsuccessful. Nevertheless, recent advances have produced encouraging results. Thus a *S. aureus* vaccine that stimulates the production of

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antibodies against the pseudocapsule appears to be effective. This pseudocapsule interferes with the ability of milk leukocytes to phagocytose *S. aureus.* Antibodies induced by the vaccine promote opsonization and destruction of the bacteria. A vaccine designed to stimulate antibody production against staphylococcal α toxin, as well as the pseudocapsule, is also effective. Encouraging results have also been obtained by the use of a J5 mutant vaccine against coliform bacteria (Chapter 26). The vaccine is given to cattle at drying off, 30 days later, and at calving.

Colostrum is rich in macrophages and lymphocytes. These macrophages can process antigen, and when cultured, their supernatants can enhance IgA production from blood lymphocytes. Milk lymphocytes survive for a short time in the intestine and transfer cell-mediated immunity to the newborn animal (Chapter 23).

Immunity in the Respiratory Tract

The respiratory tract differs from other surfaces in that it is in intimate connection with the interior of the body yet is required by its very nature to allow unhindered access of air to the alveoli. The system obviously requires a filter. Particles suspended in inhaled air are largely removed by turbulence that directs them onto its mucus-covered walls, where they adhere. The turbulence is caused by the conformation of the turbinate bones, the trachea, and the bronchi. This turbulence filter serves to remove particles as small as 5 μm before they reach the alveoli (Fig. 22.2).

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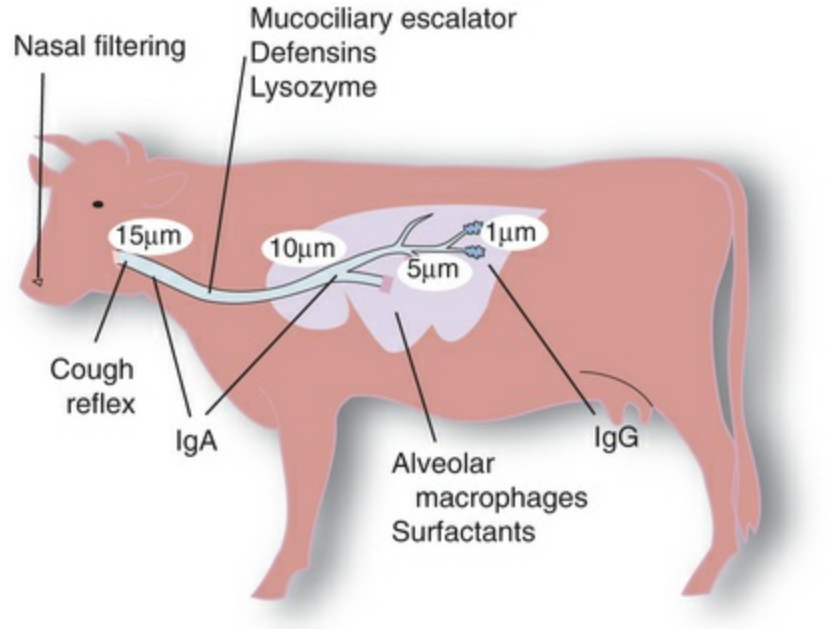


FIG. 22.2 Some of the innate mechanisms involved in the protection of the respiratory tract against infection and the influence of particle size on the site of deposition of particles within the respiratory tract. Note that only the smallest particles can penetrate deeply and gain access to the alveoli.

A blanket of mucus gel produced by goblet cells lines the upper respiratory tract. The mucus contains soluble host defense molecules such as lysozyme, lactoferrin, surfactant proteins, and cationic peptides such as the defensins and cathelicidins. Most microorganisms that enter the mucus layer are likely to be killed rapidly. There are four major surfactant proteins in lung fluid (SP-A, -B, -C, -D) produced by alveolar type II cells. SP-B and -C are extremely hydrophobic. Their function is to reduce surface tension at the alveolar surface so that a thin film of fluid forms on the surface and prevents lung collapse. SP-A and -D, in contrast, are C-type lectins that bind to microbial surface carbohydrates and act as opsonins. SP-A and -D also activate macrophages, promote chemotaxis, enhance the respiratory burst, and the production of inflammatory cytokines. These surfactant proteins also enhance the clearance of apoptotic cells from the lung. This is especially important in the resolution of inflammation, since apoptotic neutrophils should be removed by macrophages as promptly as possible. Surfactant proteins can also modulate the functions of dendritic cells and T cells. SP-A inhibits the maturation of dendritic

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cells, whereas SP-D enhances their uptake and presentation of antigen. Both SP-A and SP-D inhibit T cell proliferation.

The mucus layer is in continuous flow, being carried from the bronchioles up the bronchi and trachea by ciliary action or backward through the nasal cavity to the pharynx. Here the dirty mucus is swallowed and digested in the intestinal tract. Particles that bypass this mucociliary escalator and reach the alveoli are phagocytosed by alveolar macrophages. Once these cells have successfully ingested particles, they migrate to the mucus escalator and are also carried to the pharynx.

The respiratory tract contains bronchial lymphoid nodules as well as lymphocytes distributed diffusely throughout the lung and airways (Fig. 22.3). The mucosa of the larynx contains many immunologically active cells, including large numbers of T cells. Antigen-sampling M cells may be associated with these lymphoid nodules as well as with nasal mucosal lymphoid tissues. These tissues mainly produce secretory IgA, especially in the upper regions of the respiratory tract. This IgA binds to mucus through secretory component and so enhances the clearance of adherent bacteria. The polymeric immunoglobulin receptor (pIgR) is expressed at low levels on bronchial epithelial cells. In the bronchioles and alveoli, however, the secretions contain a large proportion of IgG, the concentration of which is intermediate between the levels in the trachea and in serum. IgE is also synthesized in significant amounts in the lymphoid tissues of the upper respiratory tract. As on other body surfaces, IgA in the respiratory tract probably protects by immune exclusion, whereas IgG and IgE act by immune elimination (Fig. 22.4).

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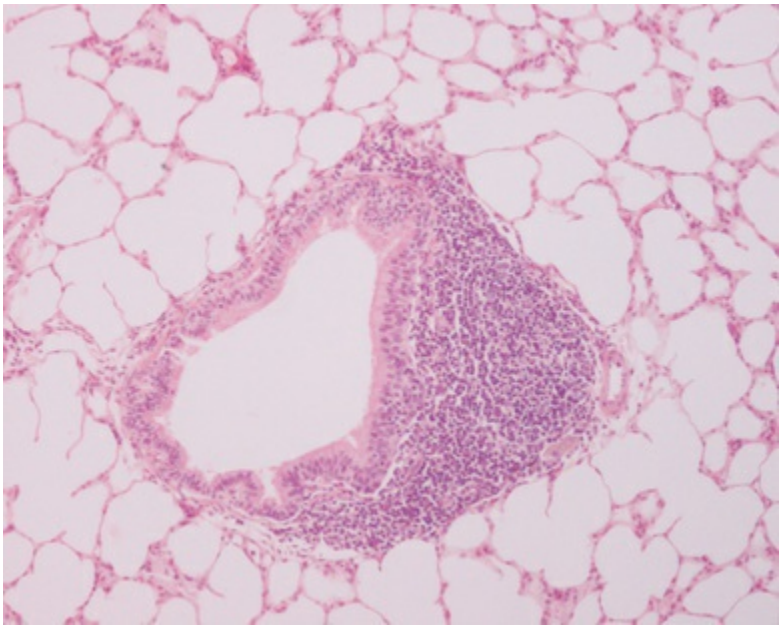


FIG. 22.3 Lymphoid follicle found in the bifurcation of an airway in a section of calf lung. This type of bronchus-associated lymphoid tissue is a key component of the defenses of the respiratory tract. (From a specimen provided by Drs. N.H. McArthur and L.C. Abbott.)

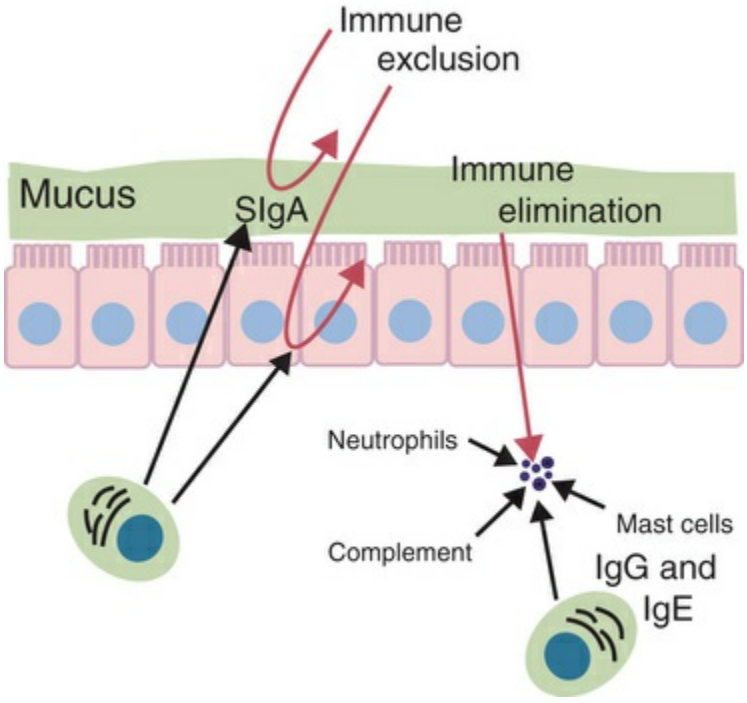


FIG. 22.4 Two key defensive mechanisms are employed on mucosal surfaces. The most important is immune exclusion, an effect primarily mediated by IgA. If antigens gain access to the mucosa, they are destroyed through IgG- and IgE-mediated processes by immune elimination.

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Many cells may be washed out of the airways of the lung with saline. In normal dogs, about 80% of bronchoalveolar cells obtained in this way are macrophages, and 13% are lymphocytes, of which about half are T cells (Table 22.1). In healthy horses, about 50% of the cells in bronchoalveolar washes are macrophages, 40% are lymphocytes, and 2% are neutrophils. In sheep, B cells are less than 10% of the lung lymphocyte population. Lung T cells can produce cytokines, and alveolar macrophages are activated following infection with *Listeria monocytogenes.* Cell-mediated immune reactions are therefore readily provoked among the cells within the lower respiratory tract.

TABLE 22.1
Composition of Cells in Canine Bronchoalveolar Lavage Fluid

Cell	Percentage (Range)
Macrophages	79.4 (71-87)
Lymphocytes	13.5 (7-20)
Eosinophils	3.6 (0-14)
Mast cells	2.1 (0-5)
Epithelial cells	0.8 (0-6)
Neutrophils	0.6 (0-2)
Lymphocyte Percentages	
T cells	52.0 (34-69)
$CD4^+$	21.9 (10-32)
$CD8^+$	17.8 (6-25)
CD4/CD8 ratio	1.3 (0.8-2.4)

From Vail DM, Mahler PA, Soergel SA: Differential cell analysis and phenotypic subtyping of lymphocytes in bronchoalveolar lavage fluid from clinically normal dogs, *Am J Vet Res* 56:282-285, 1995.

Alveolar macrophages reside on alveolar surfaces where they are in direct contact with the air. When they respond to invaders it is essential that they do not interfere with gas exchange. Thus a full-scale inflammatory response is to be avoided whenever possible. For this reason, in the absence of infection, alveolar macrophages are quiescent and tend to suppress local cytokine production. They are, however, highly phagocytic. They are also the major producers of type I interferons that in turn induce inflammation.

The lungs of most domestic species (pigs, horses, sheep, goats, cattle, cats) differ from rodent, human, or dog lungs in that they contain large numbers of intravascular macrophages (Chapter 6). It has been estimated that these macrophages cover 16% of the lung capillary surface in young pigs. As a result, the lungs of these

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species can remove more bacteria from the blood than can the liver and spleen. There is debate as to whether lung macrophages are effective antigen-presenting cells. A dense network of dendritic cells is found within airway epithelium and alveoli.

Although it has long been believed that the respiratory tract is sterile, it too has a normal bacterial microbiota. The composition of this microbiota has a direct influence on the development of allergic respiratory disease (Chapter 21). The lung is exposed to an array of microorganisms with the very first breath an animal takes. Despite this, it does not develop extensive inflammation. The early defense of the lung is almost totally dependent on innate immune pathways. The inhaled organisms trigger signaling cascades that result in the generation of surfactants, defensins, interferons, lactoferrin, and oxidants. These are critical protective pathways.

Immunity in the Urogenital Tract

In the urinary system, the flushing action and low pH of urine generally provide adequate protection; however, when urinary stasis occurs, urethritis resulting from the unhindered ascent of pathogenic bacteria is not uncommon.

The female reproductive tract can be divided into a lower part (vagina and cervix) and an upper part (uterus and fallopian tubes). The lower part is covered by stratified squamous epithelium, the upper by columnar epithelium. All are covered by microbiocidal mucus. Within the lower part, the keratinocytes express PRRs and produce cytokines and antimicrobial peptides. The upper part contains large numbers of macrophages, dendritic cells, and innate lymphoid cells. The predominant immunoglobulin in cervicovaginal mucus is IgA, whereas within the uterus, it is IgG. If bacteria such as *Campylobacter fetus* infect the genital tract, vaginal IgA antibodies immobilize and agglutinate the organisms. If the mucous membrane becomes inflamed, IgG antibodies from serum will also assist in protection. Surfactant protein A is also important in protecting the vagina from infection. *C. fetus* infections are associated with the presence of many mononuclear cells as well with delayed skin reactions (type IV hypersensitivity) so that cell-mediated immunity is also involved in resistance to this local

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infection. Similar local immune responses may also be directed against other organisms that infect the cervix and vagina, and the presence of agglutinating antibodies in vaginal mucus may be used as a diagnostic test for brucellosis, campylobacteriosis, and trichomoniasis. (The local immune response to trichomoniasis is largely mediated by IgE; see Chapter 28.) IgG also reaches the uterine lumen and the vagina by active transport mediated by FcRn. This receptor is pH dependent. It binds IgG in the tissues where the pH is high and releases it in the vagina where the pH is very low.

A type I interferon, designated interferon-ε, is expressed in the epithelial cells of the female reproductive tract. IFN-ε induces typical IFN-regulated genes. It is not, however, induced by the conventional pattern recognition pathways involving receptors such as the TLRs. Instead, it is constitutively expressed and hormonally regulated. IFN-ε–deficient mice show increased susceptibility to sexually transmitted infections such as herpes simplex 2 and *Chlamydia muridarum,* suggesting that this interferon plays a role in protecting the female reproductive tract. IFN-ε has been identified in humans, cattle, pigs, and dogs.

Antimicrobial peptides are found in testes, seminal vesicles, and prostate. Epithelial cells lining the urethra express PRRs and macrophages and dendritic cells are abundant. IgG is the predominant immunoglobulin in seminal plasma and IgA is also present. The B cells that produce these immunoglobulins are mainly found in the penile urethra and prostate. T cells are also abundant in the urethra, testes and prepuce. Preputial washings of bulls infected with *C. fetus* may contain IgG1 antibodies with some IgM and IgA. IgA is present in small amounts in normal urine, produced presumably by B cells in the walls of the urinary tract.

Immunity in the Gastrointestinal Tract

The layer of enterocytes lining the gastrointestinal tract is the largest surface between the body and the external environment. Thus the gastrointestinal tract, from mouth to anus, is a potential route of microbial invasion. This includes not only potential pathogens but also the commensals of the normal microbiota. Thus

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a mucosal "firewall" must be established to discourage invasion.

Saliva is rich in IgA and hence protects the mouth against infections. Small amounts of IgG are secreted into the crevicular groove between the gums and the base of the teeth. As a result, it has proved possible to make a vaccine against caries-causing bacteria (Box 22.1). Immunization of dogs with these organisms reduces microbial colonization of this area and prevents plaque formation and periodontitis. The flushing activity of saliva may be complemented by the generation of peroxidases from streptococci. The tonsils also produce much IgA, but because of the thin epithelium over the tonsillar clefts, they are very vulnerable to microbial invasion (Fig. 22.5).

Box 22.1

Periodontal Disease

Gingivitis and progressive chronic periodontitis are common diseases in animals. The tissue damage that occurs in periodontal disease is mediated by cells of the immune and inflammatory systems. The disease is triggered by many oral bacteria, the most important of which is *Porphyromonas gingivalis*. But the tooth microbiota are diverse and the composition of the population varies over time. These bacteria form biofilms on the surface of dental plaque. The PAMPs from these bacteria act through TLRs and other PRRs to attract neutrophils. The neutrophils, however, cannot phagocytose biofilms, so they undergo abortive phagocytosis and release their contents into the tissues. These released enzymes, especially collagenases, initiate progressive tissue destruction. In addition, local mast cells secrete TNF-α that contributes to neutrophil immigration. The gingival fluid contains complement components, especially C3a and C5a. The biofilm ensures that inflammation persists and chronic inflammation leads to chronic tissue destruction. Neutrophils are followed by macrophages and lymphocytes by about four to seven days. By day 21, 70% of the cellular infiltrate consist of lymphocytes, mainly T cells. $CD4^+$ cells increase progressively through the course of the disease and the lesion resembles a delayed hypersensitivity reaction (Chapter 33). Th1 and Th17 cells predominate. The Th17

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cells produce a cytokine mixture that promotes bone resorption. IL-17 especially promotes osteoclast activation, while IL-10 and TGFβ1 are downregulated. IL-1β and IFN-γ from the Th1 cells also promote osteoclast production and bone resorption. Since both Treg and Th17 cells are present in inflamed periodontal tissues, it is believed that an imbalance in these T cell populations, especially a deficiency in Treg cells, contributes to the chronic inflammatory process. For example, both IL-17 and IL-35 are expressed in inflamed periodontal tissue, and it has been demonstrated that if Treg populations in dogs with experimental periodontal disease are enhanced, the progress of disease is slowed. The bone resorption and soft tissue destruction are mediated by host matrix metalloproteases and collagenases. The matrix metalloproteases degrade extracellular matrix, especially type I collagen, and basement membrane components in the periodontal supporting tissues. Oxidative and proteolytic activation cascades destroy both tissues and bone and give rise to the gross lesions associated with periodontal disease.

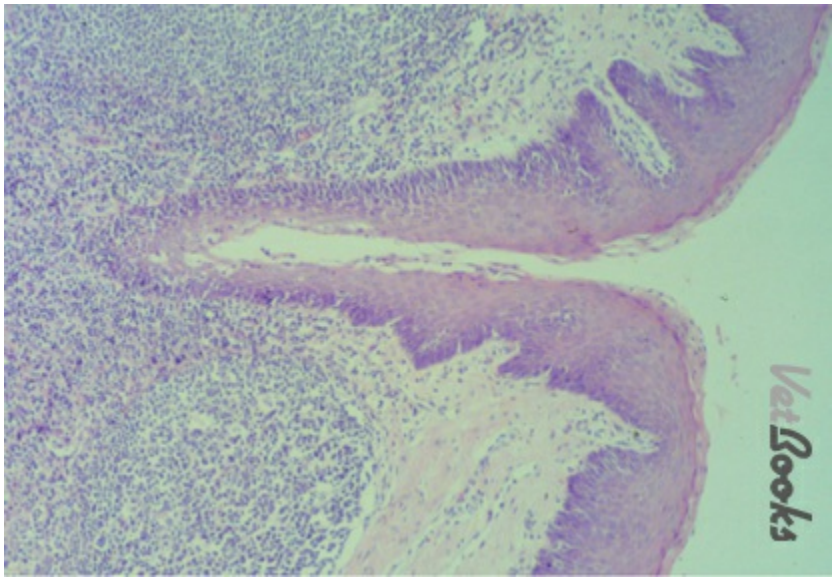


FIG. 22.5 A section of pig tonsil showing a tonsillar crypt. Note how thin the epithelium is at the base of the crypt. This is an easy invasion route for many organisms. Original magnification ×150.
(Courtesy Dr. S. Yamashiro.)

In single-stomached animals, the gastric pH may be sufficiently low to have an antimicrobial effect, although this varies among

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species and among meals. The dog, for instance, has a low gastric pH relative to that of the pig. Similarly, the pH in the center of a mass of ingested food may not necessarily drop to low levels, and some foods such as milk are potent buffers. In addition to antimicrobial peptides, lysozyme is synthesized in the gastric mucosa and in macrophages within the intestinal mucosa. As a result, it is found in large quantities in intestinal fluid.

In the small intestine, separation of the microbiota and enterocytes is maintained by a layer of mucus containing multiple antimicrobial proteins. In the large intestine this separation is maintained by two distinct layers of mucus. The inner layer is almost bacteria free. The outer loose layer contains large numbers of bacteria. In the developing animal it takes time for these mucus layers to develop, and this provides a window of opportunity for organisms such as segmented filamentous bacteria (SFB) to reach and bind enterocytes.

The first cellular barrier to microbial invasion is the intestinal epithelium. This consists of enterocytes, goblet cells, and Paneth cells (Fig. 22.6). Collectively, these cells form an effective physical barrier by having tight junctions between cells and a coating of attached mucin glycoproteins that form a glycocalyx. In addition to the goblet cells that produce the mucus, enterocytes can produce a diverse mixture of antimicrobial peptides that limit microbial exposure. In the small intestine, these are predominantly α-defensins and RegIIIα and β, while in the colon these are β-defensins and cathelicidins.

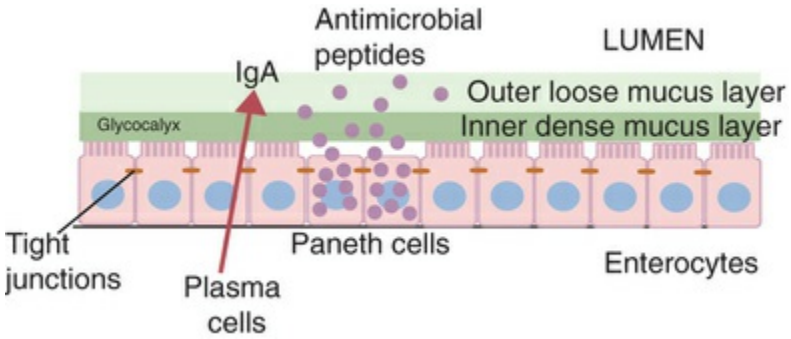


FIG. 22.6 Some of the mechanisms involved in the protection of mucosal surfaces. Paneth cells produce antimicrobial peptides and plasma cells produce IgA, while enterocytes, mucus layers and the glycocalyx form a protective barrier.

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Paneth cells are specialized intestinal epithelial cells that express TLRs (Fig. 22.7). When triggered by microbiota-associated molecular patterns (MAMPs), they secrete large quantities of α-defensins. Most of these defensins are amphipathic molecules that act like detergents. They insert themselves into microbial cell walls and cause bacterial lysis and interfere with bacterial lipid synthesis. One defensin, the α-6 defensin, acts in a very different manner. It does not have direct antibacterial effects but when released from the Paneth cell granules, it assembles spontaneously into elongated molecular networks— nanonets that surround, entangle, and entrap bacteria. Presumably, by so doing, they help protect the intestinal surface from bacterial invasion.

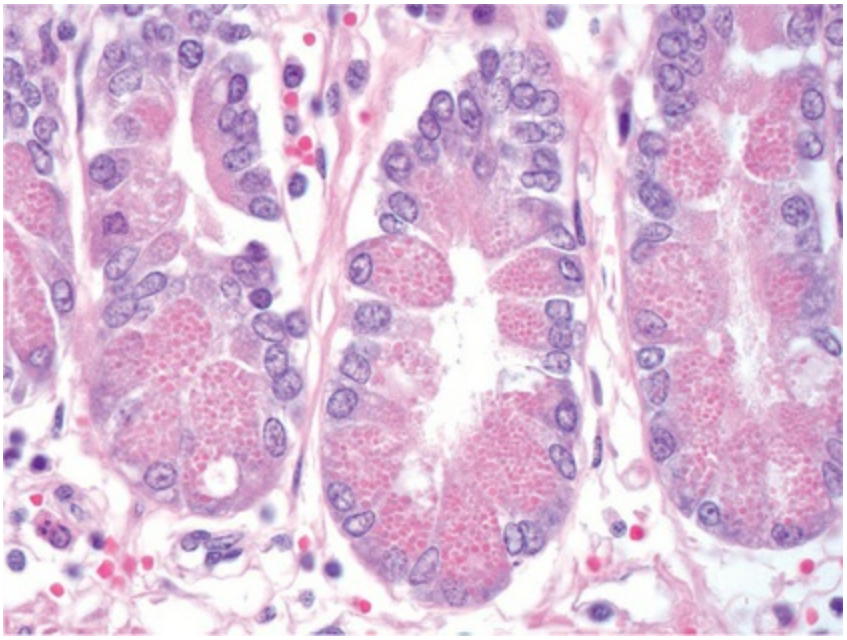


FIG. 22.7 Paneth cells from the intestine of a horse. The cells are filled with large eosinophilic granules and are the major source of intestinal defensins. Original magnification ×60. (Courtesy Dr. B. Porter.)

Enteric defensins accumulate within intestinal crypts and achieve very high concentrations in the mucus layer closest to the epithelium. They serve a barrier function since they prevent commensals from entering the crypt space and so reduce microbial contact with enterocytes. The defensin mixture selectively kills some bacterial species and as a result also regulates the composition of the microbiota. In cattle, expression of defensin genes occurs

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throughout the small intestine and colon. Bovine defensins are secreted as active molecules, as opposed to the human and mouse molecules that are secreted as inactive precursors and subsequently activated by trypsin within the intestine. Parasitic or other intestinal infections may increase production of α- and β-defensins.

Enterocytes possess a complete set of PRRs and can sense signals coming from the microbiota. They can then pass on these signals to the immune cells in the lamina propria and are actively involved in shaping the intestinal immune environment. They help maintain balance between antiinflammatory and proinflammatory signals. They also block access of intact antigens to the lamina propria. Tight junctions between enterocytes help maintain this barrier. (Molecules larger than about 2 kDa are excluded.) Thus they keep the intestinal microbes away from the intestinal immune system. Enterocytes secrete or respond to regulatory cytokines. Thus they produce thymic stromal lymphopoietin (TSLP) that is required for the generation of Tregs. They also produce TGF-β and the B cell stimulating molecules, BAFF and APRIL (see Box 15.2). The enterocytes form a major barrier against microbial invasion. If an enterocyte becomes infected, it can respond by releasing antimicrobial peptides and increasing mucin and cytokine production. It can also respond by undergoing inflammasome-mediated cell death. This mechanism is used by enterocytes to block invasion of *Salmonella enterica* serovar Typhimurium. Inflammasomes are protein activation complexes, some of which can trigger a form of cell death called pyroptosis. When *S.* Typhimurium invades enterocytes, the number of intracellular bacterial colonies increases for the first 12 hours and then begins to decline at 18 hours. This decline is correlated with the extrusion of infected enterocytes into the lumen. At the same time, inflammasome activation also results in cytokine secretion that in turn activates Th17 cells and promotes local inflammation.

The gastrointestinal mucus layer is also critical to the exclusion of both commensals and pathogens. The layer consists of a gel made of mucins, glycoproteins, and lipids that prevents bacteria from contacting the epithelium. This mucus acts as a lubricant, blocks chemical insults, and can capture and then expel pathogens. The mucus forms an inner and outer layer. The inner layer next to the

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enterocytes consists of firm mucus rich in defensins and lysozyme and contains few bacteria. The looser mucus in the intestinal lumen is composed primarily of mucins produced by intestinal goblet cells. Many bacteria are embedded in this mucus, where it prevents their washout. Its thickness and composition vary, but it tends to be thickest where the microbiota is abundant. The enterocyte brush border is also covered by a glycocalyx, a layer of acidic polysaccharides and glycoproteins that binds to the apical surface of the cells and serves as a protective barrier while still permitting the absorption of nutrients.

Goblet cells are the source of intestinal mucus. Both the inflammasome and autophagy pathways control the production of this mucus. In the absence of inflammasomes, mucus production is reduced and mice develop severe colonic inflammation. Autophagy proteins also regulate mucus production. Upon consideration, this should not come as a surprise to most people, especially veterinarians. Mucus secretion increases greatly in response to mucosal inflammation—it's called a “runny nose”!

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Mucosal Lymphoid Tissues

Because of the importance of preventing invasion through the mucosa, these surfaces contain large amounts of lymphoid tissue. Mucosal lymphoid tissues fall into two groups: sites where antigens are processed and immune responses are initiated (inductive sites), and sites where antibodies and cell-mediated responses are generated (effector sites).

Inductive Sites

The mucosa-associated lymphoid tissues (MALTs) possess the three cell types required to initiate adaptive immune responses: T cells, B cells, and dendritic cells. These tissues include lymphoid tissues in the eyelids, nasal mucosa, tonsils, pharynx, tongue, and palate (collectively called Waldeyer's ring); Peyer's patches; solitary lymphoid nodules; the appendix in the intestine; and numerous lymphoid nodules in the lung. These lymphoid tissues are known by their acronyms. Thus GALT (gut-associated lymphoid tissue) is the collective term for all the lymphoid nodules, Peyer's patches, and individual lymphocytes found in the intestinal walls. Similarly, BALT is the acronym used for the bronchus-associated lymphoid tissue in the lungs. These organized lymphoid tissues, unlike lymph nodes, do not react to foreign antigens delivered through afferent lymph but rather sample them directly from the surface.

The tonsils are especially important in inducing immunity on mucosal surfaces. Some organisms, however, can overcome the defenses of the tonsils and use them as a portal of entry into the body. For example, pathogens such as bovine herpesvirus-1, *Mannheimia hemolytica, S. suis,* and *Mycobacterium tuberculosis* can persist indefinitely within the tonsils.

Peyer's patches are the most significant of the mucosal lymphoid tissues. A newborn calf normally has about 100 Peyer's patches, and these may cover as much as half of the ileal surface. In ruminants and pigs, there are two types of Peyer's patch that differ in location, structure, and function. The ileocecal Peyer's patches may be primary lymphoid organs in some species, whereas the jejunal

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Peyer's patches are secondary lymphoid organs (see Fig. 12.6). In lambs, the ileocecal patches increase in size from birth to 6 months of age and then regress, leaving only a small scar. In contrast, the jejunal patches persist throughout adult life and continue to play a major role in intestinal defense.

Both types of Peyer's patch consist of masses of lymphocytes arranged in follicles and covered with an epithelium that contains M cells. M cells are specialized epithelial cells involved in antigen transportation. They have microfolds (M) rather than microvilli on their surface (Fig. 22.8). The mucus layer tends to thin out over Peyer's patches so that the M cells protrude into the lumen. M cells endocytose the proteins and microbes they encounter, but rather than destroy them, they transport the antigens to their underlying lymphoid tissue. M cells may also transport soluble macromolecules such as IgA, small particles, and even whole organisms. (Some pathogens, such as salmonellae, *Yersinia* and *Listeria* species, *M. tuberculosis,* and the reoviruses may take advantage of the M cells and use them to gain access to the body.) The proportion of M cells in the follicle-associated epithelium varies from less than 10% in humans and mice to 50% in rabbits and to 100% in the terminal ileum of pigs and calves.

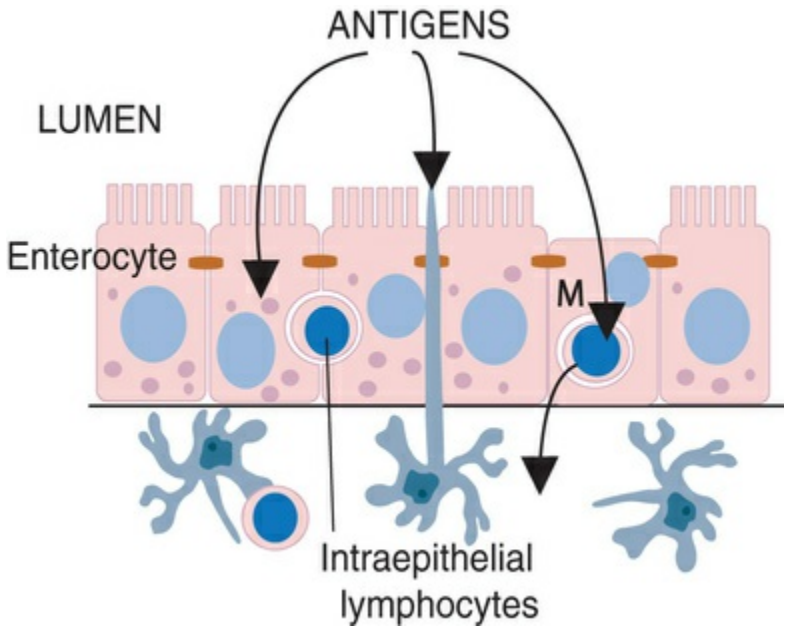


FIG. 22.8 The role of M cells and dendritic cells as antigen-processing cells in the intestinal wall. Antigen that enters enterocytes is usually rapidly degraded in lysosomes. Antigen that

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enters M cells is not degraded. It may be presented directly to intraepithelial lymphocytes within the M cell or, alternatively, permitted to pass along the intercellular space to the tissue fluid. From here, it will be carried to the draining lymph nodes.

Artiodactyls possess gut-associated lymphoid tissues that develop at the extreme end of the ileum during fetal life. They contain ~90% B cells with very few T cells and involute within weeks after birth. A similar lymphoid organ called the sacculus rotundus develops in rabbits. It was long believed that these lymphoid organs in the lower hind gut are the primary lymphoid organs where B cells develop. However, recent studies on piglets have cast doubt on this. Removal of the piglet ileal Peyer's patch does not result in a B cell deficiency such as would occur in a bursectomized bird. There are no differences in B cell diversity, distribution, or repertoire in piglets where this organ has been removed. In addition, there is no evidence that B cell diversification occurs within this organ. Evidence now suggests that in pigs at least, the ileal Peyer's patch is a secondary mucosal lymphoid organ that regulates the initial microbial colonization of the lower bowel. There may be no discrete organ equivalent to the bursa in these mammals.

Many dietary components have a profound effect on the organization and maintenance of intestinal immune tissues. For example, vitamin A metabolites play a role in the differentiation and functioning of B and T cell subsets. Likewise, dietary vitamin D alters B and Th responses, inhibiting Th1 and enhancing Th2 activity. Dietary lipids influence prostaglandin and leukotriene synthesis and so modify inflammatory responses. Collectively, these and other dietary components play a key role in ensuring that the intestinal lymphoid tissues function optimally.

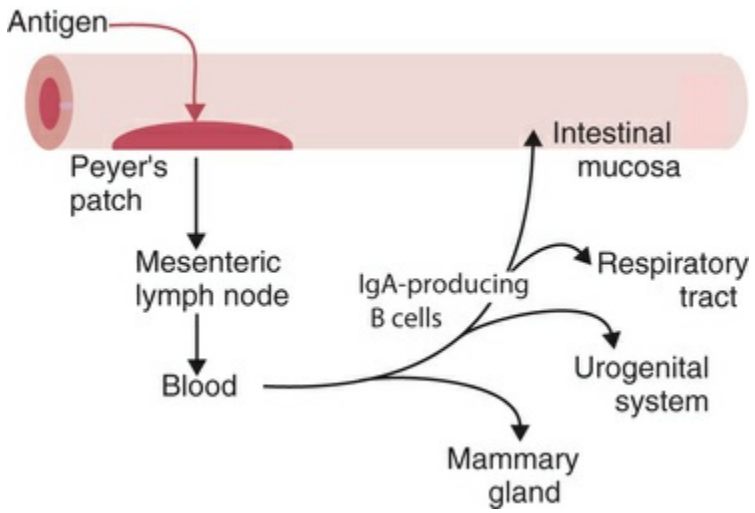
Effector Sites

Although Peyer's patches are full of lymphocytes, most IgA is produced in diffuse lymphoid nodules and in isolated plasma cells scattered throughout the walls of the intestine, in bronchi, in salivary glands, and in the gallbladder.

B Cells

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B cells respond to antigen that penetrates the enterocyte barrier. Some of these responding B cells migrate to regional lymph nodes and into intestinal lymphatics, from which they reach the thoracic duct and enter the bloodstream. These circulating IgA-positive B cells have an affinity for all body surfaces. As a result, they colonize not only in the intestinal tract but also at the respiratory tract, urogenital tract, and mammary gland. Thus antigen priming at one location will permit antibodies to be synthesized and secondary responses to occur at locations remote from the priming site, reflecting the existence of a common mucosal immune system (Fig. 22.9). The movement of IgA-positive B cells from the intestine to the mammary gland is especially important since it provides a route by which antibodies directed against intestinal pathogens can be transferred to the newborn through milk. Oral administration of antigen to a pregnant animal will thus result in the appearance of IgA antibodies in its milk. In this way, antibodies directed against intestinal pathogens will flood the intestine of the newborn animal. T cells originating within the Peyer's patches will also home specifically to the intestinal mucosa by using specialized vascular adhesive molecules. For example, mucosal addressin cell-adhesion molecule-1 (MAdCAM-1) is an adhesion molecule expressed on the high endothelial venules of Peyer's patches and on venules in intestinal lamina propria and the mammary gland. Its ligand is the lymphocyte integrin $\alpha 4/\beta 7$. B and T cells that express this integrin migrate preferentially to the intestine and the mammary gland.



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FIG. 22.9 When stimulated by antigen, IgA-producing B cells are produced in inductive sites, such as the Peyer's patches. They then leave the intestine and circulate in the bloodstream. They all eventually settle on other surfaces, such as the lung, the mammary gland, and other regions of the gastrointestinal tract effector sites, reflecting the existence of a common mucosal immune system. This transfer of antibody-producing cells in the mammary gland ensures that milk contains IgA antibodies directed against intestinal pathogens.

T Cells

Both α/β and γ/δ T cells are found in the intestinal wall but in two very different locations. Thus α/β T cells are mainly scattered throughout the lamina propria and in Peyer's patches. γ/δ T cells, in contrast, are situated between enterocytes immediately below the mucosal surface, where they are known as intraepithelial lymphocytes (IELs) (Fig. 22.10). Their location suggests that they play an early role in the defense of the mucosa. IELs regulate host-microbial interactions at the intestinal mucosal surface and are critical components in preventing invasion by the commensal microbiota. IEL function is in turn regulated by many different cytokines including IL-2, -7, -15, and TGF-β.

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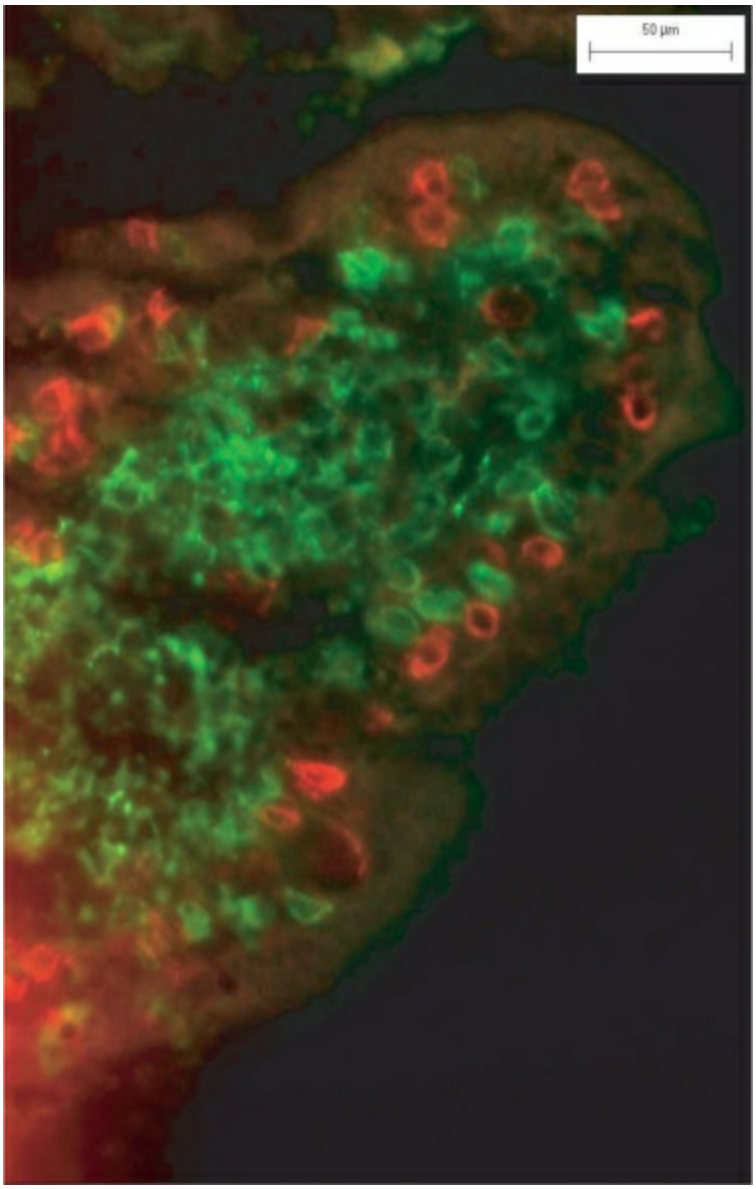


FIG. 22.10 Double-color immunofluorescence showing a canine duodenal villous tip stained with monoclonal antibodies to α/β TCR and γ/δ TCR. The α/β T cells are stained green and are located in the interior of the villus. The γ/δ T cells are stained red and are clearly located within the intestinal epithelium. (From German AJ, Hall EJ, Moore PF, et al: The distribution of lymphocytes expressing alpha/beta and gamma/delta T-cell receptors, and the expression of mucosal addressin cell adhesion molecule-1 in the canine intestine, *J Comp Pathol* 121:249-263, 1999.)

γ/δ IELs originate in the bone marrow and mature within cryptopatches, clusters of cells located just under the enterocytes. Cryptopatches each contain several hundred immature T cells. Located between epithelial cells, IELs can recognize antigens directly, possibly through the TLR-MyD88 pathway as well as their antigen receptors (TCRs), and secrete cytokines such as IFN-γ in response. The interferon in turn stimulates macrophages and

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nearby enterocytes to secrete protective nitric oxide.

There are major species differences in the properties of IELs. Five percent of IELs in humans, 50% in mice, and up to 90% in ruminants carry γ/δ TCR. A high proportion of the IELs are $CD8^+$ (85% in humans, 77% in pigs, 24% in sheep). These CD8 molecules are α/α homodimers, in contrast to the CD8 α/β heterodimers found on conventional α/β T cells. IELs tend to use unusual TRGV and TRDV genes to form the TCR antigen-binding site. These genes are not expressed in other lymphoid organs, suggesting that the intraepithelial T cells are specialized for epithelial surveillance. IELs are MHC class II positive and may act as antigen-presenting cells. They regulate B cell IgA responses. Some have natural killer (NK) cell activity, whereas others are cytotoxic T cells that may attack parasites within the intestinal lumen. They also play a role in the repair of damaged epithelia.

Some of the γ/δ T cells in the intestine can produce IL-17. There are two different sets of these cells. ILC3s that produce IL-17 spontaneously and Th17 cells that produce it following antigen exposure and are presumably proinflammatory (Chapter 21).

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Adaptive Protective Mechanisms

Both antibody- and cell-mediated immune processes protect body surfaces. The antibodies produced on mucosal surfaces include IgA, IgM, IgE, and IgG. Some of these, most notably IgA and possibly IgM, act by immune exclusion (see Fig. 22.4). The others, especially IgE and IgG, destroy antigen within the surface tissues by immune elimination.

Immune Exclusion

Immunoglobulin A

IgA predominates in surface secretions. At least 80% of all plasma cells are found in the lamina propria, and together they produce more immunoglobulin A than all other immunoglobulin isotypes combined. IgA is found in enormous amounts in saliva, intestinal fluid, nasal and tracheal secretions, tears, milk, colostrum, urine, and the secretions of the urogenital tract (Fig. 22.11; Table 22.2).

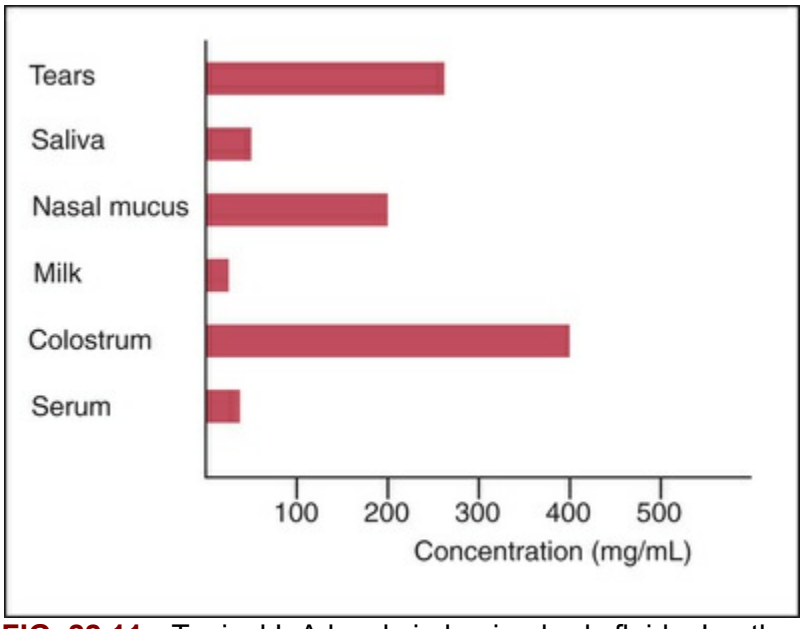


FIG. 22.11 Typical IgA levels in bovine body fluids. In other species, milk and colostral IgA concentrations may be considerably higher.

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TABLE 22.2

Approximate IgA Levels in the Serum and Various Secretions of Domestic Animals

SECRETION (mg/dL)						
Species	**Serum**	**Colostrum**	**Milk**	**Nasal Mucus**	**Saliva**	**Tears**
Horse	60-350	500-1500	50-1000	160	140	150
Cow	10-50	100-700	10-50	200	56	260
Sheep	10-50	100-700	5-12	50	90	160
Pig	50-200	1000	300-500	2-14	25-75	—
Dog	20-150	500-2200	110-620	—	—	—
Cat	30-150	150	240-620	—	54	—
Chicken	50	—	—	—	20	15

To undergo a class switch and thus produce IgA, mucosal B cells must also receive signals from other cells as well as the microbiota and retinoic acid from dietary vitamin A (Chapter 21). Some IgA production is T cell independent and so requires cytokines from dendritic cells and epithelial cells. Other B cells require help from Th2 cells to make the switch to IgA production.

T-dependent IgA production mainly occurs in Peyer's patches. Dendritic cells take antigen from M cells and use it to generate Tfh cells. CD40-ligand (CD40L) and IL-21 from these Tfh cells induce expression of activation-induced cytidine deaminase (AID). This promotes IgA class switch recombination. This pathway is stimulated by bacteria such as the SFBs attached to the enterocytes. The B cells generated by this pathway are persistent. They can re-enter germinal centers and undergo somatic mutation. Thus this IgA may be of very high affinity.

T-independent IgA production mainly occurs in the lamina propria and isolated lymphoid follicles under the influence of BAFF and APRIL. ILC3 cells also contribute to this process. The IgA produced is usually of low affinity and is directed primarily at the microbiota.

Follicular dendritic cells secrete soluble B cell–stimulating cytokines including BAFF and APRIL (Chapter 15). The dendritic cells themselves are activated by intestinal neuropeptides, such as vasoactive intestinal peptide (VIP). Eosinophils are required for the maintenance of plasma cells in the lamina propria. They are also a major source of APRIL. As a result, they directly support the B cell class switch and are required for both T-independent and T-dependent generation of IgA. These signals, together with CD40-

CD154 interactions, trigger B cell IgA production in the absence of antigen. Enterocytes and intraepithelial lymphocytes also produce APRIL and promote B cell differentiation. B cells that require both antigen and Th2 cells to make the switch to IgA are stimulated by TGF-β (Fig. 22.12). Other Th2 cytokines that promote this switch include IL-4, IL-5, IL-6, and even IL-10. The IgA response is relatively slow to develop and has a very high threshold for induction (10^9 bacteria). When boosted, the IgA response does not increase exponentially but rather in an additive manner. The plasma cells that synthesize IgA have other unique properties in that they also produce TNF-α and nitric oxide synthase and thus share some properties with monocytes. These additional products appear to contribute to the defenses of the intestine.

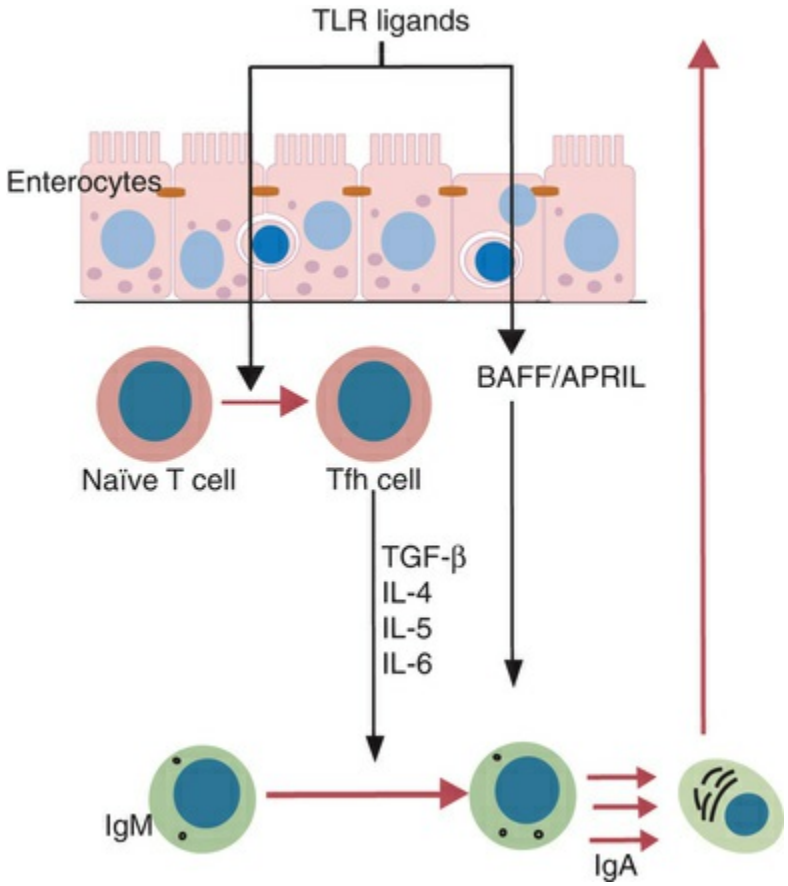


FIG. 22.12 The control of IgA production. Multiple Th2 cytokines, especially TGF-β, are primarily responsible for the IgM-to-IgA switch. Co-stimulation is provided by BAFF and APRIL from epithelial cells and dendritic cells as well as CD40-CD154 interactions.

IgA monomers are about 160 kDa in size and are typical four-chain, Y-shaped molecules (see Fig. 16.6). They are usually secreted as dimers or larger polymers linked by a J chain. IgA has several extra cysteine residues in its heavy chains. As a result, the short interchain disulfide bonds compact the chains and shield vulnerable bonds from proteases.

IgA is synthesized and secreted by plasma cells in the intestinal submucosa, especially in the crypt region. This dimeric IgA binds to a glycoprotein receptor (pIgR) on the basal surface of enterocytes (Fig. 22.13). The receptor binds covalently to the Cα2 domain of one of the IgA monomers. The membrane-bound IgA-pIgR complex is then endocytosed and actively transported across the enterocyte. When it reaches the exterior surface, this endocytic vesicle fuses with the plasma membrane and exposes the complex to the intestinal lumen. The extracellular domains of the pIgR are then cleaved by proteases so that the IgA, with the receptor peptide still attached (secretory IgA), is released into the lumen. The receptor peptide is called secretory component. The production, transport, and secretion of secretory component occur even in the absence of IgA so that free secretory component is found in high concentrations in intestinal contents.

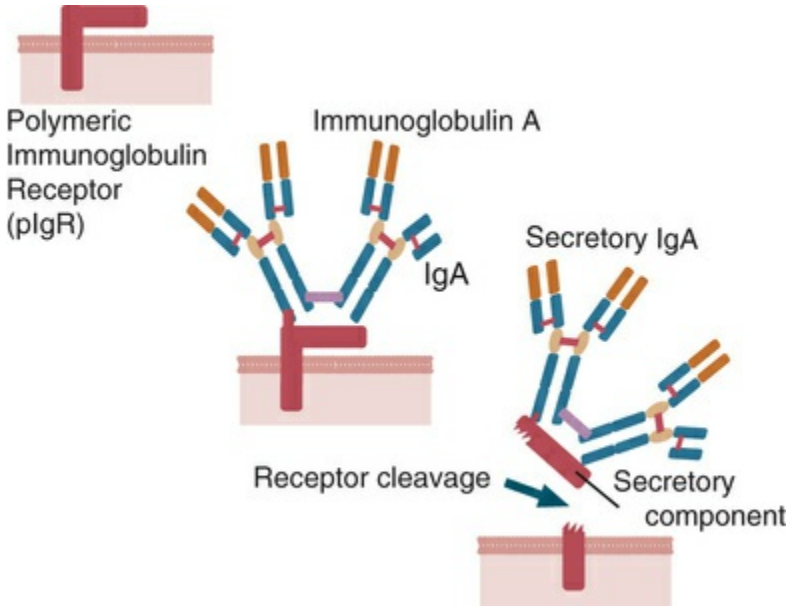


FIG. 22.13 IgA is secreted by mucosal plasma cells and binds to receptors (pIgR) on the interior surface of intestinal enterocytes. The bound IgA is taken into the enterocytes and passed in vesicles

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to the cell surface. Once in the intestinal lumen, the pIgR is cleaved from the cell and remains bound to the IgA. In this state it is called secretory component and serves to protect the IgA from degradation.

IgA is not bactericidal and does not activate complement. It can neutralize viruses and some viral and bacterial enzymes. IgA does not agglutinate bacteria within the gut. However, it cross-links daughter cells preventing their separation after division. As a result, the bacteria are chained together and their clearance accelerated. IgA-antigen complexes bind to monocytes and macrophages, neutrophils, and eosinophils through a low-affinity receptor, FcαR1 (CD89). When IgA-opsonized particles bind to this receptor, they trigger superoxide production, opsonization, antibody-dependent cell-mediated cytotoxicity, and the release of inflammatory mediators. Its most important function is to prevent the adherence of bacteria and viruses to epithelial surfaces—immune exclusion. If bacteria or viruses cannot adhere to enterocytes, they simply pass along with the intestinal contents and are expelled without doing any harm.

The presence of the gut microbiota also drives IgA production. Different commensals differ in their ability to induce secretory IgA. The gut microbiota is constantly being sampled at the inductive sites and this triggers the generation of new IgA-secreting plasma cells. Thus the IgA system is constantly responding to the microbiota. Conversely, most, but not all commensals are coated with IgA, and this IgA affects their composition. It is likely that the IgA-coated organisms are potential mucosal pathogens. For example, it preferentially coats the Enterobacteriaceae while ignoring the Bacteroides and Lactobacilli. SFBs are especially potent at triggering IgA responses.

Because IgA is transported through enterocytes, it can also act inside these cells (Fig. 22.14). Thus IgA can bind to newly synthesized viral proteins inside these cells and interrupt viral replication. In this way, the IgA can prevent viral growth before the integrity of the epithelium is damaged. This is a unique example of antibodies acting in an intracellular location. The second unique function of intracellular IgA is to excrete foreign antigens. Thus IgA can bind to antigens that have penetrated to the submucosa. Once

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bound, the IgA-antigen complexes will bind to pIgR and be actively transported back across the enterocytes into the intestinal lumen. IgA can therefore act at three different levels to exclude foreign antigens: within the submucosa, within enterocytes, and within the intestinal lumen.

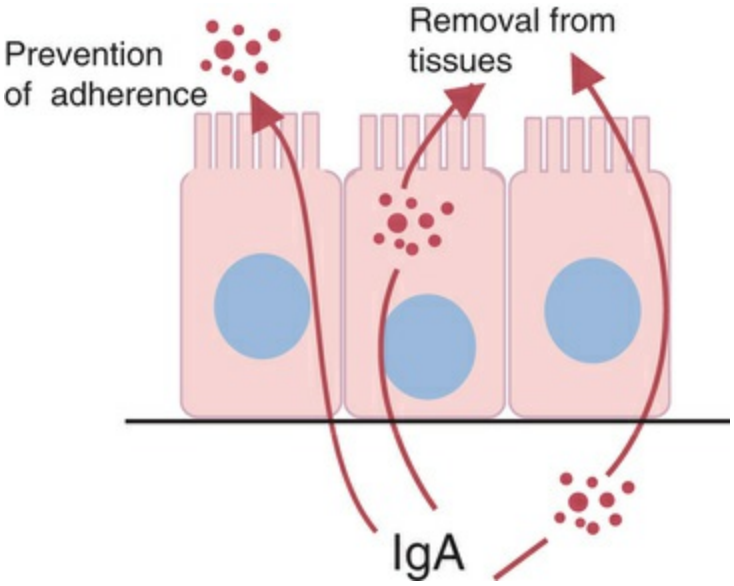


FIG. 22.14 IgA is unique in that it can act in three locations. It can bind antigen in tissue fluid or in enterocytes as well as in the intestinal lumen. The bound antigen in tissues or enterocytes is carried to the intestinal lumen.

Immunoglobulin M

The earliest immunoglobulins found in the intestine of the newborn are of the IgM class. IgM will also bind to pIgR and is carried through the enterocyte to the lumen. Because of its structure, however, secretory IgM is much more susceptible to proteases than secretory IgA.

Immune Elimination

Immunoglobulin E

Because IgA does not activate complement, it functions by immune exclusion. There is a second line of defense, however, that destroys antigen that penetrates the mucosal barrier (immune elimination).

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This is mediated by IgE. Cells producing IgE are mainly found under epithelial surfaces rather than in the lymph nodes or spleen. IgE binds to mast cell Fc receptors in the mucosa of the intestine and respiratory tract, and under the skin. If invading organisms evade the IgA and gain access to the tissues, IgE-mediated inflammation will be triggered (Fig. 22.15). These responses involve rapid degranulation of mast cells and the release of their inflammatory mediators into the surrounding tissues. As described in Chapter 29, the mast cell contents cause acute inflammation, increase the permeability of small blood vessels, and promote fluid leakage between enterocytes, leading to the outflow of mucus containing large quantities of IgG.

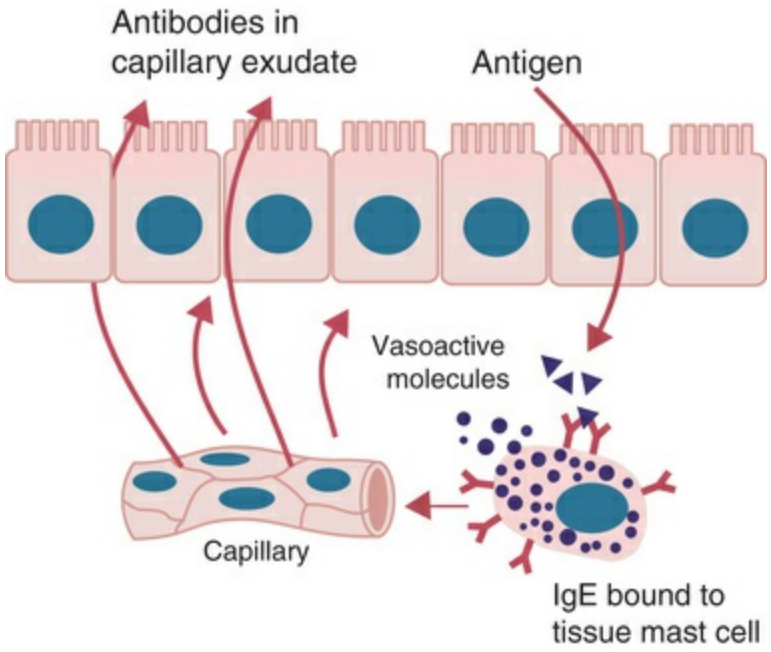


FIG. 22.15 The IgE response in the intestinal wall. Antigen reaches IgE-sensitized mast cells to cause their degranulation. As a result of this, vasoactive factors are released. These cause increased vascular permeability and exudation of serum IgG antibodies.

This process occurs, for example, when parasitic worms invade the intestinal mucosa. IgA has little effect on these invaders, so they have no difficulty in burrowing into the superficial layers of the mucosa. When sensitized mast cells encounter parasite antigens, however, the release of vasoactive molecules, together with the intense local inflammation, changes in blood flow, and intestinal

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motility may be sufficient to force the parasite to disengage—a phenomenon called "self-cure" (Chapter 28).

Thus IgA and IgE work in concert. IgA normally is the first line of defense, and IgE serves as a backup system. If IgA production is defective, the IgE response may be triggered to excess. As a result, low levels of IgA result in increased IgE production and the development of allergic responses to food and inhaled antigens.

Immunoglobulin G

In ruminants (especially cattle), IgG1, not IgA, is the major secretory immunoglobulin in colostrum and milk. This is due to selective transfer from the bloodstream into the mammary gland. On other body surfaces in ruminants, however, IgA remains the predominant immunoglobulin, although IgG1 is also present. IgG2 is also transferred into the intestine and saliva in ruminants. IgG may be of greater protective significance in the respiratory tract than in the intestine because there it is less likely to be degraded by proteases.

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Immunity to Food

Dietary antigens are not normally antigenic as a result of oral tolerance mediated by Treg cells. These peripheral Tregs (pTregs) are abundant in the intestine. Under normal conditions, dietary antigens generate small intestinal pTreg cells. These pTregs are distinctly different from the pTregs produced in response to the microbiota. For example, they are present in the intestine of germ-free mice. They are short-lived but very potent suppressive cells. There is evidence that a specialized subpopulation of intestinal dendritic cells is also required to stimulate this pTreg production and hence oral tolerance.

Secretory IgA responses are not usually generated against food antigens. Likewise, soluble food proteins are unlikely to trigger TLR responses. (Although TLR4-deficient mice readily develop food allergies.)

Another mechanism by which oral tolerance may be induced is through the production of "tolerosomes." These are exosomes produced by enterocytes. They carry MHC class II on their surface and this binds antigenic peptides sampled from the gut lumen. Purified tolerosomes fed to animals induce tolerance. It is suggested that presentation of food antigens by tolerosomes induces Treg formation.

It has been estimated that about 2% of ingested food protein is absorbed as peptide fragments large enough to be recognized by the immune system, although a very much smaller fraction of these molecules (<0.002%) is absorbed intact. This protein reaches the portal circulation, but little passes the liver and enters the systemic circulation. Presumably the Kupffer cells of the liver capture blood-borne food antigens. Antibodies produced locally may bind to this adsorbed antigen and generate immune complexes that are removed as the blood passes through the liver. If a calf is fed a defined dietary antigen such as soy protein, although it is initially well absorbed, the animal soon begins to make IgA antibodies to soy. Once antibodies are produced, immune exclusion occurs, and the amount of protein absorbed drops significantly. If another novel protein is introduced into the feed, it too will be initially absorbed

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until IgA is produced against it. Thus IgA may serve to exclude intact food antigens from the body. The extent to which normal animals make antibodies against proteins in their food has been unclear. Cats fed soy and casein produced high levels of IgG and IgA antibodies in their serum against both these proteins. For unknown reasons, proteins from canned food appear to be more immunogenic than unprocessed proteins.

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Vaccination on Body Surfaces

When animals are vaccinated against organisms that invade the intestinal or respiratory tracts, it makes sense to stimulate a mucosal IgA response. To do this, the vaccine antigen can simply be ingested or inhaled. Unfortunately, such vaccines are not always effective. Inactivated antigens fail to trigger an IgA response because they are immediately washed or sneezed off when applied to mucous membranes. Because of the abundant intestinal microbiota, intestinal IgA responses also have a high threshold, tend to lack memory, and tend to fade rapidly. The body tightly regulates antigen input across epithelial cells. Regulatory effects on IgA production constantly adapt the IgA response to the intestinal microbiota. The only way a significant IgA response can be triggered is to use live vaccines, in which the vaccine organism can invade mucous membranes. The vaccine must persist for a sufficient time to trigger an immune response yet not cause significant damage. Good examples of such vaccines are the respiratory tract vaccines against bovine or feline rhinotracheitis. Even some of these vaccines may cause a transient conjunctivitis or tracheitis. Other examples of effective live oral vaccines include polio vaccine in humans and transmissible gastroenteritis vaccine in piglets. Oral tolerance also remains a challenge for mucosal vaccines. Thus administration of some antigens to the respiratory or intestinal tracts may promote mucosal and T cell unresponsiveness.

Systemic vaccination against surface infections may provide adequate immunity (as in human influenza and polio vaccines) since some IgG may be transferred from serum to the mucosal surface. Indeed, many available vaccines simply work by stimulating high levels of IgG antibodies in blood. These are effective because once an invading organism causes tissue damage and triggers inflammation; the site of invasion is flooded by IgG. Nevertheless, this is not the most efficient way of providing immunity.

Once a protective IgA response has been generated, other difficulties may arise. For example, secondary immune responses are sometimes difficult to induce on surfaces, and multiple doses of

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vaccine may not increase the intensity or duration of the local immune response. This is not caused by any intrinsic defect but occurs because high levels of IgA can block antigen absorption and so prevent it from reaching antigen-presenting cells.

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23

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Immunity in the Fetus and Newborn

CHAPTER OUTLINE

Development of the Immune System, 248

Specific Animal Immune Systems, 248

Foal, 248

Calf, 248

Lamb, 248

Piglet, 249

Puppy, 249

Kitten, 249

The Immune System and Intrauterine Infection, 250

Immune Response of Newborn Mammals, 251

Role of the Intestinal Microbiota, 251

Innate Immunity, 251

Adaptive Immunity, 252

Transfer of Immunity From Mother to Offspring, 253

Secretion and Composition of Colostrum and Milk, 253

Absorption of Colostrum, 254
MicroRNAs and Intestinal Development, 255
Failure of Passive Transfer, 256
Production Failure, 256
Ingestion Failure, 256
Absorption Failure, 256
Diagnosis of Failure of Passive Transfer, 256
Management of Failure of Passive Transfer, 257
Cell-Mediated Immunity and Colostrum, 257
Development of Adaptive Immunity in Neonatal Mammals, 258
Local Immunity, 258
Systemic Immunity, 258
Vaccination of Young Animals, 259

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Describe the progressive development of the immune system in the developing mammal.
- Understand the state of development of the innate and adaptive immune systems at birth.
- Explain how newborn mammals are temporarily protected against infection by transfer of immunoglobulins from their mother.
- Describe how immunoglobulins are derived from the mother either by direct

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transfer across the placenta as in primates, or by ingestion of immunoglobulin-rich colostrum.
- Understand the immunological properties of colostrum and milk.
- Describe the absorption of colostrum from the neonatal intestine.
- Understand how colostrum protects against septicemic infections while milk protects against enteric infections.
- Understand the reasons for failure of passive transfer.
- Understand the principles of diagnosing and treating failure of passive transfer.
- Define colostrum and passive transfer.
- Understand why young mammals cannot be successfully vaccinated.
- Understand the role of the microbiota in the development of the neonatal immune system.

When a mammal is born, it emerges from the sterile uterus into an environment where it is immediately exposed to a host of microorganisms. Its surfaces, such as the gastrointestinal tract, acquire a complex microbial flora within hours. If it is to survive, the newborn animal must be able to control this microbial colonization. In practice, the adaptive immune system takes some time to become fully functional, and innate mechanisms are responsible for the initial resistance to infection. In some species with a short gestation period, such as mice, the adaptive immune system may not even be fully developed at birth. In animals with a long gestation period, such as large domestic mammals, the adaptive immune system is fully developed at birth but cannot function at adult levels for several months. The complete development of adaptive immunity depends on antigenic stimulation. The development of B cells and B cell receptor (BCR) diversity requires clonal selection and antigen-driven cell multiplication (Chapter 15). Thus newborn mammals are vulnerable to infection for the first few weeks of life. They need assistance in defending themselves at this time. This temporary help is provided by the mother's milk in the form of antibodies and possibly T cells. The passive transfer of immunity from mother to newborn is essential for survival.

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Development of the Immune System

The development of the immune system in the mammalian fetus follows a consistent pattern. The thymus is the first lymphoid organ to develop, followed by the secondary lymphoid organs. B cells appear soon after the development of the spleen and lymph nodes, but antibodies are not usually detectable until late in fetal life. The ability of the fetus to respond to antigens develops very rapidly once the lymphoid organs appear, but all antigens are not equally capable of stimulating fetal lymphoid tissue. The immune system develops in a series of steps, with each step permitting the fetus to respond to more antigens. These steps are driven by a gradual increase in the use of gene conversion or somatic mutation to increase antibody diversity. The ability to mount cell-mediated immune responses develops at the same time as antibody production. T cell receptor (TCR) diversity is also limited in the fetus and neonate, and their cytokine production may be low. This may simply be due to their lack of exposure to foreign or microbial antigens.

Specific Animal Immune Systems

Foal

The gestation period of the mare is about 340 days. Lymphocytes are seen first in the thymus at about 60 to 80 days post-conception. They are found in the mesenteric lymph node and intestinal lamina propria at 90 days and in the spleen at 175 days. Blood lymphocytes appear at about 120 days. A few plasma cells may be seen at 240 days. Graft-versus-host disease, a cell-mediated response, has developed in immunodeficient foals transplanted with tissues from a 79-day-old fetus. The equine fetus can respond to coliphage T2 at 200 days post-conception and to Venezuelan equine encephalitis virus at 230 days. VDJ sequence diversity increases as the fetus develops and as the foal develops into an adult. Newborn foals have detectable quantities of IgM and IgG and occasionally IgG3 in their serum, but IgE production in the horse does not begin until foals are 9 to 11 months of age. Like other large herbivores, the foal

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has a well-developed ileal Peyer's patch that serves as a primary lymphoid organ and eventually involutes. Major B cell markers are detectable by 90 to 120 days gestation. *IGHM* and *IGLC* transcripts are expressed in liver, bone marrow, and spleen at all ages. Thus gene recombination and immunoglobulin class switching occur during equine fetal life. As a result, small amounts of IgM and IgG are detectable at birth. Despite this competence, B cell functions may be actively suppressed by Treg cells during the first few months of a foal's life.

A significant increase in immunoglobulin diversity occurs during fetal life in the horse, especially through the last two-thirds of gestation. In the fetus, most diversity begins increasing about day 20 gestation and occurs in the third heavy chain complementarity determining region (CDR). In neonatal foals the diversity begins to increase in the second CDR as well. In adult animals, variation in the first CDR increases belatedly, although it is much less diverse than that in CDRs 2 and 3. Some V, D, and J genes are used predominantly throughout life, and changes in relative gene usage does not occur.

Calf

Although the gestation period of the cow is 280 days, the fetal thymus is recognizable by 40 days post-conception. The bone marrow and spleen appear at 55 days. Lymph nodes are found at 60 days, but Peyer's patches do not appear until 175 days (Fig. 23.1). Blood lymphocytes are seen in fetal calves by day 45, IgM^+ B cells by day 59, and IgG^+ B cells by day 135. The time of appearance of serum antibodies depends on the sensitivity of the techniques used. It is therefore no accident that the earliest detectable immune responses are those directed against viruses, using highly sensitive virus neutralization tests. Fetal calves have been reported to respond to rotavirus at 73 days, to parvovirus at 93 days, and to parainfluenza 3 virus at 120 days. Fetal blood lymphocytes can respond to mitogens between 75 and 80 days, but this ability is temporarily lost near the time of birth as a result of high steroid production. T cell subpopulations are present in calves at levels comparable to adults, but B cell numbers increase significantly during the first 6 months after birth. Calves acquire innate and IgM-

mediated immune competence within the first week of life. *IGHA* and *IGHG* expression reach adult levels by 14 to 28 days.

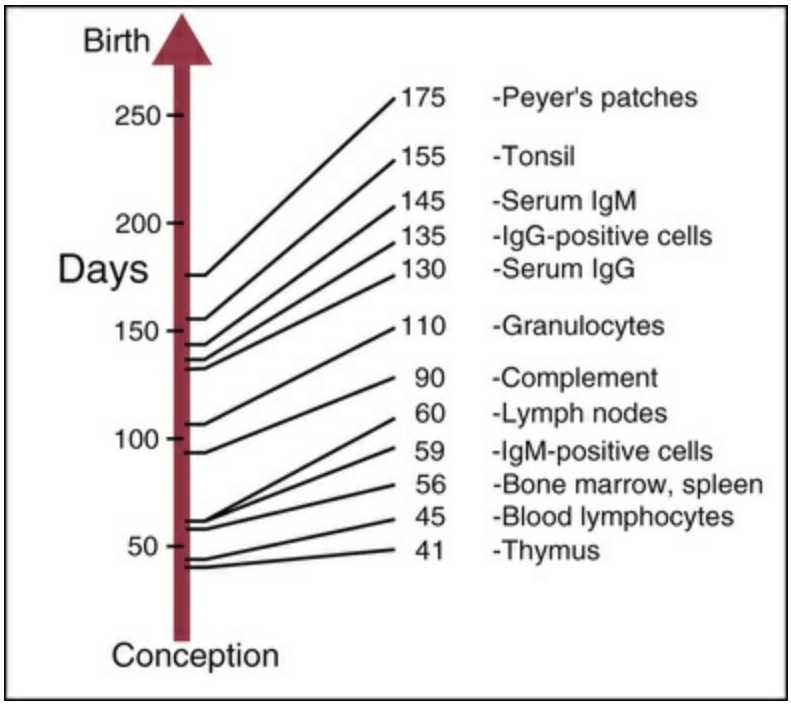


FIG. 23.1 The stages in the progressive development of the immune system in the fetal calf.

Lamb

The gestation period of the ewe is about 145 days. Major histocompatibility complex (MHC) class I^+ cells can be detected by day 19, and MHC class II^+ cells can be found by day 25. The thymus and lymph nodes are recognizable by 35 and 50 days post-conception, respectively. Gut-associated follicles appear in the colon at 60 days, jejunal Peyer's patches at about days 75 to 80, and ileal Peyer's patches at days 110 to 115. Blood lymphocytes are seen in fetal lambs by day 32, and $CD4^+$ and $CD8^+$ cells appear in the thymus by 35 to 38 days. B cells are detectable at 48 days in the spleen and by that time have already begun to rearrange their *IGLV* genes. C3 receptors appear by day 120, but Fc receptors do not appear until the animal is born. Fetal liver lymphocytes can respond to phytohemagglutinin by 38 days. Lambs can produce antibodies to phage ΦX174 at day 41 and reject skin allografts by

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day 77. Some fetal lambs can produce antibodies to Akabane virus by as early as 50 days post-conception. Antibodies to Cache Valley virus can be provoked by day 76, to SV40 virus by day 90, to T4 phage by day 105, to bluetongue virus by day 122, and to lymphocytic choriomeningitis virus by day 140. The proportions of α/β and γ/δ T cells change as lambs mature. Thus 1 month before birth, 18% of blood T cells are γ/δ positive. By 1 month after birth, they constitute 60% of blood T cells.

Piglet

The gestation period of the sow is about 115 days. B cells appear in the yolk sac at day 20, in the fetal liver by day 30, and in the bone marrow by day 45. The first $SWC3^+$ leukocytes can be found in the yolk sac and liver on day 17. The thymus develops by 40 days post-conception and is colonized by two waves of T cell progenitors beginning on day 38. γ/δ T cells appear first in the thymus and in peripheral blood about 10 days later. α/β T cells develop by day 55, but their numbers grow rapidly so that they predominate late in gestation. The intestinal lymphoid tissues are devoid of T cells at birth. $CD4^+$ T cells appear in the intestine at 2 weeks of age, and $CD8^+$ T cells appear at 4 weeks. Their proliferation appears to be driven by the intestinal microbiota. IgM^+ B cells can be found in the liver at 40 days, spleen by day 50, and bone marrow by day 60. Fetal piglets can produce antibodies to parvoviruses at 58 days and can reject allografts at about the same time. Blood lymphocytes can respond to mitogens between 48 and 54 days. Natural killer (NK) cell activity does not develop until several weeks after birth, although cells with an NK phenotype can be identified at 45 days' gestation in spleen and umbilical blood.

B cells are the first lymphocytes to appear in peripheral blood. The number of circulating B cells rises significantly between 70 and 80 days' gestation. The response to antigens in the fetus is of the IgM type, but newborn and fetal piglets also produce a small immunoglobulin that may not have light chains. It is interesting to note that B cells can be found in the thymus of newborn pigs.

The molecular development of the antibody repertoire has been followed in the developing pig. Thus VDJ rearrangement is first seen in the fetal liver at day 30. However, the fetal piglet does not

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initially use all its *IGHV* or *IGHD* genes. Likewise, N-region addition does not occur before day 40, suggesting that the onset of terminal deoxynucleotidyltransferase activity occurs after that time. IgM, IgA, and IgG transcripts are present from 50 days in all major lymphoid organs. Piglets are thus born with relatively limited B cell diversity. B cell numbers increase for the first 4 weeks after birth, but their antigen-binding repertoire does not begin to expand until 4 to 6 weeks of age.

The production of IgA is controlled by exposure to the intestinal microbiota (Box 23.1). A limited amount of B cell class switching occurs in the developing fetus so that, as a result, newborn piglets already possess some intestinal IgA. Thus piglets at birth are exposed to new antigens from the growing microbiota as well as from colostrum and milk. It is these antigens that initiate IgA production. At weaning, the animal is exposed to new dietary antigens and must also develop oral tolerance.

Box 23.1

The Inheritance of Fecal IgA Levels

In inbred mice, fecal IgA levels are highly variable. Some have high—while others have low—fecal IgA. If the microbiota of low-fecal IgA mice are transferred to high-fecal IgA mice, the recipients' fecal IgA levels promptly drop. So the quantity of IgA in the feces is determined by the microbiota. The bacteria from IgA-low mice appear to be able to degrade both secretory component and IgA. It is generally recognized that a young animal receives its microbiome from its mother. As a result, fecal IgA levels are vertically transmitted from the mother and just appear to be inherited. If this process occurs in domestic mammals, it may explain many of the enteric disease problems associated with intensive production of pigs and other species.

Lambda chain rearrangements can be detected in the developing yolk sac of piglets between 20 and 50 days' gestation well before kappa rearrangements. Junctional diversity within VDJ regions is limited at all stages of development. B cell lymphogenesis and gene rearrangements continue for at least 5 weeks postpartum. All of

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these features appear to be somewhat unique to the pig.

Puppy

The gestation period of the bitch is about 60 days. The thymus differentiates between days 23 and 33, and fetal puppies can respond to phage ΦX174 by day 40. Blood lymphocytes can respond to phytohemagglutinin by 45 days post-conception, and these cells can be detected in lymph nodes by 45 days and in the spleen by 55 days. The ability to reject allografts also develops at about day 45, although rejection is slow at this stage, and fetal puppies may be made tolerant by intrauterine injection of an antigen before day 42. Thymic seeding of T cells to the secondary lymphoid organs and the development of humoral immune responses are therefore relatively late phenomena in the dog compared with the situation in the other domestic mammals.

Kitten

Data on the ontogeny of the kitten are limited. Lymphocytes are seen in the blood at 25 days post-conception. B cells are seen in the fetal liver at 42 days. Fetal kittens do make some IgG that can be detected in their serum before suckling, although this may be due to antibodies crossing the placenta.

The Immune System and Intrauterine Infection

Although a fetus is not totally defenseless, it is less capable than an adult of combating infection. Its adaptive immune system is not fully functional; as a result, some infections may be mild or unapparent in the mother but severe or lethal in the fetus. Examples include bluetongue, infectious bovine rhinotracheitis (bovine herpesvirus 1 [BHV-1]), bovine viral diarrhea, rubella in humans, and toxoplasmosis. Fetal infections commonly trigger an immune response as shown by lymphoid hyperplasia and elevated immunoglobulin levels. For this reason the presence of any immunoglobulins in the serum of a newborn, unsuckled animal suggests infection *in utero.*

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In general, the response to these viruses is determined by the state of immunological development of the fetus. For example, if live bluetongue virus vaccine, which is nonpathogenic for normal adult sheep, is given to pregnant ewes at 50 days post-conception, it causes severe lesions in the nervous system of fetal lambs, including hydranencephaly and retinal dysplasia, whereas if it is given at 100 days post-conception or to newborn lambs, only a mild inflammatory response is seen. Bluetongue vaccine virus given to fetal lambs between 50 and 70 days post-conception may be isolated from lamb tissues for several weeks, but if given after 100 days, reisolation is not usually possible. Akabane virus acts in a similar fashion in lambs. If given before 30 to 36 days post-conception, it causes congenital deformities. If given to older fetuses, it provokes antibody formation and is much less likely to cause malformations. Piglets infected by parvovirus before 55 days post-conception will usually be aborted or stillborn. After 72 days, however, piglets develop high levels of antibodies to the parvovirus and survive. Prenatal infection of calves with BHV-1 results in a fatal disease, in contrast to postnatal infections, which are relatively mild. The transition between these two types of infection occurs during the last month of pregnancy.

The effects of the timing of viral infection are well seen with bovine viral diarrhea virus (BVDV). If a cow is infected early in pregnancy (up to 50 days), she may abort. On the other hand, infections occurring between 50 and 120 days, before the fetus develops immune competence, lead to asymptomatic persistent infection because the calves develop tolerance to the virus (Fig. 23.2). These calves are viremic yet, because of their tolerance, fail to make antibodies or T cells against the virus. Some of these calves may show minor neurologic problems and failure to thrive, but many are clinically normal. If the cow is infected with BVDV between 100 and 180 days post-conception, calves may be born with severe malformations involving the central nervous system and eye, as well as jaw defects, atrophy, and growth retardation. Calves infected after 150 to 180 days' gestation are usually clinically normal.

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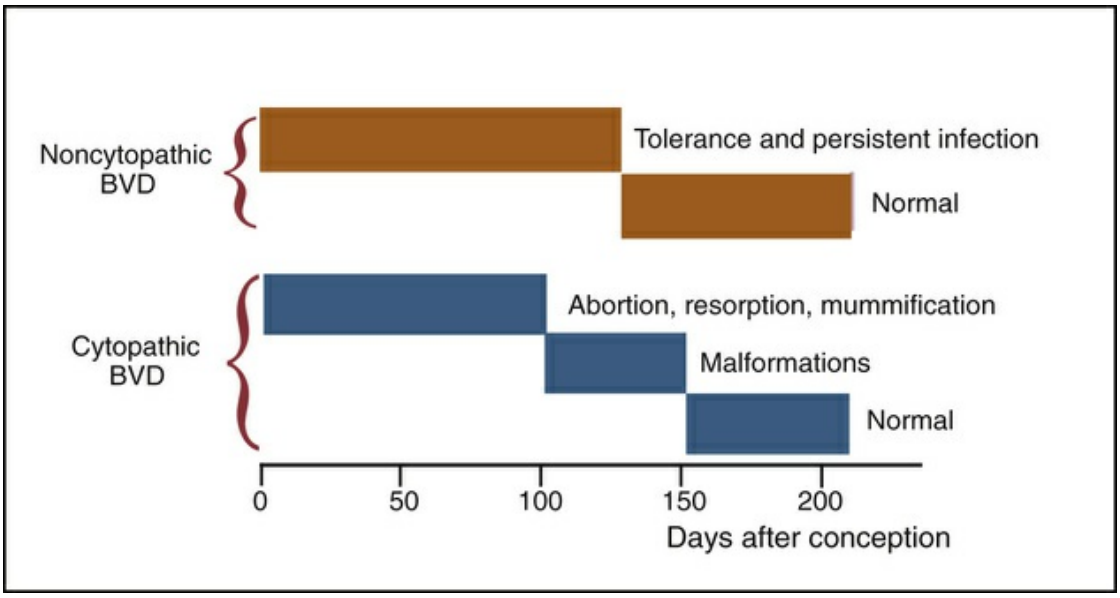


FIG. 23.2 The effects of bovine viral diarrhea virus infection on development of the fetal calf depend on the timing of infection. As with adult animals, there is considerable individual variation in resistance to infection. Persistently infected calves may show minor neurological problems or failure to thrive.

Since they are tolerant to BVDV, persistently infected calves shed large quantities of the virus in secretions and excretions. These calves may also produce neutralizing antibodies if immunized with a live BVDV vaccine of a serotype different from that of the persistent virus. Despite this, the original virus will persist in these animals. These persistently infected calves grow slowly and often die of opportunistic infections such as pneumonia before reaching adulthood. (BVDV has a tropism for lymphocytes and is immunosuppressive.)

BVDV occurs in two distinct biotypes: cytopathic and non-cytopathic. (The name derives from their behavior in cell culture, not their pathogenicity in animals.) Noncytopathic strains suppress type I interferon (IFN-α) production but permit type III (IFN-λ) production by plasmacytoid DCs. This type III interferon suppresses T cell responses and enables the virus to survive in calves and cause persistent infections by depleting $WC1^+$ γ/δ T cells. Cytopathic strains induce type I IFN production and cannot cause persistent infection. These cytopathic strains, however, do cause mucosal disease (MD), a severe enteric disease leading to profuse diarrhea and death (Fig. 23.3). Mucosal disease develops as a result of a mutation in a nonstructural viral gene that changes the BVDV

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biotype from noncytopathic to cytopathic while the animal fails to produce neutralizing antibodies or T cells. The cytopathic strain can spread between tolerant animals and lead to a severe mucosal disease outbreak. Both cytopathic and noncytopathic viruses can be isolated from these animals. Recombination may also occur between persistent noncytopathic strains and cytopathic strains in vaccines and lead to MD outbreaks. Although some of the lesions in MD are attributable to the direct pathogenic effects of BVDV, glomerulonephritis and other immune-complex–mediated lesions also develop. This may reflect superinfection or the production of non-neutralizing antibodies. Because persistently infected calves can reach adulthood and breed, it is possible for BVD infection to persist indefinitely within carrier animals and their progeny. Between 0.4% and 1.7% of cattle in the United States are persistently infected in this way.

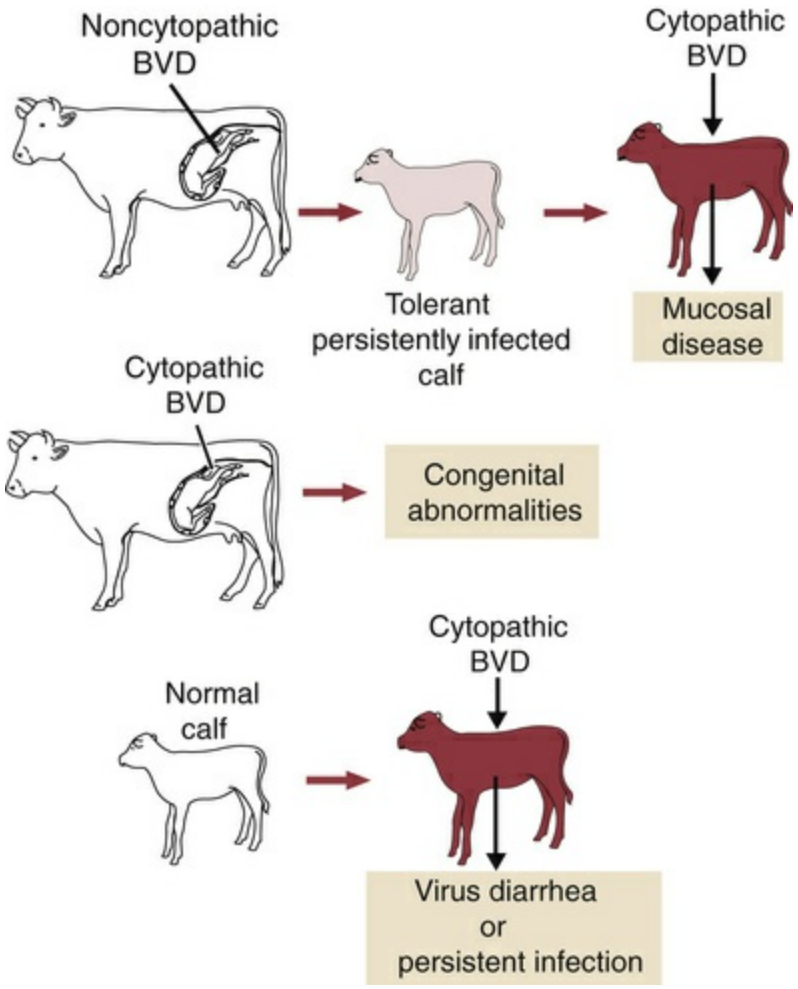


FIG. 23.3 The relationship of mucosal disease to persistent

infection with bovine viral diarrhea virus (BVDV) in tolerant cattle. Calves persistently infected with noncytopathic BVDV and then superinfected with cytopathic BVDV develop mucosal disease.

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Immune Response of Newborn Mammals

After developing in the sterile environment of the uterus, newborn mammals first encounter a diverse population of microbes at the time of birth (Box 23.2). They must be able to combat any attempted invasion immediately. Thus neonatal mammals are capable of mounting both innate and adaptive immune responses at birth. However, any adaptive immune response mounted by a newborn must be a primary response with a prolonged lag period and low antibody levels. Innate immune responses are therefore critical for survival in the first weeks of life.

Box 23.2

The Microbiota of the Placenta

It has been a widely held belief that the internal organs of the body are sterile. However, the rapid development of the microbiota in newborn infants in less than a week after birth has raised the possibility that they are somehow seeded by bacteria inside the body prior to birth. One possible source is the placenta. Gram-positive and –negative intracellular bacteria can be seen in the tissue layer at the maternal-fetal interface. Studies on placental tissues using DNA-based metagenomic studies have also detected a small, but unique placental microbiome. It has generally been assumed that these bacteria originate in the lower genital tract and ascend into the uterus. However, this microbiota appears to be more closely related to the oral microbiota in nonpregnant subjects! *E. coli* was the single most prevalent species in the placenta. It is no coincidence that *E. coli* can be found in meconium and is a major cause of early onset sepsis in newborns.

Aagaard K, Ma J, Antony KM, et al: The placenta harbors a unique microbiome, *Sci Transl Med* 6:237-246, 2014.

Role of the Intestinal Microbiota

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The development of the newborn immune system is driven by the microbiota that colonize body surfaces, especially the gastrointestinal tract and skin (Chapter 21). These early-life microbial exposures have a profound effect on the development of the immune system. In their absence, "germ-free" mammals fail to fully develop their mucosal lymphoid tissues. The microbiota generates a complex mixture of pathogen-associated molecular patterns (PAMPs) that act through enterocyte toll-like receptors (TLRs). Likewise, microbial antigens are taken up by dendritic cells and presented to $CD4^+$ T cells. These signals collectively promote the functional development of the immune system. The intestinal and skin microbiota also plays a key role in determining any Th1 or Th2 bias in immune function. This affects blood levels of IgE in adult life and the development of allergies. This is the basis of the "hygiene hypothesis," the concept that the development of allergies is influenced by microbial exposure in early life (Chapter 30).

Innate Immunity

Newborns can produce a diverse array of antimicrobial molecules, including pentraxins and collectins, peptides such as the defensins, and lactoferrin and lysozyme. Surfactant proteins A and D as well as β-defensin 1 and TLR4 are produced in the pre-term lamb lung. As a result, invaders can be killed relatively efficiently. TLRs are present and functional in the newborn. In the fetal pig, neutrophils at 90 days post-conception are fully capable of phagocytizing bacteria such as *Staphylococcus aureus.* However, they are deficient in bactericidal activity, which only reaches adult levels 10 days later. Near birth, the phagocytic and bactericidal capacity of these neutrophils declines as a result of increased steroid production. The neutrophils of newborn foals move relatively slowly compared with their dams. The serum of newborn mammals, however, is deficient in some complement components, resulting in a poor opsonic activity. Serum C3 increases rapidly after birth in newborn piglets and reaches adult levels by 14 days of age.

Changes also occur in the distribution of macrophages. Newborn piglets have few pulmonary intravascular macrophages. During the first few days after birth, blood monocytes adhere to the pulmonary

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capillary endothelium and differentiate into macrophages. In the newborn piglet, 75% of particles are removed from the blood by the liver and spleen, but by 2 months of age, 75% are removed by the lungs. The alveolar macrophages of newborn pigs have poor phagocytic activity, but this is effectively acquired by 7 days. These alveolar macrophages produce high levels of IL-10 in newborn piglets.

Newborn calves have fewer NK cells than adults, but these respond more strongly to stimulation with IL-2 or IL-15 and are more cytotoxic! Age-dependent changes occur in acute-phase proteins in newborn calves. Serum amyloid A, lipopolysaccharide-binding protein, haptoglobin, and α_1-acid glycoprotein are present in high concentrations immediately after birth but gradually decline by 21 days. In developing piglets, IL-8 and tumor necrosis factor-α (TNF-α) production by blood monocytes increases significantly during the postnatal period, whereas monocyte production of IL-1β does not. Absolute numbers of γ/δ T cells increase greatly from birth until 20 to 25 weeks of age, and there are progressive changes in helper cell phenotypes from $CD8a^-$, $SLA\text{-}DR^-$, $CD27^+$ to $CD8a^+$, $SLA\text{-}DR^+$, and $CD27^-$ during this time.

Adaptive Immunity

The early development of the adaptive immune system has been well analyzed in newborn foals since some are highly susceptible to lethal *Rhodococcus equi* infection (Box 23.3). Newborn mammals, including foals, mount adaptive responses skewed toward type 2 rather than type 1 responses. Thus they favor antibody responses over cell-mediated immunity. This imbalance results from the late development of IL-12–producing DC1 cells and the activities of IL-4 and IL-13 from DC2 cells. Mononuclear cells from newborn foals are unable to express IFN-γ. Their Th2 cells rapidly differentiate, whereas neonatal Th1 cells are slow to develop. Excess IFN-γ may cause placental damage, so this skewing is not accidental. IFN-γ production gradually increases through the first 6 months of life to reach adult levels within a year, when the acquired responses revert to the balanced adult pattern.

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Box 23.3

Rhodococcus equi

Rhodococcus equi is a ubiquitous bacterium found in soil and animal bedding. Newborn foals are exposed to *R. equi* soon after birth. Most foals then mount a protective immune response, but a few develop severe pneumonia at a time when maternal antibodies are waning.

Multiple defects have been identified in the immune systems of neonatal foals. Foal neutrophils have a phagocytic ability similar to that in adult horses, but their serum opsonic activity is low. Their killing activity is also low since they mount a weak inflammatory burst. Their dendritic cell function is defective, owing to decreased expression of MHC class II molecules and of CD1b.

Because this disease is associated with declining maternal antibody levels, it is possible that antibodies are critical in determining resistance or susceptibility to *R. equi*. Foals generally lack mucosal IgA for the first 28 days of life. While production of IL-8, IL-10, IL-12, and IL-23 in newborns is comparable to that in older foals, interferon-γ (IFN-γ) and IL-6 production are impaired. On stimulation with *R. equi*, neonatal foal mononuclear cells increase their production of IFN-γ, IL-6, and IL-23, but much less so than cells from older foals. *R. equi* growth is greater in 3-month-old foals than in macrophages from younger foals or from adult horses. Expression of IL-4 mRNA is higher in monocytes, while expression of IL-6, IL-18 and TNF-α is higher in bronchoalveolar macrophages. Induction of IL-1β, IL-10, IL-12 p40 and IL-8 mRNA is highest in cells from 1- to 3-day-old foals. Thus the persistence of this organism within macrophages probably plays a role in its tissue tropism and in determining age susceptibility.

In prospective studies, foals destined to develop *R. equi* pneumonia were born with fewer leukocytes, fewer segmented neutrophils, a lower proportion of $CD4^+$ T cells, and a lower CD4/CD8 ratio than normal foals. It has long been accepted that a Th1 response is essential for protection against *R. equi*. Since it is an intracellular organism, activation of phagocytic cells by IFN-γ is required for its elimination. Unfortunately, foals younger than 3 to 4 months have reduced IFN-γ expression compared with adults. It

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is likely that in susceptible foals, a combination of multiple immune defects permits this otherwise innocuous organism to cause disease.

Berghaus LJ, Giguère S, Sturgill TL: Effect of age and macrophage lineage on intracellular survival and cytokine induction after infection with *Rhodococcus equi, Vet Immunol Immunopathol,* 160:41-50, 2014.

During the first 3 months of life, puppies have a higher lymphocyte count than adult dogs. Much of the difference is due to $CD21^+$ B cells. The proportion of puppy $CD8^+$ T cells is low at birth but gradually climbs to reach adult levels. A similar gradual increase in lymphocyte numbers is seen in newborn kittens. Thymic involution begins at around 6 months of age in both dogs and cats.

Unless additional immunological assistance is provided, however, organisms that present little threat to an adult may kill newborn mammals. This immunological assistance is provided by antibodies transferred from the mother to her offspring through colostrum. Maternal lymphocytes may also be transferred to the fetus through the placenta or to newborn mammals through colostrum.

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Transfer of Immunity From Mother to Offspring

The route by which maternal antibodies reach the fetus is determined by the structure of the placenta. In humans and other primates, the placenta is hemochorial; that is, the maternal blood is in direct contact with the trophoblast (Fig. 23.4). This type of placenta allows maternal IgG but not IgM, IgA, or IgE to transfer directly to the fetus. Maternal IgG can enter the fetal bloodstream, and the newborn human infant has circulating IgG levels comparable to those of its mother.

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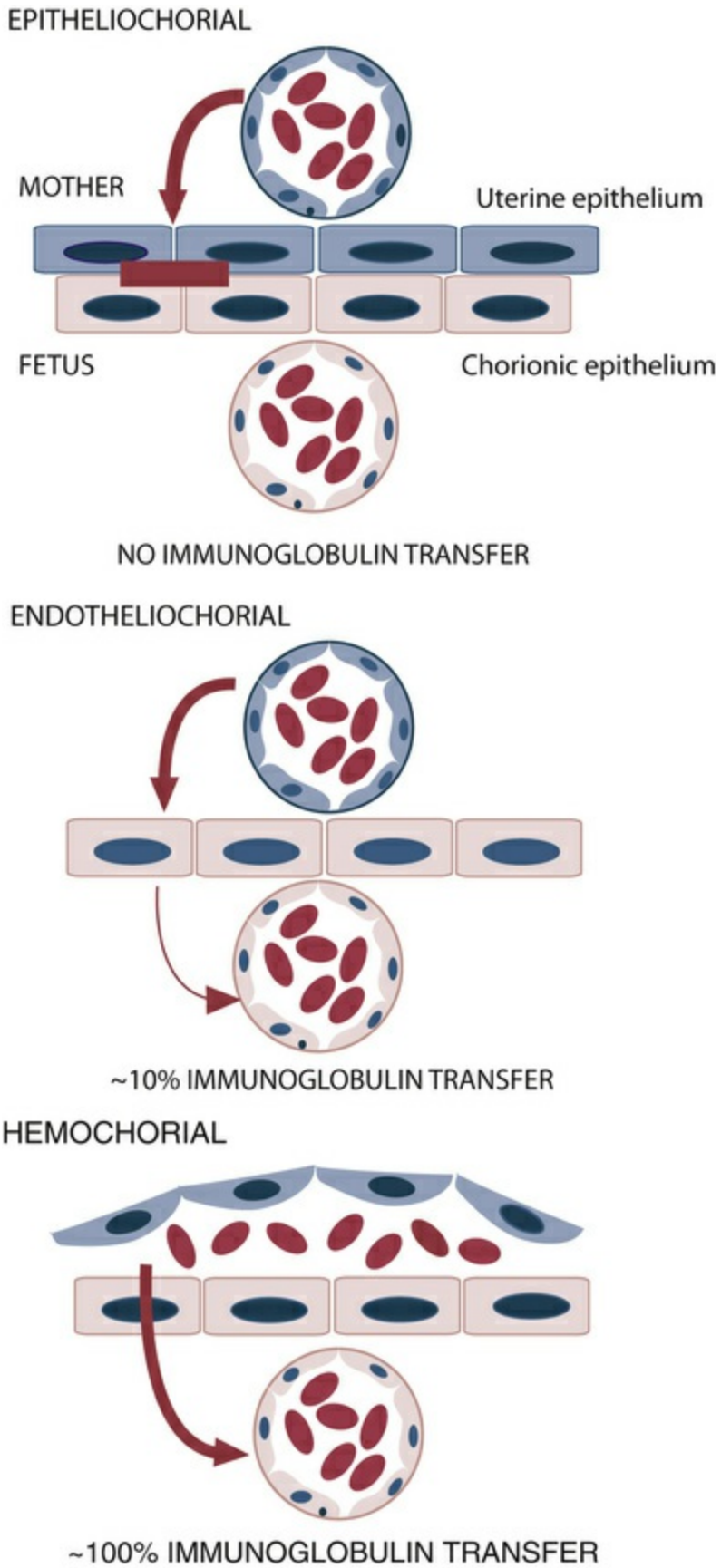


FIG. 23.4 The placentation of domestic mammals and their relationship to immunoglobulin transfer. The *blue* cells are on the maternal side of the placenta. Antibodies freely cross a hemochorial

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placenta from mother to fetus; are largely prevented from crossing in an endotheliochorial species such as dogs and cats; and are totally prevented from crossing in an epitheliochorial species such as horses or pigs.

Dogs and cats have an endotheliochorial placenta in which the chorionic epithelium is in contact with the endothelium of the maternal capillaries. In these species, 5% to 10% of IgG is directly transferred from the mother to the puppy or kitten, but most must be obtained through colostrum. (It is interesting to note that elephants also have an endotheliochorial placentation and there is significant prenatal passive transfer of immunity in this species.)

The placenta of ruminants is syndesmochorial; that is, the chorionic epithelium is in direct contact with uterine tissues, whereas the placenta of horses and pigs is epitheliochorial and the fetal chorionic epithelium is in contact with intact uterine epithelium. In mammals with both these types of placenta, the transplacental passage of immunoglobulin molecules is totally prevented. Thus their newborns are entirely dependent on antibodies received through the colostrum.

Secretion and Composition of Colostrum and Milk

Colostrum contains the accumulated secretions of the mammary gland over the last few weeks of pregnancy together with proteins actively transferred from the bloodstream under the influence of estrogens and progesterone. Neonatal immunoglobulin receptors (FcRn) are expressed in mammary gland ductal and acinar cells and mediate the transfer of IgG from serum into colostrum. Colostrum is therefore rich in IgG and IgA and contains some IgM and IgE (Table 23.1). The predominant immunoglobulin in the colostrum of most domestic mammals is IgG, which may account for 65% to 90% of its total antibody content; IgA and the other immunoglobulins are usually minor but significant components. As lactation progresses and colostrum changes to milk, differences among species emerge. In primates, IgA predominates in both colostrum and milk. In pigs and horses, IgG predominates in colostrum, but its concentration drops rapidly as lactation proceeds so that IgA

predominates in milk. In ruminants, IgG1 is the predominant immunoglobulin in both milk and colostrum (Fig. 23.5).

TABLE 23.1

Colostral and Milk Immunoglobulin Levels in Domestic Animals

IMMUNOGLOBULIN (mg/dL)						
Species	**Fluid**	**IgA**	**IgM**	**IgG**	**IgG3**	**IgG6**
Horse	Colostrum	500-1500	100-350	1500-5000	500-2500	50-150
	Milk	50-100	5-10	20-50	5-20	0
Cow	Colostrum	100-700	300-1300	2400-8000		
	Milk	10-50	10-20	50-750		
Ewe	Colostrum	100-700	400-1200	4000-6000		
	Milk	5-12	0-7	60-100		
Sow	Colostrum	950-1050	250-320	3000-7000		
Bitch	Colostrum	500-2200	14-57	120-300		
	Milk	110-620	10-54	1-3		
Queen	Colostrum	150-340	47-58	4400-3250		
	Milk	240-620	0	100-440		

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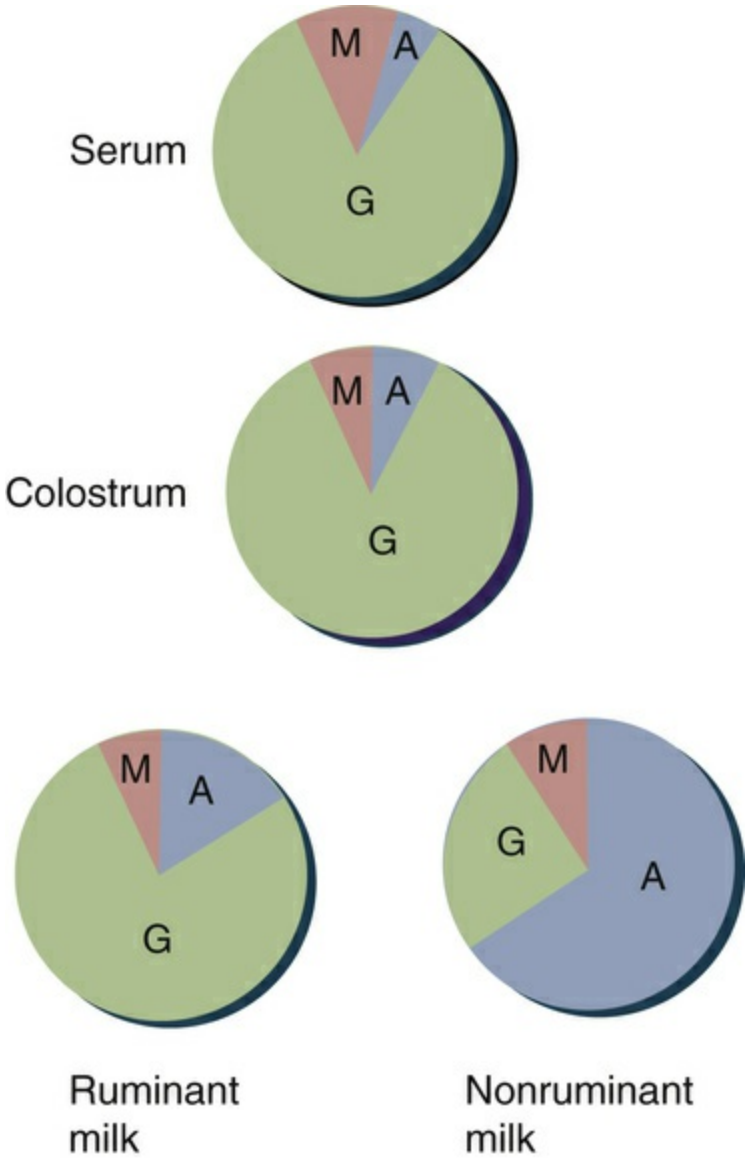


FIG. 23.5 The relative concentrations of the major immunoglobulin classes in serum, colostrum, and the milk of ruminants and nonruminants.

All of the IgG, most of the IgM, and about half of the IgA in bovine colostrum are transferred from the bloodstream. In milk, in contrast, only 30% of the IgG and 10% of the IgA are so derived; the rest is produced locally by lymphoid tissue within the udder. Colostrum also contains secretory component both in the free form and bound to IgA. Colostrum contains many cytokines. For example, bovine colostrum contains significant amounts of IL-1β, IL-6, TNF-α, and IFN-γ. Equine colostrum has been shown to contain IL-4, IL-6, TNF-α and IFN-γ. These cytokines may promote the development of the immune system in the young animal.

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Absorption of Colostrum

Young mammals that suckle soon after birth ingest colostrum. Naturally suckled calves ingest an average of 2 L of colostrum, although individuals may ingest as much as 6 L. In these young mammals, protease activity in the digestive tract is low and is further reduced by trypsin inhibitors in colostrum. Therefore, colostral proteins are not degraded but can reach the small intestine intact. These colostral immunoglobulins are endocytosed by enterocytes and bind to FcRn within their endosomes (Fig. 23.6). Once bound to endosomal FcRn, immunoglobulin molecules are transported across the enterocytes and transferred to the lacteals and possibly the intestinal capillaries. Eventually the absorbed immunoglobulin reaches the bloodstream, and newborn mammals obtain a massive transfusion of maternal immunoglobulins.

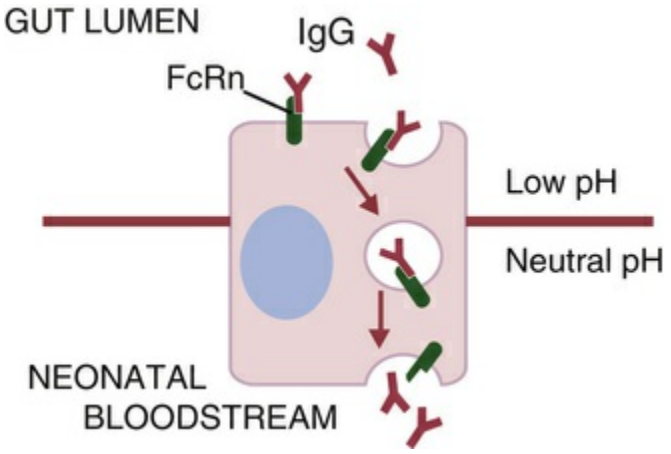


FIG. 23.6 The role of the neonatal Fc receptor (FcRn) in absorbing immunoglobulin from the intestine. IgG binds FcRn at low pH in the intestinal lumen and unbinds at neutral pH within the newborn animal's bloodstream.

Newborn mammals differ in the selectivity and duration of their intestinal permeability. In the horse and pig, protein absorption is selective. IgG and IgM are preferentially absorbed, whereas IgA mainly remains in the intestine. In ruminants, immunoglobulin absorption is unselective, and all classes are absorbed, although IgA is gradually excreted. Young pigs and probably other young mammals have large amounts of free secretory component in their intestine. Colostral IgA, and to a lesser extent IgM, can bind this

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secretory component, which may then prevent their absorption. The duration of intestinal permeability also varies among species and immunoglobulin classes. In general, permeability is highest immediately after birth and declines after about 6 hours because of the replacement of FcRn-bearing enterocytes by cells that do not express this receptor. As a rule, absorption of all immunoglobulin classes drops to a very low level after about 24 hours. Feeding colostrum tends to hasten this closure, whereas a delay in feeding results in a slight delay in closure (up to 33 hours). In piglets, the ability to absorb immunoglobulins may be retained for up to 4 days if milk products are withheld. The presence of the mother may be associated with increased immunoglobulin absorption. Thus calves fed measured amounts of colostrum in the presence of the mother will absorb more immunoglobulins than calves fed the same amount in her absence. In laboratory studies in which measured amounts of colostrum are fed, there is a great variation (25%–35%) in the quantity of immunoglobulins absorbed. Management should ensure that foals or calves ingest at least 1 L of colostrum within 6 hours of birth.

Unsuckled mammals normally have very low levels of immunoglobulins in their serum. The successful absorption of colostral immunoglobulins immediately supplies them with serum IgG at a level approaching that found in adults (Fig. 23.7). Peak serum immunoglobulin levels are normally reached between 12 and 24 hours after birth. After absorption ceases, these passively acquired antibodies decline through normal metabolic processes. The rate of decline differs among immunoglobulin classes, and the time taken to decline to non-protective levels depends on their initial concentration. In calves the serum half-life of colostral-derived IgG is about 28 days.

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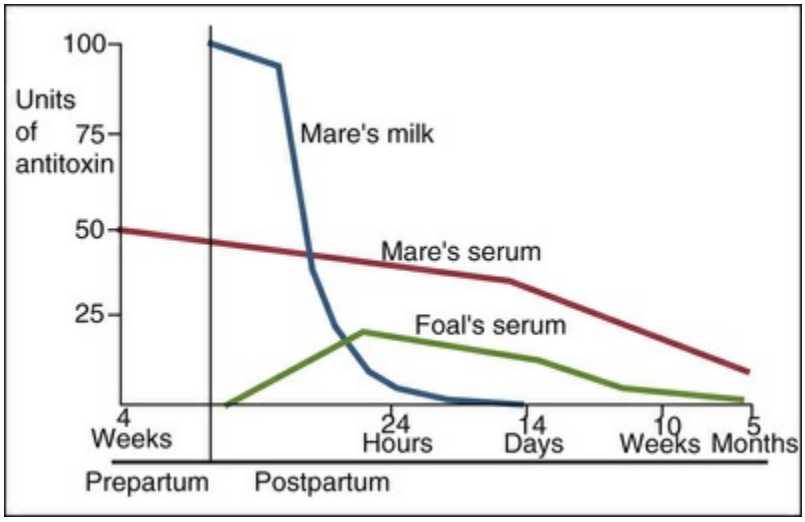


FIG. 23.7 *Clostridium perfringens* antitoxin levels in serum, colostrum, and milk of six pony mares and in the serum of their foals from birth to 5 months. (Jeffcott LB: Studies on passive immunity in the foal. 1. γ-globulin and antibody variations associated with the maternal transfer of immunity and the onset of active immunity. *J Comp Pathol* 84:93-101, 1974.)

Neonatal mammals develop proteinuria. This is due to intestinal absorption of very small proteins such as β-lactoglobulin that can be excreted in the urine. In addition, the glomeruli of newborn mammals are permeable so that the urine of neonatal ruminants contains intact immunoglobulin molecules. This proteinuria ceases with the termination of intestinal absorption. Urine from puppies collected 24 hours after birth contains relatively large amounts of IgG, IgM, and IgA. The amount declines over time so that IgM is undetectable by 14 days, although there may still be significant amounts of IgG and IgA present. Over the first 2 weeks of life, the puppy's glomeruli mature and acquire the ability to retain macromolecules.

The secretions of the mammary gland gradually change from colostrum to milk. Ruminant milk is rich in IgG1 and IgA. Nonruminant milk is rich in IgA. For the first few weeks in life, while protease activity is low, these immunoglobulins can be found throughout the intestine and in the feces of young mammals. As the digestive ability of the intestine increases, eventually only secretory IgA molecules remain intact. The amount of IgA provided by milk can be large; for instance, a 3-week-old piglet may receive 1.6 g daily from sow's milk.

Although IgE is present in mare's milk and transmitted to the

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suckling foal, its level in foal serum drops to a very low level by 6 weeks of age. IgE synthesis by foals begins at about 9 to 11 months of age, and at that time a pattern of relatively high or low IgE levels is established. These levels are not correlated with the levels resulting from suckling. Total levels of IgE in young horses (and their susceptibility to allergies) are mainly determined by genetic factors and the microbiota. IgE is also transferred in sheep colostrum. Colostral IgE levels are significantly higher than in ewe's serum. IgE is absent from pre-suckling lamb serum but can rise to adult levels by 2 days after birth. It then declines steadily over several weeks.

The IgG transferred through a mother's colostrum represents the results of her history of antigen exposure, B cell responses, and somatic mutation. This maternal IgG in effect represents the immunological experiences of the mother. Maternal antibodies act on the immune system of the newborn during a critical imprinting period and exert a lifelong influence on the newborn's immune development. This influence may be stronger than some genetic predispositions! Thus maternal antibodies can enhance the newborn immune responses to some antigens and suppress their responses to others. They may also influence Th1/Th2 polarization and the subsequent development of allergies.

MicroRNAs and Intestinal Development

MicroRNAs (miRNAs) are small single-stranded RNA molecules about 22 nucleotides in length that regulate gene expression. They bind to the 3'untranslated region or the coding regions of messenger RNAs and promote their degradation. miRNAs regulate many different biological processes including embryonic development, cell differentiation, and apoptosis. Their expression patterns and regulatory functions may be tissue specific. Some miRNAs regulate gut development and mucosal immunity in newborns. miRNAs are present in many body fluids including milk and colostrum. They are shed into the colostrum in microvesicles and exosomes. These colostral miRNAs are absorbed from the intestine and enter the circulation of newborn mammals. These miRNAs influence the development of the mucosal immune

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system, especially during the first week of life. The miRNAs likely regulate IL-6 and IL-17 signaling as well as T cell differentiation in the jejunum and ileum at this early stage. The commensal microbiota also influences miRNA expression and coordinates intestinal colonization and immune system development. Thus the expression of miRNAs in the small intestine of neonatal dairy calves is correlated with the total bacterial load and the presence of specific bacterial groups such as Lactobacilli and Bifidobacteria.

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Failure of Passive Transfer

The absorption of IgG from colostrum is required for the protection of a newborn against septicemic disease. The continuous intake of IgA or IgG1 from milk is required for protection against enteric disease (Fig. 23.8). Failure of these processes predisposes a young animal to infection.

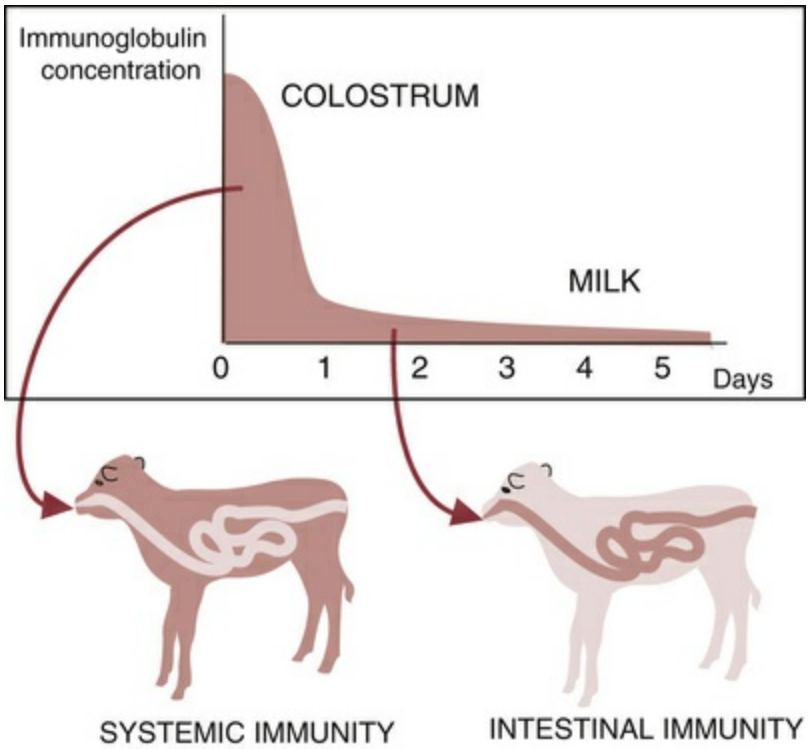


FIG. 23.8 Colostrum intake is required to protect young animals against septicemic disease. The prolonged intake of milk is necessary to ensure protection of the gastrointestinal tract against enteric infection.

There are three major reasons for failure of passive transfer through colostrum. First, the mother may produce insufficient or poor-quality colostrum (production failure). Second, there may be sufficient colostrum produced but inadequate intake by the newborn animal (ingestion failure). Third, there may be a failure of absorption from the intestine despite an adequate intake of colostrum (absorption failure).

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Production Failure

Since colostrum represents the accumulated secretions of the udder in late pregnancy, premature births may mean that insufficient colostrum has accumulated. Valuable colostrum may also be lost from mammals as a result of premature lactation or excessive dripping. Cows milked continuously (without a drying-off period) produce colostrum with reduced immunoglobulin concentrations. Colostral IgG levels also vary among individuals, with up to 28% of mares producing low-quality colostrum. It is not possible to assess colostral quality simply by looking at it. Its IgG content should be assessed using a colostrometer (a modified hydrometer) to measure its specific gravity. This is normally in the range of 1.060 to 1.085, equivalent to an IgG concentration of 3000 to 8500 mg/dL. Colostrum with an IgG level of less than 3000 mg/dL may be inadequate to protect a foal, and feeding supplemental, high-quality colostrum may become necessary.

Ingestion Failure

In sheep or pigs, an inadequate intake may result from multiple births simply because the amount of colostrum produced does not rise in proportion to the number of newborn. It may be due to poor mothering, an important problem among young, inexperienced mothers. It also may be due to weakness in the newborn, to a poor suckling drive, or to physical problems such as damaged teats or jaw defects.

Absorption Failure

Failure of intestinal absorption is a major cause for concern in any species. It is especially important in horses not only because of the value of many foals but also because even with good husbandry, about 25% of newborn foals fail to absorb sufficient quantities of immunoglobulins. Alpacas also appear to experience a disproportionate number of cases of failure of passive transfer. Foals require serum IgG concentrations of at least 800 mg/dL 18 to 24 hours after receiving colostrum to ensure protection. Foals that have less IgG than this are at increased risk for infection. If their

IgG level fails to reach 400 mg/dL, severe infections are assured (Fig. 23.9).

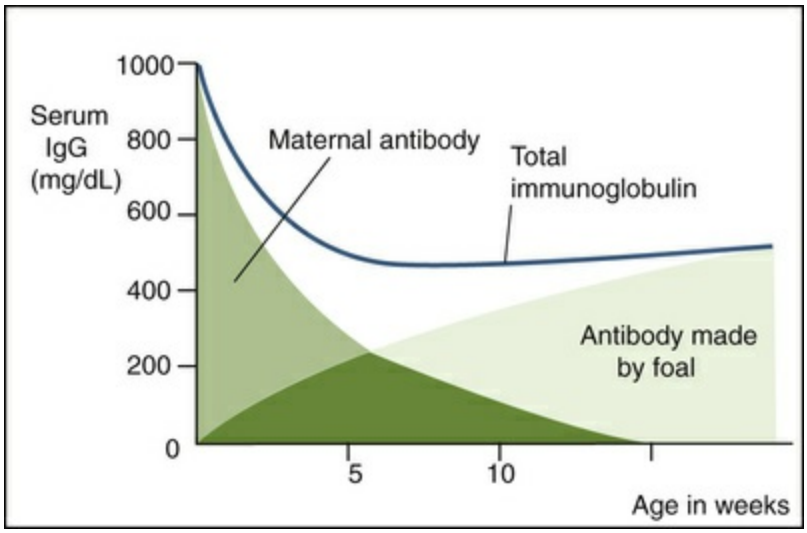


FIG. 23.9 Immunoglobulin levels in newborn serum during the first 15 weeks of life indicating the relative contributions of maternal antibody and antibody synthesized by the newborn animal.

Diagnosis of Failure of Passive Transfer

The success of passive transfer cannot be evaluated in a foal until 18 to 24 hours after birth, when antibody absorption is essentially complete. Several assays for serum immunoglobulins are available (Table 23.2). The most rapid and economic procedure is the zinc sulfate turbidity test, which involves mixing a zinc sulfate solution with foal serum. Zinc sulfate precipitates globulins and the amount of precipitate is proportional to the immunoglobulin concentration. In total failure of transfer, the reaction mixture remains clear. In sera with an IgG level of more than 400 mg/dL, the mixture becomes cloudy. As an alternative to visual inspection, the optical density of the mixtures can be read in a spectrophotometer and the IgG concentration read off a standard curve. The relative amount of precipitate can also be read in a hematocrit capillary (immunocrit). Similar techniques include precipitation by ammonium sulfate, glutaraldehyde, or sodium sulfite.

TABLE 23.2

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Tests for Failure of Passive Transfer in Horses

Test Employed	Relative Sensitivity (False Negatives)	Relative Specificity (False Positives)
Glutaraldehyde coagulation	100	59
Latex agglutination	72	79
Membrane filter ELISA	90	79
Turbidimetric immunoassay	81	86
Infrared spectroscopy	93	97

From Crisman MV, Scarratt WK: Immunodeficiency disorders in horses, *Vet Clin North Am Equine Pract* 24:299-310, 2008.

Single radial immunodiffusion is a more accurate method in that it is both quantitative and specific for IgG. As described in Chapter 42, known standards are compared with the test serum by measuring the diameter of precipitation produced in agar gel containing an antiserum to equine IgG. A diagnosis of failure of passive transfer is made in foals if IgG levels are less than 400 mg/dL and partial failure of passive transfer if IgG levels are between 400 and 800 mg/dL. Unfortunately, radial immunodiffusion is slow. It takes 18 to 24 hours to give a result and is thus impractical when a rapid diagnosis is required.

A third method of measuring IgG levels is by use of a latex agglutination test. The latex particles are coated with antiequine IgG. In the presence of IgG, they agglutinate. This test can be performed in about 10 minutes using either whole foal blood or serum. It appears to be reliable and rapid but somewhat insensitive.

It is also possible to use a semiquantitative membrane-filter enzyme-linked immunosorbent assay (ELISA) test to measure IgG in a foal's serum. The color intensity of the reaction on the test filter is compared with color calibration spots. A variant technique uses a dipstick ELISA. Less satisfactory techniques include serum protein electrophoresis and refractometry. (Refractometry is an acceptable, rapid and convenient test of colostral IgG in calves but is less reliable in foals, in which the wide range of values leads to inaccuracy. It may severely underestimate serum IgG levels.) Point-of-care portable analyzers also provide acceptable results in a timely manner.

Management of Failure of Passive Transfer

In foals, an IgG concentration higher than 800 mg/dL is preferred, but foals with immunoglobulin levels higher than 400 mg/dL will

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generally remain healthy and do not require treatment. About 75% of foals with IgG levels between 200 and 400 mg/dL will also remain healthy. However, they should be watched and treated with antibiotics at the first signs of bacterial infection. Any foals with total failure of passive transfer or foals younger than 3 weeks with partial failure of passive transfer should be treated. Foals with plasma IgG concentrations less than 200 mg/dL, foals that have not nursed within 6 hours of birth, and foals that have received colostrum with IgG of less than 1000 mg/dL (specific gravity less than 1.050) should receive additional colostrum. Two to three liters of good-quality colostrum (IgG of more than 7000 mg/dL) should be given by bottle or nasogastric tube in three or four doses at hourly intervals. The colostrum must be free of antibodies to the foal's erythrocytes (Chapter 31). Colostrum may be obtained from mares that have more than is needed for their own young. It can be stored frozen at -15° to -20° C for up to 1 year. If stored colostrum is unavailable, fresh colostrum from primiparous mares can be used. If colostrum is not available, serum or plasma may be administered orally. A large volume (up to 9 L) may be required since serum IgG is rapidly catabolized so that within 12 hours its concentration is much less than that found in colostrum-fed foals. The reasons for this are unknown.

In foals that are older than 15 hours, oral absorption ceases and intravenous plasma infusion must be given. Ideally the dose used can be calculated to attain an IgG level of at least 400 mg/dL. Frozen horse plasma is available commercially, although this may not contain antibodies against local pathogens. Alternatively, the plasma may be obtained from local donors. Blood should be collected aseptically with heparin or sodium citrate. The plasma is collected after the erythrocytes settle and is stored frozen until used. The plasma must be prechecked for antierythrocyte antibodies and must be free of bacterial contamination. The transfusion should be given slowly while the foal is monitored for untoward reactions. All foals receiving supplemental colostrum or plasma should have their IgG levels rechecked 12 to 24 hours later.

Considerations similar to those described previously apply to failure of passive transfer in the calf. Calves with serum IgG of less than 1000 mg/dL at 24 to 48 hours of age have mortality rates more

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than twice those of calves with higher IgG levels. A minimum of 150 to 200 g of colostral IgG is required for optimal passive transfer. Three liters of colostrum should be administered by oropharyngeal tube to calves within 2 hours of birth. Substantially larger quantities of IgG must be administered after 2 hours to achieve optimal protection. A plasma transfusion may be of benefit. Commercially available colostrum may be enriched in specific antibodies to protect the calf against potential pathogens such as K99 *Escherichia coli,* rotaviruses, and coronaviruses, the major causes of calf diarrhea.

Colostral transfer of immunity is essential for the survival of young mammals, but it may also cause disease. If a mother becomes immunized against the red cells of her fetus, colostral antibodies may cause erythrocyte destruction in the newborn, a condition called hemolytic disease (Chapter 31).

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Cell-Mediated Immunity and Colostrum

Colostrum is full of lymphocytes, but milk is not. Sow colostrum contains between 1×10^5 and 1×10^6 lymphocytes/mL. Of these, 70% to 80% are T cells. Within 2 hours after receiving colostrum that contained labeled cells, maternal lymphocytes appeared in the bloodstream of piglets. Piglets that had received these colostral cells showed enhanced responses to mitogens compared with control mammals. Transcriptome analysis of colostral T cells in sows has indicated that they are more activated than peripheral blood T cells. Bovine colostrum also contains between 3×10^4 and 1×10^5 lymphocytes/mL, about half of which are T cells. Colostral lymphocytes may survive up to 36 hours in the intestine of newborn calves, and some may penetrate the epithelium of Peyer's patches and reach the lacteal ducts or the mesenteric lymph nodes. Cell-containing and cell-free colostrum have been compared for their ability to protect calves against enteropathic *E. coli.* The calves receiving colostral cells excreted significantly fewer bacteria than those receiving cell-free colostrum. The concentration of IgA- and IgM-specific antibodies against *E. coli* in the serum of neonatal calves was higher in those that received colostral cells than in those that did not. The calves that received colostral cells had better responses to the mitogen concanavalin A and to foreign antigens such as sheep erythrocytes. The mechanisms of this protective effect are unclear.

The $CD8^+$ T cells in bovine colostrum can produce large quantities of IFN-γ that may influence the early development of Th1 responses in neonatal calves. Thus ingestion of maternal colostral cells appears to accelerate the development of activated calf lymphocytes. The monocytes of calves that received colostral cells are more capable of processing and presenting antigens.

Transfer of cell-mediated immunity by bovine milk lymphocytes has been demonstrated. Pregnant cows were vaccinated against BVDV. Blood lymphocytes from calves that received cell-free colostrum from these cows were unresponsive to BVDV antigen. In

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contrast, lymphocytes from calves that received colostrum containing live cells showed enhanced responses to BVDV antigen at 1 and 2 days after colostral ingestion. The lymphocytes of calves that received whole colostrum showed enhanced mitogenic responses to maternal and unrelated leukocytes after 24 hours. They also responded to the nonspecific stimulant staphylococcal enterotoxin B. In contrast, the lymphocytes of calves that received acellular colostrum did not. Clearly, ingestion of maternal colostral leukocytes immediately after birth stimulates the development of the neonatal immune system.

Mononuclear cells in mare's colostrum are mainly $CD4^+$ and $CD8^+$ T cells. $CD8^+$ cells in colostrum are enriched when compared to blood. When stimulated with phorbol myristate acetate (PMA) and ionomycin they produce more IL-17, similar IFN-γ, and less IL-4 and IL-10 when compared to blood cells. Thus these cells are polarized towards IFN-γ and IL-17 production. This phenotype is generally considered to promote inflammation.

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Development of Adaptive Immunity in Neonatal Mammals

Local Immunity

The intestinal lymphoid tissues of neonatal mammals respond rapidly to ingested antigens. For example, calves vaccinated orally with coronavirus vaccines at birth are resistant to virulent coronavirus within 3 to 9 days. Likewise, piglets vaccinated orally 3 days after birth with transmissible gastroenteritis virus vaccines develop neutralizing antibodies in the intestine 5 to 14 days later. Much of this early resistance is attributable to innate production of IFN-α/β, but there is an early intestinal IgM response that switches to IgA by 2 weeks. In the young animal, the IgA response appears earlier and reaches adult levels well before the other immunoglobulins.

Systemic Immunity

The antibodies acquired by a young animal as a result of ingesting its mother's colostrum inhibit the ability of the newborn to mount its own immune response (Fig. 23.10). As a result, very young animals are unable to respond to active immunization. This inhibition is B cell specific, and T cell responses are largely unaffected.

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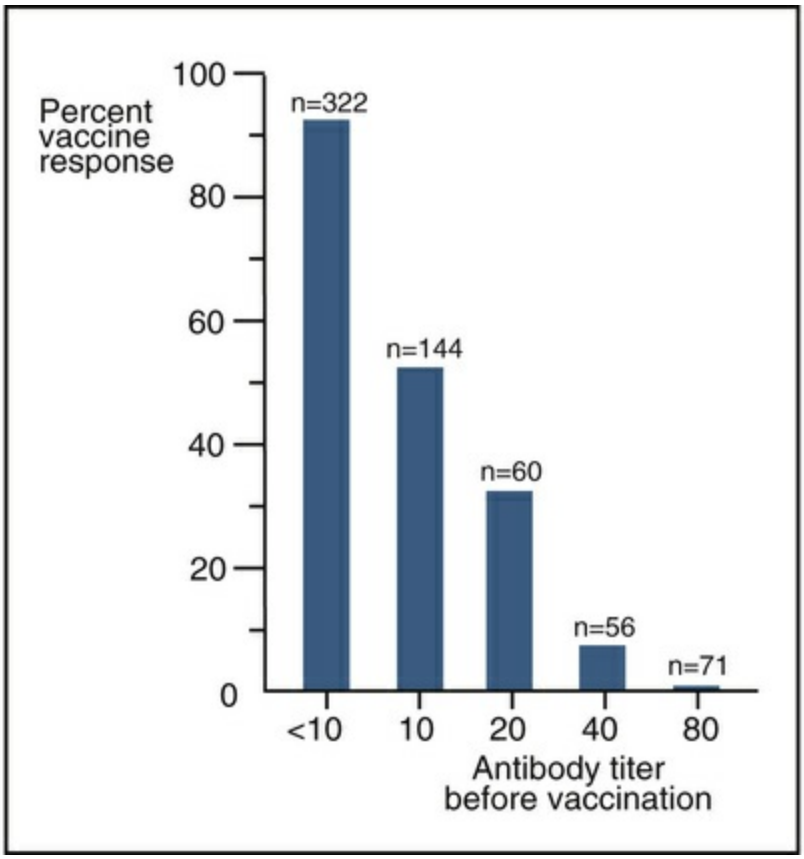


FIG. 23.10 Effect of the presence of maternal antibodies to canine parvovirus in 653 puppies on their response to a modified live parvovirus vaccine. The pre-vaccination antibody titer profoundly inhibits the response of the puppies to the vaccine. (From Carmichael LE: *Compend Contin Educ Prac Vet* 5:1043-1054, 1983.)

Several different mechanisms of this suppression have been suggested. The simplest is the rapid neutralization of live viral vaccines by the maternal antibody. This would prevent viral replication and provide insufficient antigen to prime neonatal B cells. However, data from human infants and domestic mammals indicate that sufficient antigen is present to prime T cells. Likewise, this mechanism could not account for inhibition of the immune response to nonliving vaccines.

A second proposed mechanism suggests that the inhibition results from antibodies binding to B cell Fc receptors (CD32) and blocking BCR signaling (see Fig. 20.9). However, studies of mice whose Fc receptors have been deleted (FcR knockout mice) have shown that the ability of maternal antibodies to inhibit antibody responses is unaffected. This clearly cannot be the mechanism involved.

A third suggested mechanism is that maternal antibodies simply

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mask the epitopes on vaccine antigens, preventing their recognition by the animal's B cells. This suggestion is compatible with the selective inhibition of B cell responses, the lack of inhibition of T cell responses, and the evidence that, at least in humans and mice, high doses of antigen can overcome maternal immunity. Thus for a given vaccine dose, an immune response can be elicited only when maternal antibody titers fall below a critical threshold.

In the absence of maternal antibodies, the newborn animal is able to make antibodies soon after birth. For example, if calves fail to suckle and are therefore hypogammaglobulinemic, their endogenous IgM is detectable in blood by 4 days but only reaches functional levels by 8 days. Serum IgG and IgA reach functional levels by 16 to 32 days. In calves that have suckled and thus possess maternal antibodies, antibody synthesis does not commence until about 4 weeks of age. Likewise, colostrum-deprived piglets respond to pseudorabies virus by 2 days after birth, but if they have suckled, antibody production does not begin until 5 to 6 weeks after birth. Colostrum-deprived lambs synthesize IgG1 at 1 week and IgG2 by 3 to 4 weeks. In colostrum-fed lambs, however, IgG2 synthesis does not occur until 5 to 6 weeks.

Passively acquired maternal antibodies not only protect newborns before their immune system becomes fully functional but also may shape the B cell repertoire of the offspring. Mouse pups nursed by mothers producing antibodies to vesicular stomatitis virus developed higher endogenous antibody titers of this specificity as they matured. As a result, these pups developed higher titers of protective antibody when infected as adults. Passively acquired maternal antibodies have a very significant influence on the way in which a newborn's immune system develops.

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Vaccination of Young Animals

Because maternal antibodies inhibit neonatal antibody synthesis, they prevent the successful vaccination of young animals. This inhibition may persist for many months, its length depending on the amount of antibodies transferred and the half-life of the immunoglobulins involved. This problem can be illustrated using the example of vaccination of puppies against canine distemper.

Maternal antibodies, absorbed from the puppy's intestine, reach maximal levels in serum by 12 to 24 hours after birth. Their levels then decline slowly through normal protein catabolism. The catabolic rate of proteins is exponential and is expressed as a half-life. The half-life of antibodies to distemper and canine infectious hepatitis is 8.4 days, and the half-life of antibodies to feline panleukopenia is 9.5 days. Experience has shown that, *on average,* the level of maternal antibodies to distemper in puppies declines to insignificant levels by about 10 to 12 weeks, although this may range from 6 to 16 weeks. In a population of puppies, the proportion of susceptible animals therefore increases gradually from a very few or none at birth to almost all at 10 to 12 weeks. Consequently, very few newborn puppies can be successfully vaccinated, but most can be protected by 10 to 12 weeks. Rarely, a puppy may reach 15 or 16 weeks before it can be successfully vaccinated. If virus diseases were not so common, it would be sufficient to delay vaccination until all puppies were about 12 weeks old, when success could be almost guaranteed. In practice, a delay of this type means that an increasing proportion of puppies, fully susceptible to disease, would be without immune protection—an unacceptable situation. Nor is it feasible to vaccinate all puppies repeatedly at short intervals from birth to 12 weeks, a procedure that would ensure almost complete protection; therefore, a compromise must be reached.

The earliest recommended age to vaccinate a puppy or kitten with a reasonable expectation of success is at 8 weeks. Colostrum-deprived orphan pups may be vaccinated at 2 weeks of age. Essential vaccines for normal puppies should include distemper, two adenovirus, and parvovirus vaccines. In puppies a second dose

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should be given 3 to 4 weeks after the first and a third at 14 to 16 weeks of age. Rabies is an essential vaccine that should be given at 14 to 16 weeks. In kittens, an appropriate protocol would be to use three doses of the essential vaccines (viral rhinotracheitis [herpesvirus 1], calicivirus, and panleukopenia) at 8 to 9 weeks, 3 to 4 weeks later, and at 14 to 16 weeks; feline leukemia vaccine can be given at 8 weeks and 3 to 4 weeks later; and rabies vaccine can be given at 8 to 12 weeks depending on the type of vaccine used (Fig. 23.11).

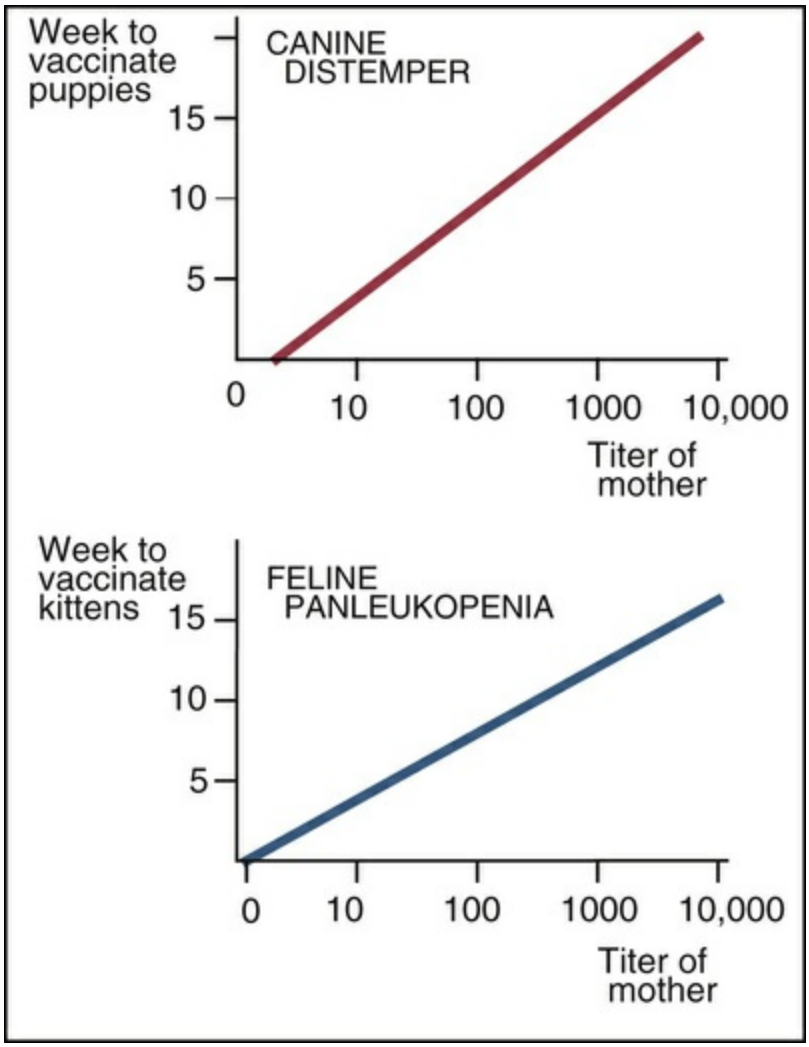


FIG. 23.11 Nomographs showing the relationship between the antibody titer of the mother and the age at which to vaccinate her offspring with modified live virus vaccine. (Data from Scott FW, Csiza CK, Gillespie JH: Maternally derived immunity to feline panleukopenia. *J Am Vet Med Assoc* 156:439-453, 1970 [FPL]; and Baker JA, Robson DS, Gillespie JH, et al: A nomograph that depicts the age to vaccinate puppies for distemper. *Cornell Vet* 49:158-167, 1959 [CD].)

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Similar considerations apply when vaccinating large farm animals. The prime factor influencing the duration of maternal immunity is the level of antibodies in the mother's colostrum. Thus in foals, maternal antibodies to tetanus toxin can persist for 6 months and antibodies to equine arteritis virus for as long as 8 months. Antibodies to BVDV may persist for up to 9 months in calves. The half-lives of maternal antibodies against equine influenza and equine arteritis virus antigens in the foal are 32 to 39 days. As in puppies, a young foal may have non-protective levels of maternal antibodies long before it can be vaccinated. Maternal antibodies, even at low levels, effectively block immune responses in young foals and calves, so premature vaccination may be ineffective. The effectiveness of vaccines increases progressively after the first 6 months of life (Fig. 23.12). A safe rule is that calves and foals should be vaccinated no earlier than 3 to 4 months of age followed by one or two revaccinations at 4-week intervals. The precise schedule will depend on the vaccine used and the species to be vaccinated. Animals vaccinated before 6 months of age should always be revaccinated at 6 months or after weaning to ensure protection.

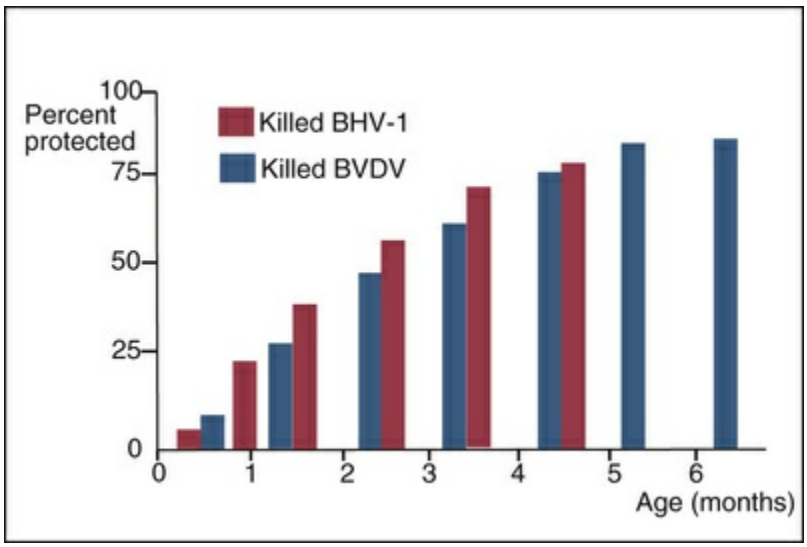


FIG. 23.12 Effectiveness to two inactivated viral vaccines in calves between birth and 6 months of age. Clearly, early vaccination is largely ineffective. (Data courtesy Dr. R.J. Schultz.)

Some live recombinant vaccines such as canarypox-vectored

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distemper in dogs or influenza in horses appear to effectively prime young animals in the face of significant maternal immunity. DNA vaccines against pseudorabies also appear to be effective in priming cell-mediated responses in the face of maternal immunity, whereas a DNA vaccine against bovine respiratory syncytial virus vaccine is not. Thus the ability of DNA vaccines to overcome maternal immunity varies among species and infections.

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24

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Vaccines and Their Production

CHAPTER OUTLINE

Types of Immunization Procedures, 261
Passive Immunization, 261
Active Immunization, 263
Living and Killed Vaccines, 264
Inactivation, 264
Attenuation, 265
Modern Vaccine Technology, 265
Antigens Generated by Gene Cloning (Category I), 265
Genetically Attenuated Organisms (Category II), 267
Live Recombinant Organisms (Category III), 267
Polynucleotide Vaccines (Category IV), 268
Prime-Boost Strategies, 269
Reverse Vaccinology, 270
Adjuvants, 270
Aluminum Salts, 270

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Saponin-Based Adjuvants, 271
Water-in-Oil Emulsions, 271
Particulate Adjuvants, 272
Immunostimulatory Adjuvants, 272
Combined Adjuvants, 273

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Describe how an animal can be made immune to infection by passive or active immunization.
- Explain how passive immunization works, as well as its benefits and disadvantages.
- Describe how active immunization works.
- Compare the benefits and disadvantages of live versus inactivated vaccines.
- List the advantages of cloned antigens.
- Explain how adjuvants, when added to vaccines, enhance their effectiveness.
- Describe the characteristics of an ideal vaccine.
- Explain the process of attenuation.
- Explain how targeted gene deletion is superior to earlier methods of attenuation.
- Explain how polynucleotide vaccines work.
- Define attenuation, adjuvants, modified live, DIVA vaccines, prime-boost, and reverse vaccinology.
- Explain the importance of identifying essential and nonessential vaccines.
- Understand what controls the duration of immunity.
- Explain the benefits and disadvantages of adjuvants.
- Explain the mechanisms by which common adjuvants work.

Vaccination is by far the most efficient and cost-effective method of controlling infectious diseases in humans and animals. The

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eradication of smallpox and rinderpest from the globe, the elimination of hog cholera and brucellosis from many countries, and the control of diseases such as foot-and-mouth disease, canine distemper, rabies, influenza, and pseudorabies would not have been possible without the use of effective vaccines. Vaccines are among the greatest triumphs of modern medicine.

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Types of Immunization Procedures

There are two procedures by which any animal may be made immune to an infectious disease (Fig. 24.1): passive and active immunization. Passive immunization produces temporary immunity by transferring antibodies from a resistant to a susceptible animal. These passively transferred antibodies (or antisera) give immediate protection, but since they are gradually catabolized, this protection wanes, and the recipient eventually becomes susceptible again.

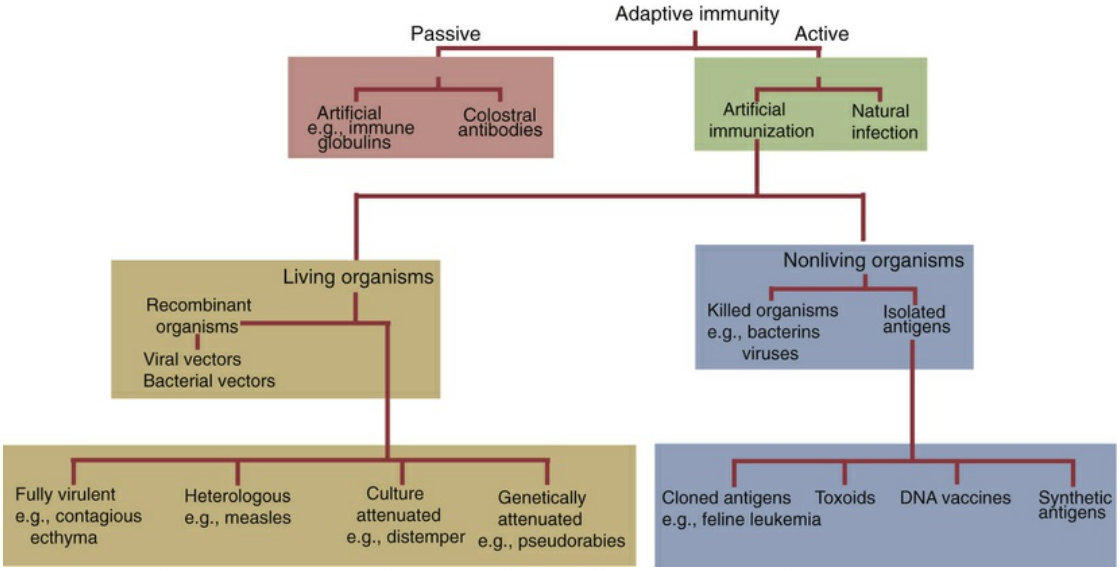


FIG. 24.1 A classification of the different types of adaptive immunity and of the methods employed to induce protection.

Active immunization, in contrast, involves administering antigen to an animal so that it responds by mounting an adaptive immune response. Reimmunization or exposure to infection in the same animal will result in a secondary response and greatly enhanced immunity. The disadvantage of active immunization is, as with all adaptive immune responses, that protection is not conferred immediately. However, once established, immunity is long lasting and capable of restimulation (Fig. 24.2).

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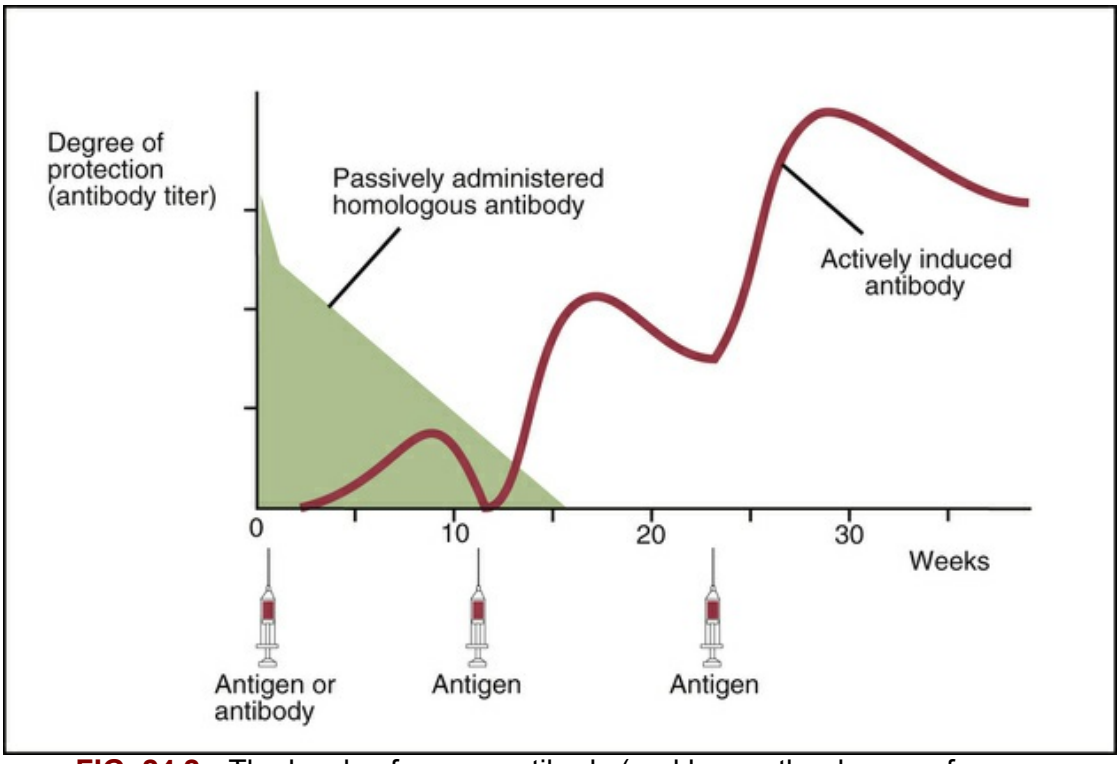


FIG. 24.2 The levels of serum antibody (and hence the degree of protection) conferred by active and passive methods of immunization.

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Passive Immunization

Passive immunization requires that antibodies be produced in donor animals by active immunization and that these antibodies be given to susceptible animals to confer immediate protection. Serum containing these antibodies (antisera or immune globulins) may be produced against a wide variety of pathogens. For instance, they can be produced in cattle against anthrax, in dogs against distemper, or in cats against panleukopenia. They are most effective at protecting animals against toxigenic organisms such as *Clostridium tetani* or *Clostridium perfringens* using antisera raised in horses. Antisera made in this way are called immune globulins and are commonly produced in young horses by a series of immunizing injections. The clostridial toxins are proteins that can be denatured and made nontoxic by treatment with formaldehyde. Formaldehyde-treated toxins are called toxoids. Donor horses are initially injected with toxoids, but once antibodies are produced, subsequent injections may contain purified toxin. The responses of the horses are monitored, and once their antibody levels are sufficiently high, they are bled. Bleeding is undertaken at intervals until the antibody level drops, when the animals are again boosted with antigen. Plasma is separated from the horse blood, and the globulin fraction that contains the antibodies is concentrated, titrated, and dispensed.

To standardize the potency of different immune globulins, comparison must be made with an international biological standard. In the case of tetanus immune globulin, this is done by comparing the dose necessary to protect guinea pigs against a fixed amount of tetanus toxin with the dose of the standard preparation of immune globulin required to do the same. The international standard immune globulin for tetanus toxin is a quantity held at the State Serum Institute in Copenhagen. An international unit (IU) of tetanus immune globulin is the specific neutralizing activity contained in 0.03384 mg of the international standard. The U.S. standard unit (AU) is double the international unit.

Tetanus immune globulin is given to animals to confer immediate protection against tetanus. At least 1,500 to 3,000 IU of

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immune globulin should be given to horses and cattle; at least 500 IU to calves, sheep, goats, and swine; and at least 250 IU to dogs. The exact amount should vary with the amount of tissue damage, the degree of wound contamination, and the time elapsed since injury. Tetanus immune globulin, 300,000 IU intravenously has been used successfully for the treatment of clinical disease in horses.

Although immune globulins give immediate protection, some problems are associated with their use. For instance, when horse tetanus immune globulin is given to a cow or dog, the horse proteins will be perceived as foreign, elicit an immune response, and be rapidly eliminated (Fig. 24.3). To reduce antigenicity, immune globulins are usually treated with pepsin to destroy their Fc region and leave intact only the portion of the immunoglobulin molecule required for toxin neutralization, the $F(ab)'_2$ fragment.

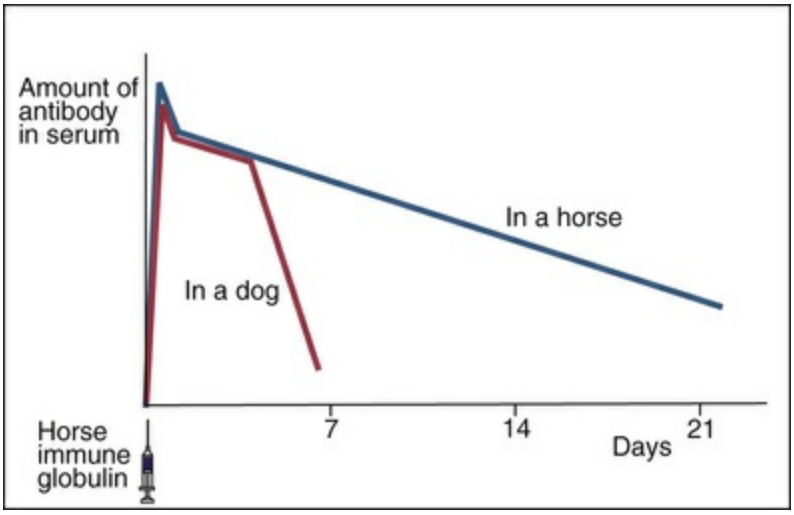


FIG. 24.3 The fate of passively administered equine immune globulin when given to a homologous species *(horse)* or to a heterologous species *(dog)*.

If circulating horse antibody is still present by the time the recipient animal mounts an immune response, the immune-complexes formed may cause a type III hypersensitivity reaction called serum sickness (Chapter 32). If repeated doses of horse immune globulin are given to an animal of another species, this may provoke immunoglobulin E (IgE) production and anaphylaxis (Chapter 30). Finally, the presence of high levels of circulating horse

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antibody may interfere with active immunization against the same antigen. This is a phenomenon similar to that seen in newborn animals passively protected by maternal antibodies.

Monoclonal antibodies are another source of passive protection for animals. These are, however, mainly made by mouse-mouse hybridomas and thus are mouse immunoglobulins. They will therefore be antigenic when given to other species. Nevertheless, mouse monoclonal antibodies against the K99 pilus antigens of *Escherichia coli* may be given orally to calves to protect them against diarrhea caused by this organism. A mouse monoclonal antibody to lymphoma cells has been used to treat dogs. Monoclonal antibodies, especially when modified to match the recipient species, are increasingly used to treat inflammatory diseases and cancer in humans and animals (Chapters 35 and 38).

Because of the need for large volumes of immunoglobulin-rich fluids when passively immunizing agricultural species, conventional methodology as described above may be unable to provide sufficient antibodies (or too expensive). Some alternatives include spray-dried blood plasma from normal pig blood used as a feed additive, which contains about 20% immunoglobulin, and surplus milk whey from cheese production where the protein fraction contains about 10% immunoglobulins.

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Active Immunization

Active immunization has several major advantages over passive immunization. These include the prolonged period of protection and the recall and boosting of this protective response by repeated injections of antigen or by exposure to infection. An ideal vaccine for active immunization should therefore give prolonged strong immunity. This immunity should be conferred on both the animal immunized and any fetus carried by it. In obtaining this strong immunity, the vaccine should be free of adverse side effects. (In effect, it should stimulate adaptive immunity without triggering the inflammation associated with innate immunity.) The ideal vaccine should be cheap, stable, and adaptable to mass vaccination; ideally, it should stimulate an immune response distinguishable from that due to natural infection so that immunization and eradication may proceed simultaneously.

In addition to these requirements, effective vaccines must have other critical properties. First, antigen must be delivered efficiently so that antigen-presenting cells can process antigen and release appropriate cytokines. Second, both T and B cells must be stimulated so that they generate large numbers of memory cells so that protection will last for as long as possible. Third, helper and effector T cells must be generated to several epitopes in the vaccine so that individual variations in MHC class II polymorphism and epitope properties are minimized. Fourth, the immune response triggered by the vaccine must be appropriate to the infectious agent, in other words, antibodies or cell-mediated immunity as appropriate.

Living and Killed Vaccines

Unfortunately, two of the prerequisites of an ideal vaccine, high antigenicity and absence of adverse side effects, are sometimes incompatible. Modified live viruses infect host cells and undergo replication. The infected cells then process endogenous antigen. In this way, live viruses trigger a response dominated by $CD8^+$ cytotoxic T cells, a type 1 response. This may be hazardous because

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the vaccine viruses may themselves cause disease or persistent infection (due to residual virulence). Killed organisms, in contrast, act as exogenous antigens. They commonly stimulate type 2 responses dominated by $CD4^+$ T cells and antibodies. This may not be the most appropriate response to some organisms, but it may be safer. Dendritic cells respond in a different fashion to live and killed bacteria.

The practical advantages and disadvantages of vaccines containing living or killed organisms are well demonstrated in the vaccines available against *Brucella abortus* in cattle. *B. abortus* is a cause of abortion in cattle, and vaccination has been used historically to control the disease. Brucella infections are best controlled by type 1 immune responses involving macrophage activation, the production of IFN-γ by Th1 cells, and cytotoxic $CD8^+$ T cells. A vaccine containing a living attenuated strain of *B. abortus* is required to induce these responses and control this infection. Older attenuated *Brucella* vaccines, especially strain 19, caused a lifelong immunity in cows and successfully prevented abortion. Unfortunately, strain 19 vaccine also caused systemic reactions: swelling at the injection site, high fever, anorexia, listlessness, and a drop in milk yield. Strain 19 could cause abortion in pregnant cows, orchitis in bulls, and undulant fever in humans. To eradicate brucellosis, serological tests are used to identify infected animals, and strain 19 causes an antibody response that is difficult to distinguish from a natural infection.

Because of the disadvantages associated with the use of strain 19, considerable efforts have been made to find a better alternative. Unfortunately, killed vaccines (strain 45/20) protected cattle for less than 1 year. An attenuated strain of *B. abortus* called RB-51 has been used in cattle in the United States. This is a rough mutant that fails to produce the lipopolysaccharide O antigen. It does produce a strong Th1 response with production of IFN-γ and $CD8^+$ cytotoxic cells. Unlike strain 19, it does not induce false-positive results in the standard diagnostic tests such as card agglutination, complement fixation, or tube agglutination. It is therefore possible to distinguish between vaccinated and infected cattle. RB-51 is less pathogenic for cattle than strain 19, and it is not shed in nasal secretions, saliva, or urine. RB-51 will not cause abortion in pregnant cattle. It will,

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however, cause disease in accidentally exposed humans, and because of its failure to stimulate antibody production, this may be difficult to diagnose.

The advantages of killed vaccines such as Brucella strain 45/20 are that they are safe with respect to residual virulence and are relatively easy to store since the organisms are already dead (Table 24.1). These advantages correspond to the disadvantages of live vaccines, such as strain 19 or RB-51. That is, some live vaccines may possess residual virulence, not only for the animal for which the vaccine is made but also for other animals. They may revert to a fully virulent type or spread to unvaccinated animals. Live vaccines always run the risk of contamination with unwanted organisms; for instance, outbreaks of reticuloendotheliosis in chickens in Japan and Australia have been traced to contaminated Marek's disease vaccines. An outbreak of bovine leukosis in Australia resulted from contamination of a batch of babesiosis vaccine containing whole calf blood. Abortion and death have occurred in pregnant bitches that received a parvovirus vaccine contaminated with bluetongue virus. Contaminating mycoplasma may also be present in some vaccines. Scrapie has been spread in mycoplasma vaccines. Finally, vaccines containing living attenuated organisms require care in their preparation, storage, and handling to avoid killing the organisms. Maintaining the cold chain can account for up to 80% of the cost of a vaccine in the tropics.

TABLE 24.1
The Relative Merits of Living and Inactivated Vaccines

Living Vaccines	Inactivated Vaccines
Fewer doses required	Stable on storage
Adjuvants unnecessary	Unlikely to cause disease through residual virulence
Less chance of hypersensitivity	Do not replicate in recipient
Induction of interferon	Unlikely to contain live contaminating organisms
Relatively cheap	Will not spread to other animals
Smaller dose needed	Safe in immunodeficient patients
Can be given by natural route	Easier to store
Stimulate both humoral and cell-mediated response	Lower development costs
Longer-lasting protection	No risk of reversion

The disadvantages of killed vaccines parallel the advantages of living vaccines. The use of adjuvants to increase effective antigenicity can cause severe inflammation or systemic toxicity, and

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multiple doses or high individual doses of antigen increase the risk of producing hypersensitivity reactions, as well as increasing costs.

Inactivation

Organisms killed for use in vaccines must remain as antigenically similar to the living organisms as possible. Therefore crude methods of killing that cause major changes in antigen structure as a result of protein denaturation are usually unsatisfactory. If chemicals are used, they must not alter the antigens responsible for stimulating protective immunity. One such chemical is formaldehyde, which cross-links proteins and nucleic acids and confers structural rigidity. Proteins can also be mildly denatured by acetone or alcohol treatment. Alkylating agents that cross-link nucleic acid chains are also suitable for killing organisms since by leaving the surface proteins of organisms unchanged, they do not interfere with antigenicity. Examples of alkylating agents include ethylene oxide, ethyleneimine, acetyl ethyleneimine, and β-propiolactone, all of which have been used in veterinary vaccines. Many successful vaccines containing killed bacteria (bacterins) or inactivated toxins (toxoids) can be made relatively simply by the use of these agents. Some vaccines may contain mixtures of these components. For example, some vaccines against *Mannheimia hemolytica* contain both killed bacteria and inactivated bacterial leukotoxin.

Attenuation

Virulent living organisms cannot normally be used in vaccines. Their virulence must be reduced so that, although still living, they can no longer cause disease. This process of reduction of virulence is called attenuation. The level of attenuation is critical to vaccine success. Underattenuation will result in residual virulence and disease; overattenuation may result in an ineffective vaccine. The traditional methods of attenuation were empirical, and there was little understanding of the changes induced by the attenuation process. They usually involved adapting organisms to growth in unusual conditions so that they lost their adaptation to their usual

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host. For example, the bacille Calmette-Guérin (BCG) strain of *Mycobacterium bovis* was rendered avirulent by being grown for 13 years on bile-saturated medium. The vaccine strain of anthrax was rendered avirulent by growth in 50% serum agar under an atmosphere rich in CO_2 so that it lost its ability to form a capsule. *B. abortus* strain 19 vaccine was grown under conditions in which there was a shortage of nutrients. Unfortunately, genetic stability cannot always be guaranteed in these attenuated strains. Back-mutation or genome reassortment using genes from related viruses may occur and attenuated organisms may redevelop virulence.

A more reliable method of making bacteria avirulent is by genetic manipulation. For example, a modified live vaccine is available that contains streptomycin-dependent *M. hemolytica* and *Pasteurella multocida.* These mutants depend on the presence of streptomycin for growth. When they are administered to an animal, the absence of streptomycin will eventually result in the death of the bacteria, but not before they have stimulated a protective immune response.

Viruses have traditionally been attenuated by growth in cells or species to which they are not naturally adapted. For example, rinderpest virus, which was normally a pathogen of cattle, was first attenuated by growth in rabbits. Eventually, a successful tissue culture-adapted rinderpest vaccine devoid of residual virulence was developed. Similar examples include the adaptation of African horse sickness virus to mice and of canine distemper virus to ferrets. Alternatively, mammalian viruses may be attenuated by growth in eggs. For example, the Flury strain of rabies was attenuated by prolonged passage in eggs and lost its virulence for normal dogs and cats.

The traditional method of virus attenuation has been prolonged tissue culture. In these cases, virus attenuation is accomplished by culturing the organism in cells to which they are not adapted. For example, virulent canine distemper virus preferentially attacks lymphoid cells. For vaccine purposes therefore, this virus was cultured in canine kidney cells. In adapting to these culture conditions, it lost its ability to cause severe disease.

Under some circumstances it is possible to use fully virulent organisms for immunization. Vaccination against contagious ecthyma of sheep is of this type. Contagious ecthyma (orf) is a viral

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disease of lambs that causes massive scab formation around the mouth, prevents feeding, and results in a failure to thrive. The disease has little systemic effect. Lambs recover completely within a few weeks and are immune from then on. It is usual to vaccinate lambs by rubbing dried, infected scab material into scratches made in the inner aspect of the thigh. The local infection at this site has no untoward effect on the lambs, and they become solidly immune. Because vaccinated animals may spread the disease, however, they must be separated from unvaccinated animals for a few weeks.

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Modern Vaccine Technology

Although both killed and modified live vaccines have been successful in controlling many infectious diseases, there is always a need to make them more effective, cheaper, and safer (Fig. 24.4). The use of modern molecular techniques can produce new and improved vaccines. These vaccines can be divided into several categories (Table 24.2).

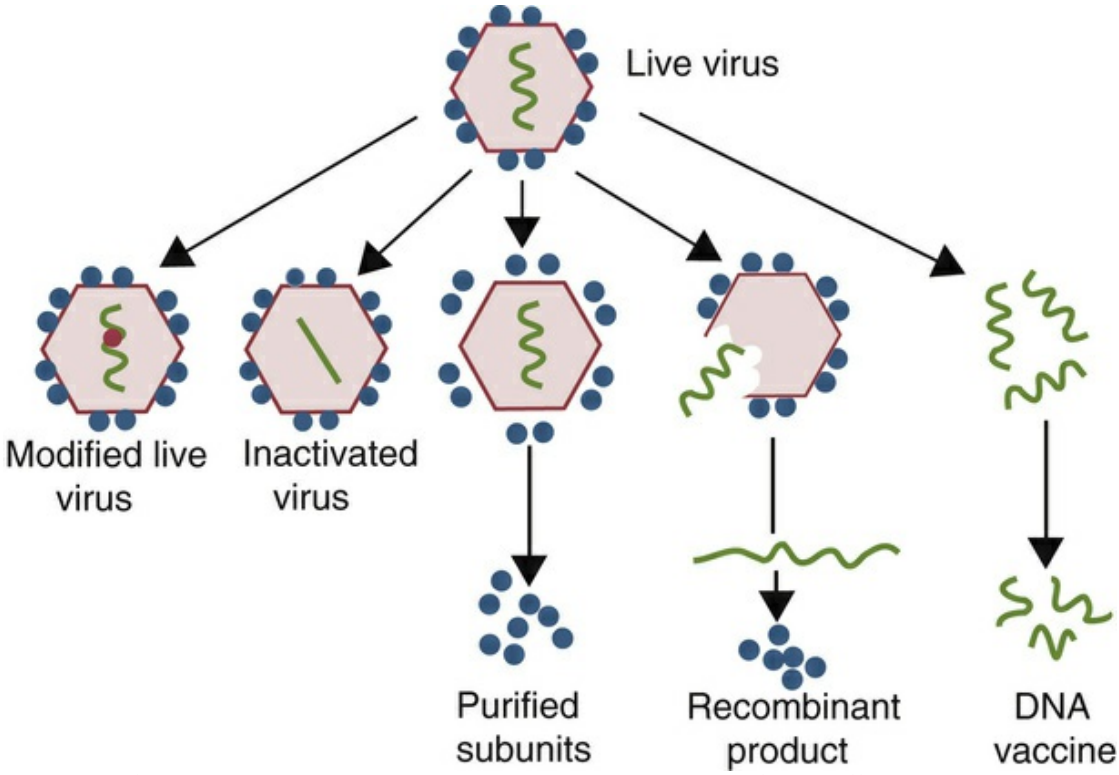


FIG. 24.4 A schematic diagram showing some of the different ways in which a virus and its antigens may be treated in order to produce a vaccine.

TABLE 24.2

USDA Classification of Genetically Engineered Veterinary Biologics

Category	Description
I	Vaccines that contain inactivated recombinant organisms or purified antigens derived from recombinant organisms
II	Vaccines containing live organisms that contain gene deletions or heterologous marker genes

III	Vaccines that contain live expression vectors expressing heterologous genes for immunizing antigens or other stimulants
IV	Other genetically engineered vaccines such as polynucleotide vaccines

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Antigens Generated by Gene Cloning (Category I)

Gene cloning can be used to produce large quantities of purified antigen in culture. In this process, DNA coding for an antigen of interest is first isolated from the pathogen. This DNA is then inserted into a bacterium or yeast in such a way that it is functional and the recombinant antigen is expressed in large amounts. The first successful use of gene cloning to prepare an antigen in this way involved foot-and-mouth disease virus (Fig. 24.5). This virus is extremely simple. The protective antigen (VP1) is well recognized, and the genes that code for this protein have been mapped. The RNA genome of the foot-and-mouth disease virus was isolated and transcribed into DNA by the enzyme reverse transcriptase. The DNA was then carefully cut by restriction endonucleases so that it only contained the gene for VP1. This DNA was then inserted into a plasmid, the plasmid inserted into *E. coli*, and the bacteria grown. The bacteria synthesized large quantities of VP1, which was harvested, purified, and incorporated into a vaccine. The process is highly efficient since 4×10^7 doses of foot-and-mouth vaccine can be obtained from 10 L of *E. coli* grown to 10^{12} organisms per milliliter. Unfortunately, the immunity produced is inferior to that produced by killed virus and requires a 1000-fold higher dose to induce comparable protection.

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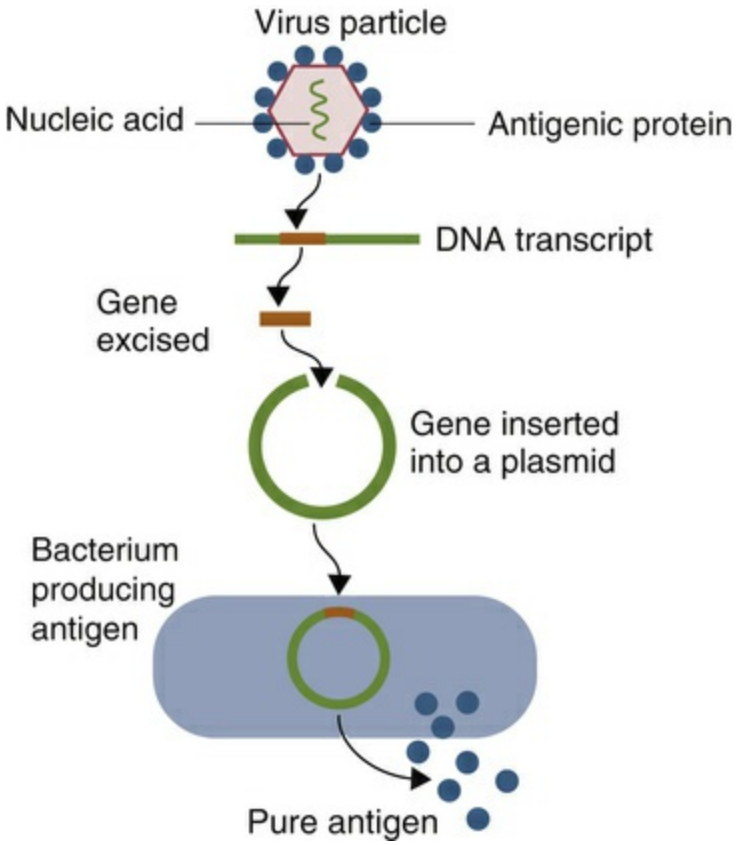


FIG. 24.5 The production of a recombinant viral protein for use in a vaccine. The gene coding for the viral antigen of interest is cloned into another organism, in this case a bacterium, and expressed and produced in very large quantities.

The first commercially available category I recombinant veterinary vaccine was made against feline leukemia virus. The major envelope protein of FeLV, gp70, is the antigen largely responsible for inducing a protective immune response in cats. Thus the gene for gp70 (a *g*lyco*p*rotein of 70 kDa) plus a small portion of a linked protein called p15e (a *p*rotein of 15 kDa from the *e*nvelope) was isolated and inserted into *E. coli,* which then synthesized large amounts of p70. This recombinant p70 is not glycosylated and has a molecular weight of just over 50 kDa. Once cloned, the recombinant protein is harvested, purified, mixed with a saponin adjuvant, and used as a vaccine.

Another example of a recombinant vaccine is that directed against the Lyme disease agent, *Borrelia burgdorferi.* Thus the gene for OspA, the immunodominant outer surface lipoprotein of *B. burgdorferi,* has been cloned into *E. coli.* The recombinant protein expressed by the *E. coli* is purified and used as a vaccine when combined with adjuvant. This vaccine is unique since ticks feeding

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on immunized animals ingest the antibody. The antibodies then kill the bacteria within the tick midgut and prevent their dissemination to the salivary glands. They thus prevent transmission by the vector.

Gene cloning techniques are useful in any situation where pure protein antigens need to be synthesized in large quantities. Unfortunately, very pure proteins are often poor antigens because they are not effectively delivered to antigen-sensitive cells and may not be correctly folded. An alternative method of delivering a recombinant antigen is to clone the gene of interest into an attenuated living carrier organism.

Genetically Attenuated Organisms (Category II)

Molecular genetic techniques make it possible to alter the genes of an organism so that it becomes irreversibly attenuated. These are classified as category II vaccines. They are available against the herpesvirus that causes pseudorabies in swine. The enzyme, thymidine kinase (TK), is required by herpesviruses to replicate in nondividing cells such as neurons. Viruses from which the *TK* gene has been removed can infect nerve cells but cannot replicate and cannot therefore cause disease (Fig. 24.6). As a result, these vaccines not only confer effective protection but also block cell invasion by virulent pseudorabies viruses, preventing the development of a persistent carrier state.

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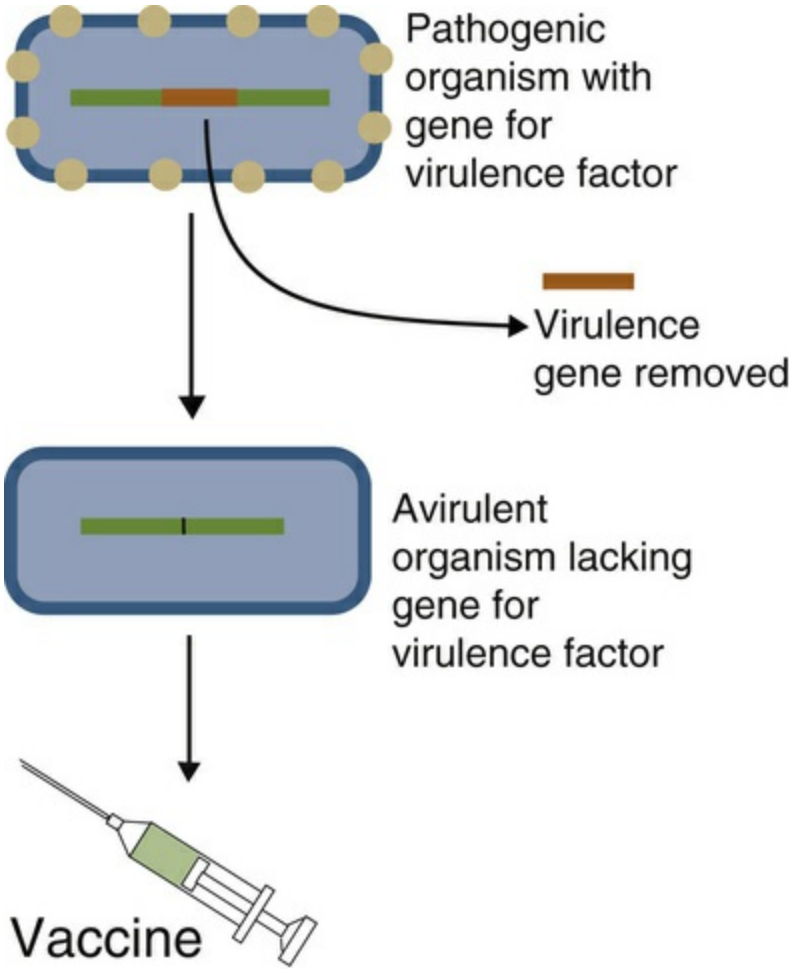


FIG. 24.6 The production of an attenuated virus by removal of a gene required for virulence. Genes coding for major antigens detected by serological techniques can also be removed, ensuring that vaccinated animals can be distinguished from naturally infected ones.

Genetic manipulation can also be used to make "marker vaccines." For example, pseudorabies virus synthesizes two glycoprotein antigens called gX and gI. These are potent antigens, yet neither is essential for viral growth or virulence. They are expressed by all field isolates of this virus, and so infected animals will make antibodies to both gX and gI. An attenuated pseudorabies vaccine has been produced that lacks these proteins. Vaccinated pigs will not make antibodies to gX or gI, but naturally infected pigs will. The vaccine will not cause positive serological reactions in assays for anti-gX or -gI, and the presence of antibodies to gX and gI in a pig is evidence that the animal has been exposed to field strains of pseudorabies virus. This type of vaccine, called a DIVA vaccine (*D*ifferentiate *I*nfected from *V*accinated *A*nimals),

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should assist in eradicating specific infectious diseases much more economically and rapidly than conventional methods. Another example of a DIVA vaccine is the insertion of an influenza B gene into an avian influenza A vaccine. Since influenza B does not infect birds, the presence of antiinfluenza B antibodies confirms vaccination.

Live Recombinant Organisms (Category III)

Genes coding for protein antigens can be cloned directly into a variety of organisms. Instead of being purified, the recombinant organism itself may then be used as a vaccine. These are classified as category III vaccines (Fig. 24.7). Experimental recombinant vaccines have used adenoviruses, herpesviruses, and bacteria such as BCG or salmonella as vectors, but the organisms that have been most widely employed for this purpose are poxviruses such as vaccinia, fowlpox, and canarypox. These viruses are easy to administer by dermal scratching or by ingestion. They have a large stable genome that makes it relatively easy to insert a new gene (up to 10% of its genome can be replaced by foreign DNA), and they can express high levels of the new antigen. Moreover, these recombinant proteins undergo appropriate processing steps, including glycosylation and membrane transport within the poxvirus. The avian poxviruses such as canarypox are especially effective vectors in mammals. They do not replicate, and antigen expression only lasts about 6 hours. As a result, these vaccines are very safe, they cannot be transmitted by arthropods, and they are not excreted in body fluids. It is of interest to note that they do not stimulate immunity to the vector virus, a feature that occurs with the use of other vectors and hence can prevent subsequent immunizations. Canarypox-vectored vaccines appear to overcome blocking by maternal antibodies and can thus prime younger animals. They cannot revert to virulence. As a result, these vaccines are widely employed for such diseases as feline leukemia, West Nile virus, canine parvovirus, canine distemper, equine influenza, and rabies. Another example of a live recombinant vaccine is vaccinia-vectored rabies. The gene for the rabies envelope glycoprotein, or G-protein, can be inserted into vaccinia. This

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glycoprotein can induce virus-neutralizing antibody and so confer protection against rabies. Infection with this rabies-vaccinia recombinant results in the production of antibodies to the G-protein and the development of immunity. This vaccine has been successfully used as an oral bait vaccine administered to wild carnivores. This form of the vaccine can be distributed by dropping from aircraft. It has been used in Ontario to prevent the spread of fox rabies, in New Jersey to prevent the spread of raccoon rabies, and in Texas to block the spread of coyote rabies. For example, since 1995, 17.5 million doses of vaccinia-vectored rabies vaccine have been air-dropped over 255,500 square miles (661,745 sq km) of Texas with great success (Box 24.1).

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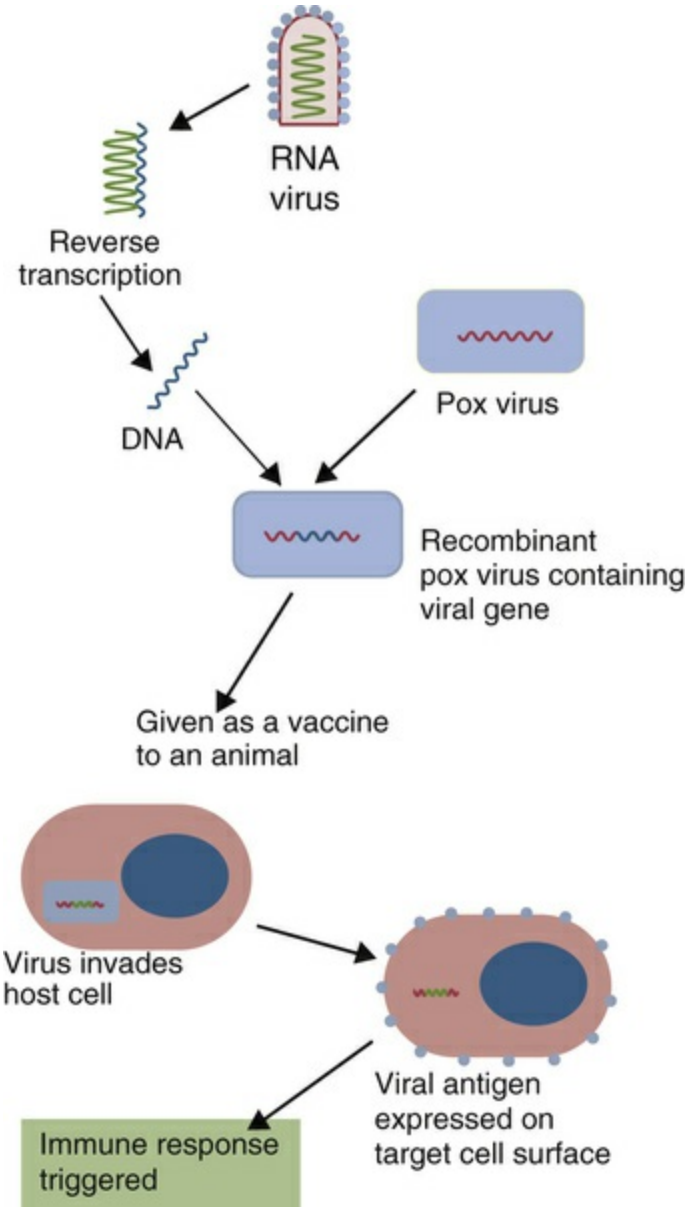


FIG. 24.7 The production of a vaccinia-vectored recombinant vaccine. Vaccinia is selected because it has room to spare in its genome, and it is easy to administer to an animal. Thus rabies-vaccinia recombinants can be given orally.

Box 24.1

Cost Benefits of Vaccination

Rabies is an expensive disease. If an unvaccinated animal bites a human, costs include those of postexposure vaccination of the victim as well as quarantine or euthanasia of the biting animal. The brain of the animal must be examined for the presence of the virus. These costs, of course, do not account for the stress and worry

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associated with this disease. In Texas, aerial vaccination by dropping vaccinia-vectored rabies vaccine enclosed in food bait has been employed to vaccinate coyotes against rabies. The costs of this were the total expenditures on the program—vaccine, food, planes, fuel, and so forth. The benefits were the savings associated with prevented human postexposure prophylaxis and animal rabies tests within the affected area. It was calculated that the rabies vaccination program cost about $26 million. The benefits were estimated at between $89 million and $346 million! Depending on the frequency of postexposure prophylaxis and animal testing, the benefit-to-cost ratio therefore ranged from 3.38 to 33.13.

Shwiff SA, Kirkpatrick KN, Sterner RT: Economic evaluation of an oral rabies vaccination program for control of a domestic dog-coyote rabies epizootic: 1995-2006, *J Am Vet Med Assoc* 233:1736-1741, 2008.

Highly effective category III vaccines were developed for rinderpest; these consisted of a vaccinia or capripox vector containing the hemagglutinin *(H)* or fusion *(F)* genes of rinderpest virus. These vaccines were so effective that their systematic use led to the global eradication of rinderpest. The recombinant capripox vaccine has also had the benefit of protecting cattle against lumpy skin disease. Another example of a category III vaccine involves the use of a yellow fever viral chimera to protect against West Nile virus. This technology uses the capsid and nonstructural genes of the attenuated yellow fever vaccine strain 17D to deliver the envelope genes of other flaviviruses such as West Nile virus. The resulting virus is a yellow fever-West Nile virus chimera that is much less neuroinvasive and hence much safer than either of the parent viruses. The margin of safety can be increased even further by introducing targeted point mutations into the envelope genes. The first category III vaccine approved by the U.S. Department of Agriculture (USDA) was against the Newcastle disease virus. The vector is a fowlpox virus, into which Newcastle disease *H* and *F* genes have been incorporated. It has the benefit of conferring immunity against fowlpox as well.

Oral vaccination has long been considered a desirable route of administration for animals but has been hindered by the ability of the gastrointestinal tract to digest and destroy oral antigens. Such

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vaccines have been delivered in the form of plant materials. Cloning of vaccine antigen genes into plants has been successfully achieved for viruses such as transmissible gastroenteritis, Norwalk virus, and Newcastle disease. The plants employed include tobacco, potato, soybean, rice, and corn. A Newcastle disease vaccine produced in suspension-cultured tobacco cells has been licensed in the United States.

Rather than administering the antigen in plants, an alternative approach is to deliver it within yeast cells. Yeast-based vaccines show high expression levels, are considered very safe, may be "self-adjuvanting," and their antigens undergo appropriate posttranslational modification. Many potential yeast-based vaccines are currently under development, especially for poultry and swine vaccination.

Polynucleotide Vaccines (Category IV)

Another method of vaccination involves injection, not of a protein antigen, but of DNA that encodes vaccine antigens. This DNA can be inserted into a bacterial plasmid, a piece of circular DNA that acts as a vector (Fig. 24.8). The vaccine antigen gene is placed under the control of a strong mammalian promoter sequence. When the genetically engineered plasmid is injected intramuscularly into an animal, it is taken up by host cells. The DNA is then transcribed into messenger RNA and translated into endogenous vaccine protein (Fig. 24.9). The plasmid, unlike viral vectors, cannot replicate in mammalian cells. Plasmid incorporation is enhanced by the use of some "adjuvants" such as lipid complexes, microcapsules, and nonionic copolymers. Aluminum phosphate seems especially effective in improving vaccine efficiency. Transfected host cells synthesize the vaccine protein and present it, as an endogenous antigen, in association with MHC class I molecules. This leads to the development not only of neutralizing antibodies but also, since the antigen is endogenous, of cytotoxic T cells. Expressed antigens have an authentic tertiary structure and posttranslational modifications such as glycosylation. The immune response is also enhanced since the bacterial DNA contains unmethylated CpG motifs that are recognized by toll-like receptor 9

(TLR9) and activate dendritic cells. These in turn promote a strong Th1 response.

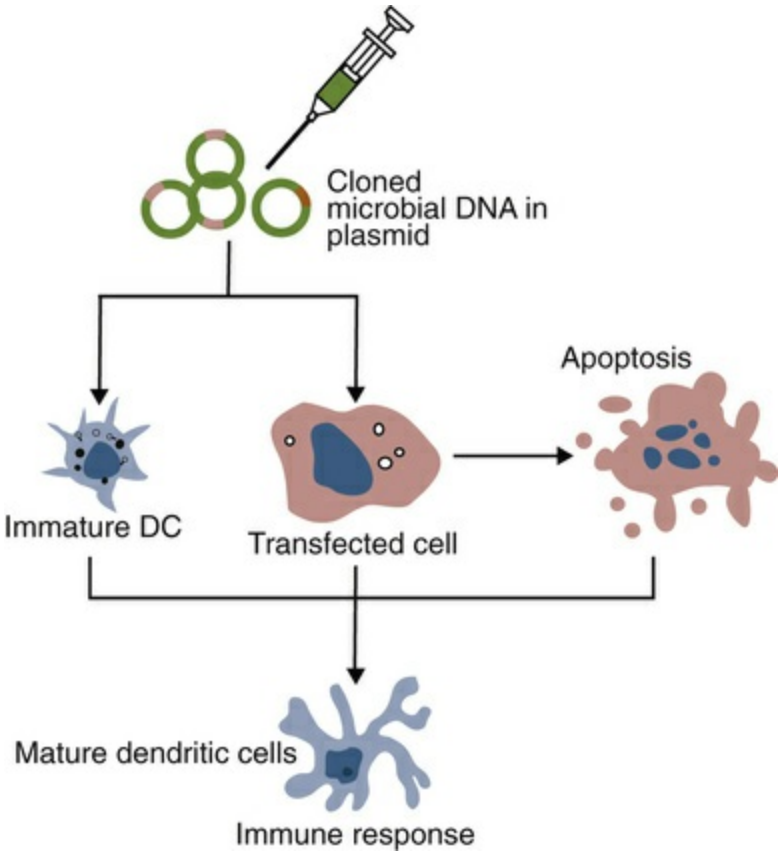


FIG. 24.8 The mechanism by which polynucleotide vaccines may work. The DNA that enters a cell is functional and can encode endogenous antigens.

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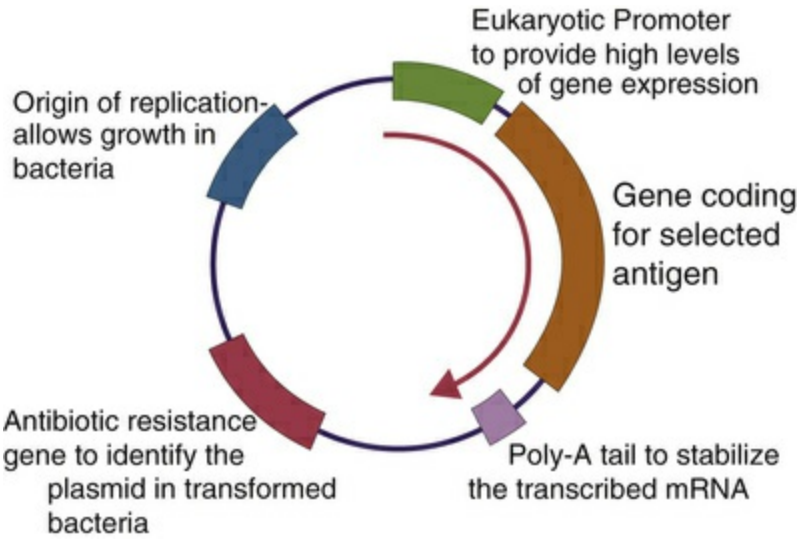


FIG. 24.9 The structure of a typical DNA plasmid used for vaccination purposes. In this case the plasmid codes for protective antigens of West Nile virus. In addition to coding for the antigen in question, the plasmid carries an antibiotic resistance marker so that its fate may be traced.

This type of vaccine is used to protect horses against West Nile virus infection. The commercial vaccine consists of a plasmid vector engineered to express high levels of the virus envelope (E) and premembrane (prM) proteins. In addition, the plasmid contains gene promoters and marker genes. Upon injection together with a biodegradable oil adjuvant, this plasmid enters cells and causes them to express the viral protein. Other DNA vaccines have been approved to prevent infectious hematopoietic necrosis in Atlantic salmon and melanomas in dogs (Chapter 35). This approach has also been applied experimentally to produce vaccines against avian influenza, lymphocytic choriomeningitis, canine and feline rabies, canine parvovirus, bovine viral diarrhea, feline immunodeficiency virus, feline leukemia virus, pseudorabies, foot-and-mouth disease virus, bovine herpesvirus-1, and Newcastle disease. Although theoretically producing a response similar to that induced by attenuated live vaccines, these nucleic acid vaccines are ideally suited to protect against organisms that are difficult or dangerous to grow in the laboratory. Some DNA vaccines can induce immunity even in the presence of very high titers of maternal antibody. Although the maternal antibodies block serological responses, the development of strong memory responses is not impaired.

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Polynucleotide vaccines must get inside target cells. This can be done by intramuscular injection or by "shooting" the DNA plasmids directly through the skin adsorbed onto microscopic gold beads fired by a "gene gun." Although intramuscular injection is very inefficient because the transfection rate is low (about 1% to 5% of myofibrils in the vicinity of an intramuscular injection site), the expression can persist for at least 2 months. The gene products are either treated as endogenous antigens and displayed on the cell surface or secreted and presented to antigen-processing cells. This processed antigen thus preferentially stimulates a Th1 response associated with IFN-γ production. The use of a gene gun is more efficient than injection since some of this DNA is taken up by dendritic cells, and it minimizes degradation. By bypassing TLR9, the DNA preferentially stimulates a Th2 response. Viral DNA in eye drops can induce an IgA response in the tears and bile of recipients.

Immunization with purified DNA allows viral antigens to be presented in their native form. They are synthesized in the same way as antigens during a viral infection. This is more effective than the use of recombinant proteins since it has proved difficult to create the proteins in the correct conformation in microbial systems. It is also possible to select only the genes for the antigen of interest rather than using a complex carrier organism with its own large gene pool and antigenic mass.

Prime-Boost Strategies

It has long been normal practice to use exactly the same vaccine for boosting an immune response as was employed when first priming an animal. This approach has many advantages, not the least of which is simplicity in manufacturing and regulating vaccine production. There is, however, no reason why different forms of a vaccine should not be used for priming and for boosting. This approach is known as a prime-boost strategy. Under some circumstances this may result in significantly improved vaccine effectiveness. The prime-boost approach is somewhat empirical, and researchers may simply test numerous vaccine combinations to determine which combination yields the best results. Prime-boosting has been most widely investigated in attempts to improve

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the effectiveness of DNA vaccines. Combinations usually involve priming with a DNA vaccine but boosting with either another DNA vaccine, perhaps in another vector, or with recombinant protein antigens.

Reverse Vaccinology

Now that many complete microbial genomes are available, it is possible to identify all the proteins of a pathogen by computer analysis. This analysis can then be used to select potential protective epitopes from this repertoire. This can lead to the identification of unique or unsuspected antigens that may then be experimentally tested; a process called reverse vaccinology (Fig. 24.10). The procedures involved include complete sequencing of the antigens of interest, followed by identification of their important epitopes, especially those that bind to common MHC molecules and are likely recognized by $CD4^+$ and $CD8^+$ T cells. These epitopes may be predicted by the use of computer models of the protein or by the use of monoclonal antibodies to identify critical protective components. Once identified, the protective epitopes may be chemically synthesized and tested in animals. Experimental T cell vaccines have been developed in this way against foot-and-mouth disease virus, canine parvovirus, and influenza A.

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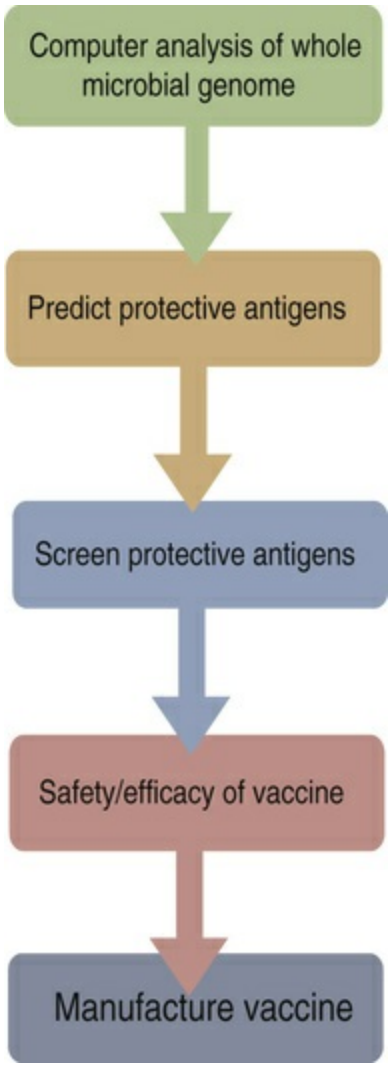


FIG. 24.10 Reverse vaccinology involves the use of our detailed knowledge of an organism's genome to predict the structure of protective epitopes. These can be synthesized and then tested.

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Adjuvants

To maximize the effectiveness of vaccines, especially those containing killed organisms or highly purified antigens, it has been common practice to add substances called adjuvants to the vaccine (*adjuvare* is the Latin verb for "to help"). Adjuvants can increase the speed or the magnitude of the body's response to vaccines, they may permit reductions in the amount of antigen injected or the numbers of doses administered, they can induce appropriate bias in the response (Th1 or Th2), they may trigger cell-mediated immunity, and they are essential if long-term memory is to be established to soluble antigens. Adjuvant use is critical for the effectiveness of subunit or recombinant vaccines. The "science" of adjuvants has historically been empirical. In other words, materials were added to vaccines to see if they improved either the strength or duration of the immune response. As a result, adjuvants appeared to be randomly selected substances and their mechanisms of action were speculative. Recently, however, it has become clear that the commonly employed adjuvants are toll-like receptor ligands. Their main mode of action is to promote antigen uptake, processing, and presentation by dendritic cells. In general, adjuvants trigger innate immune responses that in turn act on dendritic cells to enhance antigen presentation to T or B cells (Table 24.3). As our knowledge of the ways in which dendritic cells and T or B cells interact has grown, it has become possible to develop vaccines designed rationally to maximize antigen presentation. Thus the growing use of particles coated with antigen, cytokines, and co-stimulatory molecules as adjuvants has led to encouraging improvements in vaccine efficacy.

TABLE 24.3

Some Common Adjuvants

Type	Adjuvant	Mode of Action
Depot adjuvants	Aluminum phosphate	Slow-release antigen depot?
	Aluminum hydroxide	Slow-release antigen depot?
	Alum	Activate DAMPs
	Freund's incomplete adjuvant	Slow antigen release depot
Microbial adjuvants	Anaerobic corynebacteria	Macrophage stimulator

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	BCG	Macrophage stimulator
	Muramyl dipeptide	Macrophage stimulator
	Bordetella pertussis	Lymphocyte stimulator
	Lipopolysaccharide	Macrophage stimulator
Immune stimulators	Saponin	Stimulates antigen processing
	Lysolecithin	Stimulates antigen processing
	Pluronic detergents	Stimulates antigen processing
	Glucans	Macrophage stimulator
	Dextran sulfate	Macrophage stimulator
Delivery systems	Liposomes	Stimulates antigen processing
	ISCOMs	Stimulates antigen processing
	Microparticles	Stimulates antigen processing
Mixed adjuvants	Freund's complete adjuvant	Depot plus immune stimulant

BCG, Bacillus Calmette-Guérin; *DAMPs*, damage-associated molecular patterns; *ISCOMs*, immune-stimulating complexes.

Aluminum Salts

These have been used since the 1920s and they are by far the most widely employed adjuvants. They come in different forms including aluminum hydroxide gel (which is actually aluminum oxyhydroxide), aluminum phosphate gel, hydroxysulphophosphate, and aluminum potassium sulfate (alum), as well as calcium phosphate These salts have different physical characteristics and adjuvant properties.

For many years aluminum salts were added to veterinary vaccines in the belief that they formed a depot in the tissues from which antigens slowly leak and thus give a prolonged strong immune response. It is now recognized that a depot effect is not required for their adjuvant effect. Aluminum-adjuvanted vaccines induce inflammatory nodules at the injection site. These contain neutrophils with some eosinophils and lymphocytes in the first 48 hours. Recruitment of mature myeloid dendritic cells to the sites of injection is enhanced. Likewise, activated macrophages are attracted to these sites and these macrophages may develop into dendritic cells.

Removal of the injection site nodule and its associated alum depot as early as 2 hours after vaccination has no significant effect on either T or B immune responses to the vaccine. Alum appears to affect lipids in the plasma membrane and promotes dendritic cell (DC) homing to lymph nodes. Additionally, alum kills inflammatory cells such as neutrophils resulting in DNA release and enhancing DC–T cell interactions. (Fig. 24.11). Although alum has been the most widely used adjuvant in veterinary vaccines, its

mechanisms of action have remained obscure. It had been believed that it acted by promoting IL-1 release, but this has now been disproven. DNA is known to accumulate at sites of alum deposition and is apparently important since DNase treatment blocks this adjuvant activity.

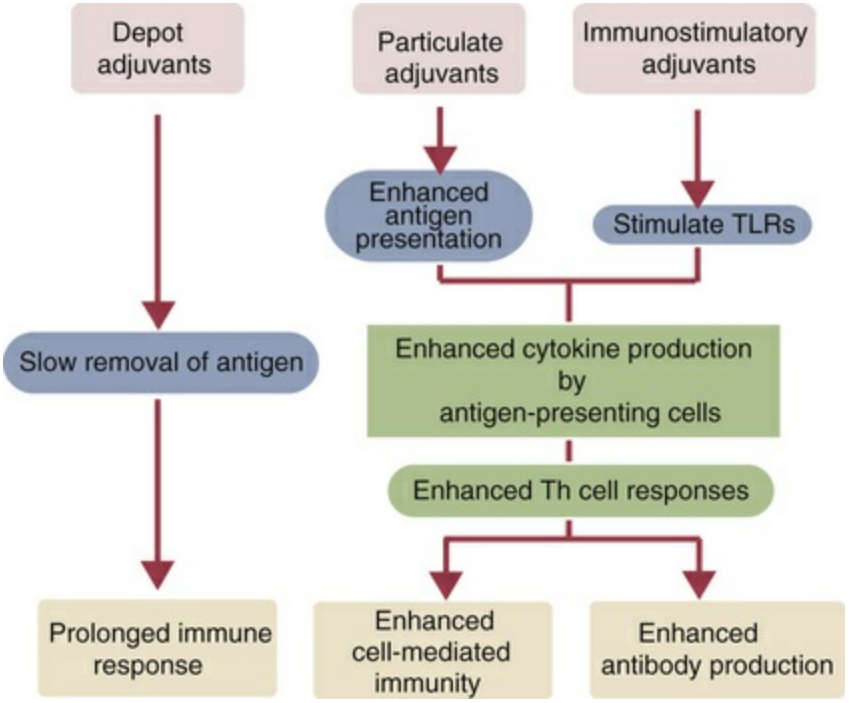


FIG. 24.11 The three major groups of adjuvants and the ways in which these may act to enhance immune responses triggered by vaccine antigens.

Aluminum adjuvants influence only the primary immune response and have little effect on secondary immune responses. They also have the disadvantage that, while promoting antibody responses, they have little effect on cell-mediated responses. Thus alum enhances Th2 responses to protein antigens and generates large numbers of B cells. However, it is not good at generating Th1 $CD8^+$ cells.

Saponin-Based Adjuvants

Saponins (triterpene glycosides), derived from the bark of the soapbark tree *(Quillaja saponaria)*, are widely used as adjuvants. Crude saponins have both toxic and adjuvant activities, although it

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is possible to purify those with potent adjuvant activity and minimal toxicity. Highly purified saponins are used in humans. Saponin-based adjuvants selectively stimulate Th1 responses since they direct antigens into endogenous processing pathways and enhance IFN-γ release by dendritic cells. The saponins activate inflammasomes. Saponin is also employed as an adjuvant for foot-and-mouth disease vaccines and recombinant feline leukemia vaccine. Toxic saponin mixtures are used in anthrax vaccines, where they destroy tissue at the site of injection so that the anthrax spores may germinate. Micelles may be constructed using protein antigens and a complex saponin mixture called Quil-A. *I*mmune *s*timulating *com*plexes (ISCOMs) are stable constructs containing cholesterol, phospholipid, saponin, and antigen. ISCOMs are effective adjuvants with few adverse side effects. They are highly effective in targeting antigens to the professional antigen-processing cells, whereas the saponin activates these cells and promotes cytokine production and the expression of co-stimulatory molecules. Depending on the antigen employed, ISCOMs can stimulate either Th1 or Th2 responses.

Water-in-Oil Emulsions

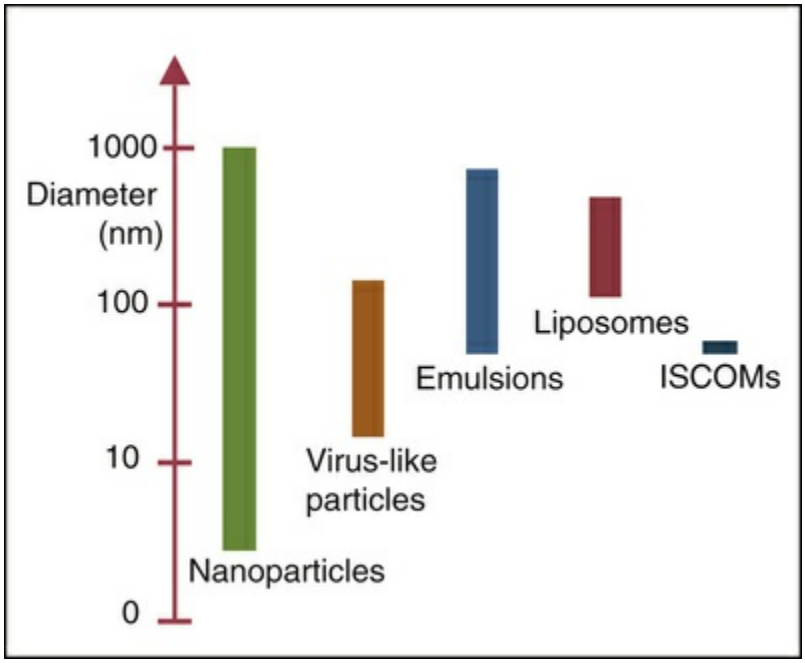
One method of forming a slow-release antigen depot is to incorporate the antigen in a water-in-oil emulsion (droplets of the aqueous phase plus a surfactant such as Tween, Span, or lecithin emulsified in an oil phase). A light mineral oil stimulates a local, chronic inflammatory response, and as a result, a granuloma or abscess forms around the site of the inoculum. The antigen is slowly leached from the aqueous phase of the emulsion. These depot adjuvants may cause significant tissue irritation and destruction. Mineral oils are especially irritating. Nonmineral oils, although less irritating, are also less effective. Tissue damage induced by adjuvants may also promote immunity since the DAMPs generated by inflammation and cell necrosis stimulate both dendritic cells and macrophages. Adjuvants with significant irritant activity are not, however, acceptable in modern vaccines, and it is essential to reduce this irritation while retaining adjuvant effectiveness. In humans, squalene based oil-in-water emulsions

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have been licensed as vaccine adjuvants. (Squalene is a triterpene oil.) They are more potent than alum-based vaccines but may induce mild local reactions by triggering the local release of TNF-α and IL-1β, but their mechanism of action remains unclear.

Particulate Adjuvants

The immune system can trap and process particles such as bacteria or other microorganisms much more efficiently than soluble antigens. As a result, successful adjuvants may incorporate antigens into readily phagocytosable particles (Fig. 24.12). These adjuvants include emulsions, microparticles, ISCOMs, and liposomes, and all are designed to deliver antigen efficiently to antigen-presenting cells. The particles are usually of similar size to bacteria and are readily endocytosed. Liposomes are lipid-based synthetic microparticles 200 to 1000 nm in size containing encapsulated antigens that are effectively trapped and processed yet are also protected from rapid degradation. ISCOMs, described above, are complex lipid-based microparticles about 40 nm in size. All of these particulate adjuvants may be made more potent by incorporating microbial immunostimulants. They are not yet widely employed in veterinary vaccines.



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FIG. 24.12 Nanoparticles come in different sizes, and this greatly influences the nature of immune responses to vaccines.

It is important to note, however, that particle size is critical. Thus conventional aluminum adjuvants employ microparticles (2-8 μm in size) and promote Th2 responses. If, however, they are reduced to nanoparticle size (200-1500 nm), they favor Th1 responses. Nanoparticle adjuvants show considerable promise in new generation vaccines. They can be made from many different compounds such as poly amino acids, polysaccharides, polystyrene, and biodegradable polymers, as well as nondegradable elements such as gold, silver, iron, and silica. They can be engineered to display a mixture of antigens and co-stimulating molecules on their surface so that the immune response is optimized.

Nanoparticles have unique immunological properties that can be manipulated by altering their size, shape, charge, and hydrophobicity. They can be coated with unique combinations of antigens, cytokines, adhesion molecules, immunomodulators, and co-stimulatory ligands, and in effect may be specifically tailored to generate key protective processes. By associating antigens with pattern recognition receptors such as the NLRs, and TLRs, nanoparticles can trigger cytotoxic lymphocyte responses to antigens that normally won't do this. Intradermal and intramuscular administration can induce strong immune responses while intravenous administration may induce tolerance.

Virus-like particles (VLPs) are nanoparticles 20 to 100 nm in size. They may be constructed using viral protein subunits to form, in effect, a viral capsid. Alternatively, they can be entirely synthetic, made by chemical synthesis of carefully designed subunits. VLPs, although they may resemble viruses, do not contain genetic material and cannot replicate and are thus very much safer than conventional virus vaccines. Other molecules can be incorporated into them such as TLR ligands or other cell-targeting molecules. VLPs and other nanoparticles are sufficiently small enough that they can penetrate tissue barriers, travel to draining lymph nodes, and are readily taken up by antigen-presenting cells. Especially when coupled to specific cell-targeting molecules, nanoparticles are much more effective than microparticles at being taken up by cells of the immune system. They promote DC activation and

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presentation of antigens to both MHC class I and class II. As a result, they can prime both $CD4^+$ and $CD8^+$ T cells.

Immunostimulatory Adjuvants

Immunostimulatory adjuvants exert their effects by promoting cytokine production. Many of them are complex microbial products designed to target specific PRRs. As a result, they activate dendritic cells and macrophages and stimulate the production of key cytokines such as IL-1 and IL-12. These cytokines in turn promote helper T cell responses and drive and focus the adaptive immune responses. Depending on the specific microbial product, they may enhance either Th1 or Th2 responses. TLR ligands alone are not usually effective adjuvants since they induce excessive inflammation. Indeed, much of the difficulty encountered in developing adjuvants is to stimulate adaptive immunity without provoking excessive innate immunity.

Double-stranded RNA (dsRNA) is the ligand for TLR3, and synthetic dsRNA (e.g., polyIC) is an effective adjuvant. TLR4 ligands such as bacterial lipopolysaccharides (or their derivatives) have long been recognized as having adjuvant activity. Their toxicity, however, has limited their use. Lipopolysaccharides enhance antibody formation if given at about the same time as the antigen. They have no effect on cell-mediated responses, but they can break T cell tolerance, and they have a general immunostimulatory activity. Killed anaerobic corynebacteria, especially *Propionibacterium acnes,* have a similar effect. When used as adjuvants, these bacteria enhance antibacterial and antitumor activity. The TLR5 ligand bacterial flagellin is an adjuvant that promotes mixed Th1 and Th2 responses. The ligand for TLR7 and TLR8 is single-stranded RNA. Unfortunately, it is rapidly degraded and therefore impractical. Some synthetic ligands, such as the imidazoquinolines, and some guanosine and adenosine analogs, may be effective adjuvants. Unmethylated CpG oligodinucleotides that bind TLR9 are potent immunostimulatory adjuvants for Th1 responses. There are multiple classes of CpG-oligodinucleotides, each of which has slightly different immunostimulatory effects. In practice, it has been found that multiple innate stimuli may be more

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effective than a single stimulus, and that combination adjuvants that have multiple mechanisms of action appear to be most effective.

Combined Adjuvants

Very powerful adjuvants can be constructed by combining a particulate or depot adjuvant with an immunostimulatory agent. For example, an oil-based depot adjuvant can be mixed with killed *Mycobacterium tuberculosis* incorporated into the water-in-oil emulsion. The mixture is called Freund's complete adjuvant (FCA). Not only does FCA form a depot, but the tubercle bacilli also contain muramyl dipeptide (*N*-acetylmuramyl-L-alanyl-D-isoglutamine), a molecule that activates macrophages and dendritic cells through NOD2. FCA works best when given subcutaneously or intradermally and when the antigen dose is relatively low. FCA promotes IgG production over IgM. It inhibits tolerance induction, favors delayed hypersensitivity reactions, accelerates graft rejection, and promotes resistance to tumors. FCA can be used to induce experimental autoimmune diseases, such as experimental allergic encephalitis and thyroiditis (Chapter 37). It also stimulates macrophage activation to M1 cells, promoting their phagocytic and cytotoxic activities.

Use of oil-based adjuvants in animals intended for human consumption is problematic since the oil may spoil the meat. Use of FCA is unacceptable in cattle, not only because of the mineral oil but also because its mycobacteria may induce a positive tuberculin skin test. FCA is highly toxic in dogs and cats.

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25

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The Use of Vaccines

CHAPTER OUTLINE

Administration of Vaccines, 275
- Multiple-Antigen Vaccines, 275
- Vaccination Schedules, 276
- *Initial Series, 276*
- *Revaccination and Duration of Immunity, 276*

Vaccination Strategies, 277
Vaccine Assessment, 278
Failures in Vaccination, 278
- Failure to Vaccinate, 278
- Failure to Respond, 279
- Correct Administration and Response, 280

Adverse Consequences of Vaccination, 280
- "Normal" Toxicity, 281
- Inappropriate Responses, 281
- Errors in Manufacture or Administration, 282
- Vaccine-Associated Autoimmune Disease, 283

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Vaccine-Induced Osteodystrophy, 283
Injection Site–Associated Sarcomas, 283
Adverse Effect Principles, 283
Production, Presentation, and Control of Vaccines, 283

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Explain how the use of any vaccine should be determined by a careful assessment of the relative risks and benefits to an animal.
- Understand why vaccines differ in their importance and thus should be ranked accordingly.
- Understand why unnecessary or over-frequent vaccination should be discouraged.
- Understand the advantages and disadvantages of multiple antigen vaccines.
- Understand and explain the critical importance of the correct timing of vaccination in young animals.
- Understand why vaccines should only be administered in the doses and by the routes recommended by the manufacturer.
- Understand why, on occasion, vaccines may cause adverse effects in animals. These are often mild but may be life threatening.
- Explain again why one cannot effectively vaccinate neonatal animals.
- Identify the major causes of vaccination failure.
- Understand the reasons why some animals will not respond to some vaccines.
- Identify the major adverse events that may be associated with vaccine use.
- Define vaccine failure, vaccine efficacy, and preventable fraction.

Although the principles of vaccination have been known for many years, vaccines and vaccination procedures are continuing to improve in efficacy and safety. The earliest veterinary vaccines were often of limited efficacy and some had significant adverse effects, although these were considered acceptable when measured

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against the risks of acquiring disease. The vaccination protocols developed at that time reflected the inadequacies of these vaccines. Ongoing developments in vaccine design and production have resulted in great improvements in both safety and effectiveness. These improvements permit a reassessment of the relative risks and benefits of vaccination. Vaccination is not always an innocuous procedure. For this reason, the use of any vaccine should be accompanied by a risk-to-benefit analysis conducted by the veterinarian in consultation with the animal's owner. Vaccination protocols should be determined for each individual animal, giving due consideration to the seriousness of the disease, the zoonotic potential of the agent, the animal's susceptibility and exposure risk, and any legal requirements relating to vaccination.

The two major factors that determine vaccine use are safety and efficacy. We must always be sure that the risks of vaccination do not exceed those associated with the chance of contracting the disease. Thus it may be inappropriate to use a vaccine against a disease that is rare, is readily treated by other means, or is of little clinical significance. Because the detection of antibodies is a common diagnostic procedure, unnecessary use of vaccines may complicate diagnosis based on serology and perhaps make eradication of a disease impossible. On the other hand, new serologic tests may make it possible to determine animal susceptibility and rationalize vaccine use decisions. The decision to use vaccines for the control of any disease must be based not only on the degree of risk associated with the disease but also on the availability of superior alternatives.

The second major consideration is vaccine efficacy. Vaccines may not always be effective. In some diseases, such as equine infectious anemia, Aleutian disease in mink, and African swine fever, poor or no protective immunity can be induced even with the best currently available vaccines. In other diseases, such as foot-and-mouth disease in pigs, the immune response is transient and relatively ineffective, and successful vaccination is sometimes difficult to achieve.

As a result of these considerations, animal vaccines should be ranked based on their importance. The first category consists of essential vaccines—those that are required because they protect

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against common, dangerous diseases and because a failure to use them would place an animal at significant risk of disease or death. Determination of which vaccines are essential may vary based on local conditions and disease threats. A second category consists of optional vaccines. These are directed against diseases for which the risks associated with not vaccinating may be low. In many cases, risks from these diseases are determined by the location or lifestyle of an animal. The use of these optional vaccines should be determined by a veterinarian on the basis of exposure risk. A third category consists of vaccines that may have no application in routine vaccination but may be used under special circumstances. These are vaccines directed against diseases of little clinical significance or vaccines whose risks do not significantly outweigh their benefits. Of course, all vaccine use should be conducted on the basis of informed consent. An animal's owner should be made aware of the risks and benefits involved before seeking approval to vaccinate.

When vaccines are used to control disease in a population of animals rather than in individuals, a veterinarian should also consider the concept of herd immunity. This herd immunity is the resistance of an entire group of animals to a disease as a result of the presence, in that group, of many immune animals. Herd immunity reduces the probability of a susceptible animal meeting an infected one so that the spread of disease is slowed or prevented. If it is acceptable to lose individual animals from disease while preventing epizootics, it may be possible to do this by vaccinating only a proportion of the population. Certainly, veterinarians should seek to ensure that as many animals as possible are vaccinated in order to maximize herd immunity.

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Administration of Vaccines

Most vaccines are administered by injection. Care must be taken not to injure or introduce infection into any animal. All needles used must be clean and sharp. Dirty or dull needles can cause tissue damage and infection at the injection site. The skin at the injection site must be clean and dry, although excessive alcohol swabbing should be avoided. Vaccines are provided in a standard dose, and this dose should not be divided to account for an animal's size. Doses are not yet formulated to account for body weight or age. There must be a sufficient antigen to trigger the cells of the immune system and provoke an immune response. This amount is not necessarily related to body size. (Unfortunately, the risk of an adverse event occurring is increased in smaller animals, so it may be necessary to make some adjustment in vaccine dose for safety reasons.) Vaccination by subcutaneous or intramuscular injection is the simplest and most common method of administration. This approach is obviously excellent for small numbers of animals and for diseases in which systemic immunity is important. In other diseases, however, systemic immunity is not as important as immunity on surfaces, and it is perhaps more appropriate to administer a vaccine at the site of potential invasions. Therefore, intranasal vaccines are available for infectious bovine rhinotracheitis, parainfluenza 3 and respiratory syncytial virus of cattle; for *Streptococcus equi* infections in horses; for feline rhinotracheitis, *Bordetella bronchiseptica,* coronavirus, and calicivirus infections; for canine parainfluenza and *Bordetella* infection; and for infectious bronchitis and Newcastle disease in poultry. These methods of administration require that each animal be dealt with on an individual basis. When animal numbers are large, other methods must be employed. For example, aerosolization of vaccines enables them to be inhaled by all the animals in a group. This technique is employed in vaccinating against canine distemper and mink enteritis on mink ranches and against Newcastle disease in poultry. Alternatively, the vaccine may be administered in the feed or drinking water, as is done with *Erysipelothrix rhusiopathiae* vaccines in pigs and against Newcastle disease, infectious

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laryngotracheitis, and avian encephalomyelitis in poultry. Alternative routes of vaccine administration that are in development or employed in humans include cutaneous vaccination using liquid-jet injectors, microinjection, or topical skin application through patches or nanoparticles. Plague vaccine-coated M&Ms have been delivered to prairie dogs and black-footed ferrets in the western United States by means of drones that can shoot the candies in three directions simultaneously in order to ensure even coverage.

Multiple-Antigen Vaccines

For convenience, it is common to employ mixtures of organisms within single vaccines. In cattle, for example, vaccines are available that contain infectious bovine rhinotracheitis (BHV-1), bovine virus diarrhea (BVDV), parainfluenza 3 (P13), and even *Mannheimia hemolytica.* Dogs may be given vaccines containing all of the following organisms: canine distemper virus, canine adenovirus 1, canine adenovirus 2, canine parvovirus 2, canine parainfluenza virus, leptospira bacterin, and rabies vaccine. These mixtures may be used when exact diagnosis is not possible and may protect animals against several diseases with economy of effort. However, it can also be wasteful to use vaccines against organisms that may not be causing problems. When different antigens in a mixture are inoculated simultaneously, competition occurs between antigens. Manufacturers of multiple-antigen vaccines take this into account and adjust their components accordingly. Vaccines should never be mixed indiscriminately since one component may dominate the mixture or interfere with the response to the other components.

Some veterinarians have questioned whether the use of complex vaccine mixtures leads to less than satisfactory protection or increases the risk for adverse side effects. They are concerned that the use of 5- or 7-component vaccines in their pets will somehow overwhelm the immune system, forgetting that our animals encounter hundreds of different antigens in daily life. The suggestion that these multiple-antigen vaccines can overload the immune system is unfounded, nor is there any evidence to support the contention that the risk for adverse effects increases

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disproportionately when more components are added to vaccines. The success of a 21-component bluetongue vaccine in sheep or a 23-component Pneumococcal vaccine in AIDS patients should serve as a reassurance that multiple component vaccines are not overwhelming. Certainly such vaccines should be tested to ensure that all components induce a satisfactory response. Licensed vaccines provided by a reputable manufacturer will generally provide satisfactory protection against all components.

Vaccination Schedules

Although it is not possible to give exact schedules for all veterinary vaccines, certain principles are common to all methods of active immunization. Most vaccines require an initial series in which protective immunity is initiated, followed by revaccination (booster shots) at intervals to ensure that this protective immunity remains at an adequate level.

Initial Series

Because maternal antibodies passively protect newborn animals, it is not usually possible to successfully vaccinate very young animals. If stimulation of immunity is deemed necessary at this stage, the mother may be vaccinated during the later stages of pregnancy, the vaccinations being timed so that peak antibody levels are achieved at the time of colostrum formation. Once an animal is born, successful active immunization is effective only after passive immunity has waned. Since it is impossible to predict the exact time of loss of maternal immunity, the initial vaccination series will generally require administration of multiple doses. Current guidelines for essential canine and feline vaccines, for example, indicate that the first dose of vaccine should be administered at 8 to 9 weeks of age, followed by a second dose 3 to 4 weeks later, and concluding at about 16 weeks of age. (These are not, strictly speaking, booster doses. They are simply designed to trigger a primary response as soon as possible after maternal immunity has waned.) All animals should then receive a booster dose 12 months later or at 1 year of age. Administration of vaccines to young animals is discussed in Chapter 23. It is unclear whether

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maternal antibodies can block responses to all intranasal vaccines. Despite high levels of circulating maternal antibodies, maternal interference does not always occur and nasal antibody production is often unimpaired.

The timing of initial vaccinations may also be determined by disease epidemiology. Some diseases are seasonal, and vaccines may be given before disease outbreaks are expected. Examples of these include the vaccine against the lungworm *Dictyocaulus viviparus* given in early summer just before the anticipated lungworm season; the vaccine against anthrax given in spring; and the vaccine against *Clostridium chauvoei* given to sheep before turning them out to pasture. Bluetongue of lambs is spread by midges *(Culicoides variipennis)* and is thus a disease of midsummer and early fall. Vaccination in spring will therefore protect lambs during the susceptible period.

Revaccination and Duration of Immunity

As pointed out in Chapter 24, it is the persistence of memory cells after vaccination that provides an animal with long-term protection. The presence of long-lived plasma cells is associated with persistent antibody production so that a vaccinated animal may have antibodies in its bloodstream for many years after exposure to a vaccine. It is these antibodies that are mainly responsible for long-term protection.

Revaccination schedules depend on the duration of effective protection (Table 25.1). This in turn depends on specific antigen content, whether the vaccine consists of living or dead organisms, and its route of administration. In the past, relatively poor vaccines may have required frequent administration, perhaps as often as every 6 months, to maintain an acceptable level of immunity. Newer, modern vaccines usually produce a long-lasting protection, especially in companion animals; many require revaccination only every 3 or 4 years, whereas for others, immunity may persist for an animal's lifetime. Even killed viral vaccines may protect individual animals against disease for many years. Unfortunately, the minimal duration of immunity has, until recently, rarely been measured, and reliable figures are not available for many vaccines. Likewise, although serum antibodies can be monitored in vaccinated animals,

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tests have not been standardized, and there is no consensus regarding the interpretation of these antibody titers. Even animals that lack detectable antibodies may have significant resistance to disease. Nor is there much information available regarding long-term immunity on mucosal surfaces. In general, immunity against feline panleukopenia, canine distemper, canine parvovirus, and canine adenovirus is considered to be relatively long-lasting (>5 years). On the other hand, immunity to feline rhinotracheitis, feline calicivirus, and *Chlamydophila* is believed to be relatively short. One problem in making these statements is variability among individual animals and among different types of vaccine. Thus recombinant canine distemper vaccines may induce immunity of shorter duration than conventional, modified live vaccines. There may be a great difference between the shortest and longest duration of immunological memory within a group of animals. Duration of immunity studies are confounded by the fact that many older animals have increased innate resistance. Different vaccines within a category may differ significantly in their composition, and although all vaccines may induce immunity in the short term, it cannot be assumed that all confer long-term immunity. Manufacturers use different master seeds and different methods of antigen preparation. The level of immunity required for most of these diseases is unknown. A significant difference exists between the minimal level of immunity required to protect most animals and the level of immunity required to ensure protection of all animals.

TABLE 25.1

Estimated Minimum Duration of Immunity (DOI) of Select Commercially Available Canine Vaccine Antigens

Vaccine	Estimated Minimum DOI	Estimated Relative Efficacy (%)
Essential		
Canine distemper (modified live virus [MLV])	>7 yr	>90
Canine distemper (recombinant [R])	>1 yr	>90
Canine parvovirus-2 (MLV)	>7 yr	>90
Canine adenovirus-2 (MLV)	>7 yr	>90
Rabies virus (killed [K])	>3 yr	>85
Optional		
Canine coronavirus (K or MLV)	N/A	N/A
Canine parainfluenza (MLV)	>3 yr	>80
Bordetella bronchiseptica (ML)	<1 yr	<70
Leptospira canicola (K)	<1 yr	<50

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Leptospira grippotyphosa (K)	<1 yr	N/A
Leptospira icterohaemorrhagiae (K)	<1 yr	<75
Leptospira pomona (K)	<1 yr	N/A
Borrelia burgdorferi (K)	1 yr	<75
Borrelia burgdorferi OspA (R)	1 yr	<75
Giardia lamblia (K)	<1 yr	N/A

From Paul MA, Appel M, Barrett R, et al: Report of the American Animal Hospital Association (AAHA) Canine Vaccine Task Force: executive summary and 2003 canine vaccine guidelines and recommendations, *J Am Anim Hosp Assoc* 39:119-131, 2003.

Annual revaccination was once the rule for most animal vaccines since this approach is administratively simple and has the advantage of ensuring that an animal is regularly seen by a veterinarian. It is clear, however, that vaccines such as those against canine distemper or feline herpesvirus induce protective immunity that can last for many years, and that annual revaccination using these vaccines is inappropriate. A growing body of evidence now indicates that most modified live viral (MLV) vaccines induce lifelong sterile immunity in dogs and cats. In contrast, immunity to bacteria is of much shorter duration and often may prevent disease but not infection. Old dogs and cats rarely die from vaccine-preventable disease, especially if they have been vaccinated as adults. Young animals, in contrast, may die from such diseases, especially if not vaccinated or if vaccinated prematurely. A veterinarian should always assess the relative risks and benefits to an animal in determining the timing of any vaccination. It is therefore good practice to use serum antibody assays such as rapid test enzyme-linked immunosorbent assay (ELISA) kits, if available, to provide guidance on revaccination intervals. Persistent antibody titers determine whether an animal requires additional protection. These tests not only identify those animals that have responded to vaccination, they can determine if an animal is a nonresponder. They can determine if an animal that previously suffered from an adverse event really requires revaccination. They can determine whether an animal with an undocumented vaccine history needs to be vaccinated and with which vaccines. They can determine which animals in a shelter undergoing a disease outbreak are susceptible and so require vaccination. They can also determine whether revaccination is really necessary at 3 years. It should be pointed out, however, that animals with low or undetectable serum antibody levels may still be protected as a result of persistence of memory B and T cells capable of responding rapidly to reinfection. “Blind”

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revaccination should be avoided if appropriate serum antibody assays are available.

Notwithstanding the previous discussion, animal owners should be made aware that protection against an infectious disease can only be maintained reliably when vaccines are used in accordance with the protocol approved by the vaccine-licensing authorities. The duration of immunity claimed by a vaccine manufacturer is the minimum duration of immunity that is supported by the data available at the time the vaccine license is approved. This must always be taken into account when discussing revaccination protocols with an owner.

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Vaccination Strategies

Although vaccination is a powerful tool for the control of infectious disease, its potential to prevent the spread of or eliminate a disease depends on selecting correct control strategies. If an infectious disease outbreak, such as one caused by foot-and-mouth virus, is to be rapidly controlled, it is vitally important to select the correct population to be vaccinated. The success of any mass vaccination program depends both on the proportion of animals vaccinated and on the efficacy of the vaccine. Neither of these factors will reach 100%, so it is essential to target the vaccine effectively. It is also the case that vaccines do not confer immediate protection, so the strategy employed will depend on the rate of spread of an infection. Vaccines may thus be given prophylactically, in advance of an outbreak, or reactively, in response to an existing outbreak. Both strategies have advantages and disadvantages. In general, prophylactic vaccination greatly reduces the potential for a major epidemic of a disease such as foot-and-mouth disease by reducing the size of the susceptible population. The effectiveness of this approach can be greatly enhanced by identifying high-risk individuals and ensuring that they are protected in advance of an outbreak.

It is generally not feasible to vaccinate an entire population of animals once a disease outbreak has occurred. However, two effective reactive vaccination strategies are ring vaccination, which seeks to contain an outbreak by establishing a barrier of immune animals around an infected area, and predictive vaccination, which seeks to vaccinate the animals on farms likely to contribute most to the future spread of disease. Reactive vaccination in this way can ensure that an epidemic is not unduly prolonged. A prolonged "tail" to an epidemic commonly results from the disease "jumping" to a new area. Well-considered, predictive vaccination may well prevent these jumps. Thus a combination of prophylactic and reactive vaccination will likely yield the most effective results.

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Vaccine Assessment

To assess the efficacy of a vaccine, animals must first be vaccinated and then challenged. The percentage of vaccinated animals that survive this challenge can then be measured. It is important, however, to determine the percentage of non-vaccinated control animals that also survive the challenge. The true efficacy of a vaccine, called the preventable fraction (PF), is calculated as follows:

$$PF = \frac{(\%\text{ of controls dying} - \%\text{ of vaccinates dying})}{\%\text{ of controls dying}}$$

For example, a challenge that kills 80% of controls and 40% of vaccinates shows that the PF of the vaccine is as follows:

$$PF = \frac{80 - 40}{80} = 50\%$$

Good, effective vaccines should have a PF of at least 80%. Obviously, less effective vaccines are acceptable if safe and if nothing better is available. In determining vaccine efficacy, however, large challenge doses may overwhelm any reasonable level of vaccine-induced immunity. It is also important to determine what degree of protection is desired. It may be much easier to prevent deaths rather than illness.

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Failures in Vaccination

There are many reasons why a vaccine may fail to confer protective immunity on an animal (Fig. 25.1).

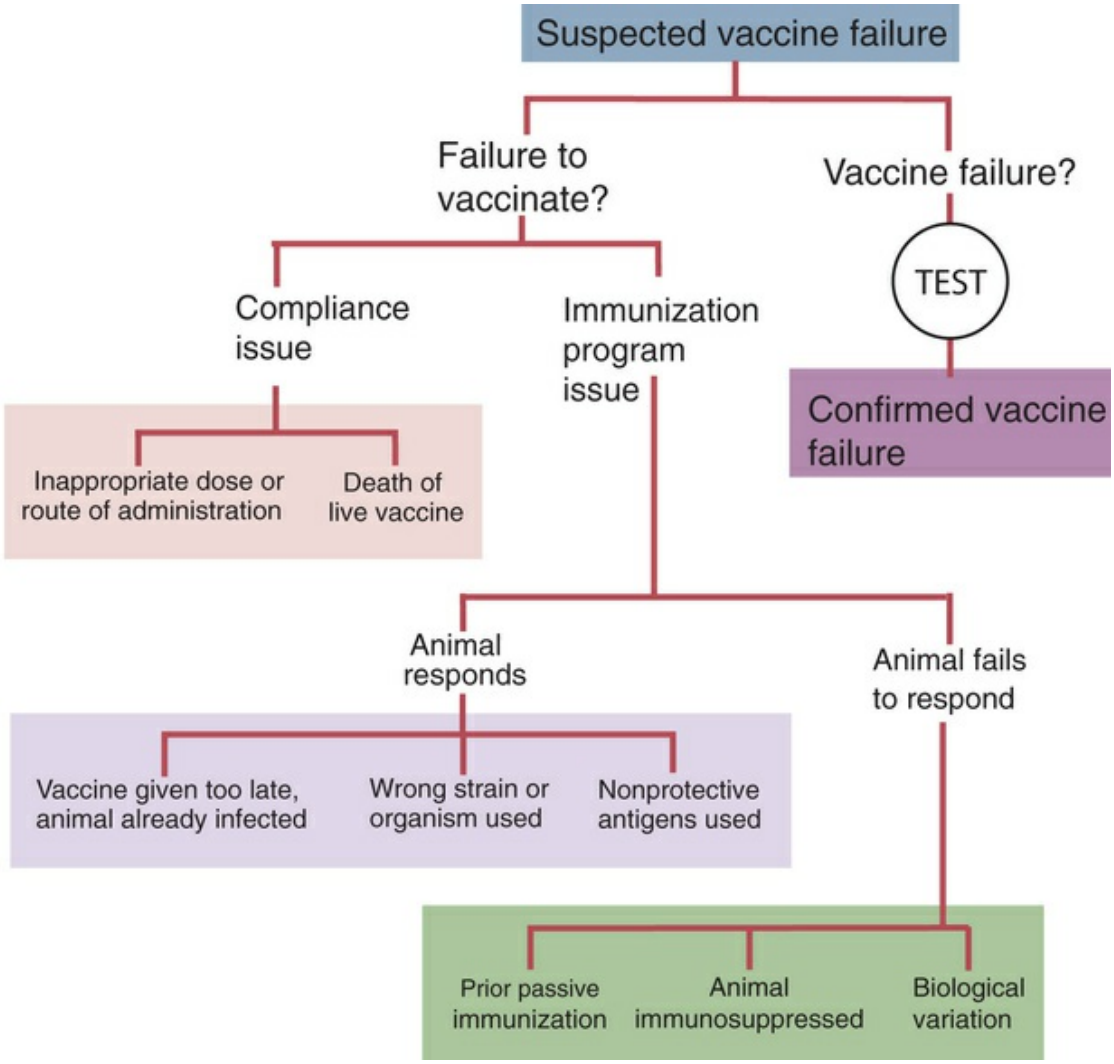


FIG. 25.1 A simple classification of the ways in which vaccination may fail to protect an animal against disease.

Failure to Vaccinate

Most cases of vaccine failure result from unsatisfactory or incomplete administration of the vaccine or noncompliance with manufacturer's recommendations. For example, a live vaccine may

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have died as a result of poor storage, the use of antibiotics in conjunction with live bacterial vaccines, the use of chemicals to sterilize the syringe, or the excessive use of alcohol swabs on the skin. Sometimes animals given vaccines by unconventional routes may not be protected. When large flocks of poultry or mink are to be vaccinated, it is common to administer the vaccine either as an aerosol or in drinking water. If the aerosol is not evenly distributed throughout a building, or if some animals do not drink, they may receive insufficient vaccine. Inappropriate vaccination of young animals prior to loss of maternal immunity remains a problem. Animals that subsequently develop disease may be interpreted as cases of vaccine failure.

Failure to Respond

Occasionally, a vaccine may actually be ineffective. The method of production may have destroyed the protective epitopes, or there may simply be insufficient antigen in the vaccine. Problems of this type are uncommon and can generally be avoided by using only vaccines from reputable manufacturers.

More commonly, an animal may simply fail to mount an immune response. The immune response, being a biological process, never confers absolute protection and is never equal in all members of a vaccinated population. Since immunity is influenced by many genetic and environmental factors, the range of immune responses in a large random population of animals follows a normal distribution. This means that most animals respond to antigens by mounting an average immune response, whereas a few will mount an excellent response, and a few will mount a poor immune response (Figs. 25.2 and 25.3). These poor responders may not be protected against infection despite having received an effective vaccine. It is impossible to protect 100% of a large outbred population of animals by vaccination. The size of this unreactive portion of the population will vary between vaccines, and its significance will depend on the nature of the disease. Thus, for highly infectious diseases against which herd immunity is poor and in which infection is rapidly and efficiently transmitted, such as foot-and-mouth disease, the presence of even a few unprotected

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animals can permit the spread of disease and disrupt control programs. Likewise, problems can arise if the unprotected animals are individually important, such as companion animals. In contrast, for diseases that are inefficiently spread, such as rabies, 70% protection may be sufficient to effectively block disease transmission within a population and may therefore be quite satisfactory from a community health viewpoint.

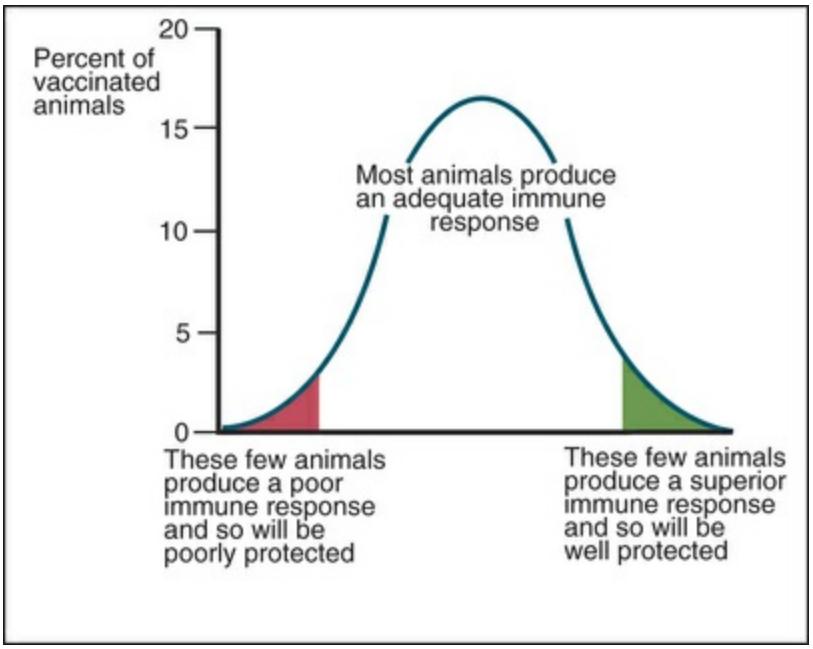


FIG. 25.2 The normal distribution of protective immune responses in a population of vaccinated animals. No vaccine can be expected to protect 100% of a population.

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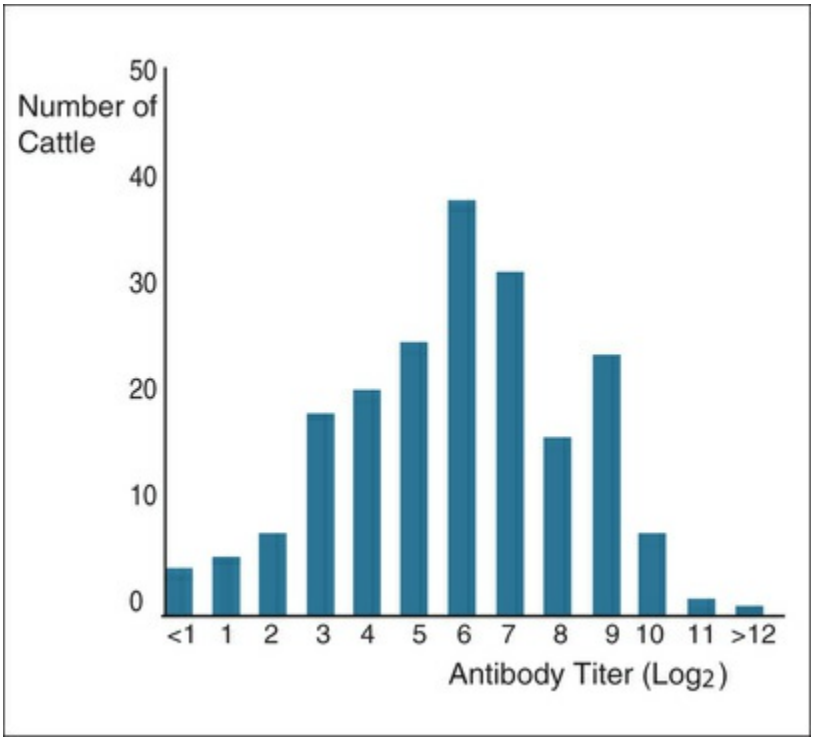


FIG. 25.3 The actual antibody responses in a herd of cattle vaccinated against bovine viral diarrhea type 1. Antibody log titers of less than 3 are not considered significant. Log titers greater than 9 may reflect a secondary response as a result of prior infection or previous vaccination. Note that these results represent a normal distribution and reflect the responses to most vaccines. Vaccination success is not guaranteed. (Data courtesy Dr. T. Hairgrove).

Another type of vaccine failure occurs when the normal immune response is suppressed. For example, heavily parasitized or malnourished animals may be immunosuppressed and should not be vaccinated. Some virus infections induce profound immunosuppression. Animals with a major illness or high fever should not normally be vaccinated unless for a compelling reason. Stress may reduce a normal immune response, probably because of increased steroid production; examples of such stress include fatigue, malnutrition, and extremes of cold and heat. Studies have shown that surgical neutering at or near the time of first vaccination does not impair the antibody responses of kittens. This type of immunosuppression is discussed in detail in Chapter 40. The most important cause of vaccine failure of this type is passively derived maternal immunity in young animals, as described in Chapter 23.

Analysis of an outbreak of influenza in racehorses has shown some interesting and important factors that appeared to determine vaccine effectiveness. Thus when the effect of age was analyzed, it

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appears that 2-year-old horses were less susceptible than other animals. Further analysis suggested that this increased resistance resulted from recent vaccination of this age cohort despite other age groups possessing similar antibody levels. There was evidence of gender differences in resistance (62% of females and 71% of males were infected). There was also some evidence that vaccination at a young age (<6 months) in the presence of maternal antibody had detrimental long-term effects on protection when compared to foals first vaccinated between 6 and 18 months of age.

Recent studies have also analyzed data from 10,483 dogs of all ages and breeds vaccinated against rabies to determine the factors that influence seroconversion. It was found that a relationship exists between a dog's size and its antibody level. Smaller dogs produced higher antibody titers than large dogs. Vaccine effectiveness also varied among breeds. Thus significant failure rates were seen in German Shepherds and Labradors. Young animals vaccinated before 1 year of age produced lower antibody levels than adults. The highest antibody titers were generated in dogs aged 3 to 4 years at time of vaccination. Primary vaccination of aged animals showed lower antibody levels and increased failure rates. Gender had no effect on failure rate or titer. Failure rates varied greatly between vaccines. They ranged from 0.2% in the worst case to 0.01% in the best, and some vaccines showed significant batch-to-batch variation in efficacy. Of the variation in antibody titers observed, 19% was due to vaccine differences, 8% was due to breed differences, 5% was attributed to size differences, and 3% to other differences. It is likely that similar variables influence the responses of animals to other vaccines. Perhaps vaccines should be reformulated to take these age, size, and breed differences into account.

Correct Administration and Response

Even animals given an adequate dose of an effective vaccine may fail to be protected. If the vaccinated animal was incubating the disease before inoculation, the vaccine may be given too late to affect the course of the disease. Alternatively, the vaccine may contain the wrong strain of organisms or the wrong (nonprotective) antigens.

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Adverse Consequences of Vaccination

Vaccination continues to be the only safe, reliable, and effective way of protecting animals against the major infectious diseases. Vaccine-related toxicity is usually rare, mild, and transient, and hypothetical side effects must not dominate our perceptions. Nevertheless, the use of vaccines is not free of risk. Residual virulence and toxicity, allergic responses, disease in immunodeficient hosts, neurological complications, and harmful effects on the fetus are the most significant risks associated with the use of vaccines (Fig. 25.4). Veterinarians should use only licensed vaccines, and the manufacturer's recommendations should be carefully followed. Before using a vaccine, the veterinarian should consider the likelihood that an adverse event will happen, as well as the possible consequences or severity of this event. These factors must be weighed against the benefits to the animal. Thus a common but mild complication will require a different consideration than a rare, severe complication.

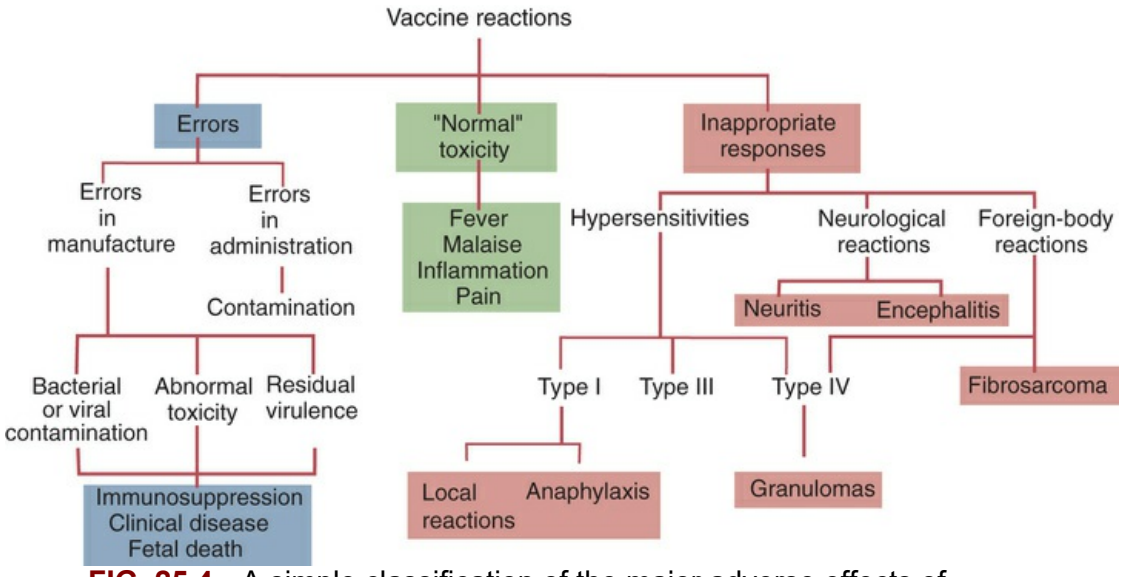


FIG. 25.4 A simple classification of the major adverse effects of vaccination.

The issue of the risk associated with vaccination remains in large

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part a philosophical one since the advantages of vaccination are well documented and extensive, whereas the risk for adverse effects is poorly documented and, in many cases, largely hypothetical. Nevertheless, established facts should be recognized, unsubstantiated allegations rebutted by sound data, and uncertainties acknowledged. For example, there is absolutely no evidence that vaccination itself leads to ill health. Although difficult to prove a negative, competent statistical analysis has consistently failed to demonstrate any general adverse effect of vaccination.

Traditionally, adverse events resulting from vaccine administration have been reported by veterinarians to manufacturers or government agencies. The resulting figures have been impossible to analyze satisfactorily for two major reasons. First, reporting is voluntary, so significant underreporting occurs. Many adverse events are regarded as insignificant, or it may be inconvenient to report them. Second, very little data has been available on the number of animals vaccinated. Although manufacturers know the number of doses of vaccine sold, they are unable to measure the number of animals vaccinated. Nevertheless, it has proved possible by examining the electronic records of a very large general practice to determine the prevalence of vaccine-associated adverse events in more than one million dogs. The use of a standardized reporting system within a very large population has permitted objective analysis of the prevalence of adverse events occurring within 3 days of vaccine administration. Out of 1,226,159 dogs vaccinated, there were 4,678 adverse events recorded (38.2/10,000 dogs); 72.8% of these events occurred on the same day the vaccine was administered, 31.7% were considered to be allergic reactions, and 65.8% were considered "vaccine reactions" and were likely due to toxicity. Additional analysis indicated that the risk of adverse events was significantly greater for small than for large dogs (Fig. 25.5); for neutered than for sexually intact dogs; and for dogs that received multiple vaccines. Each additional vaccine dose administered increased the risk of an adverse event occurring by 27% in small dogs (<10 kg) and by 12% in dogs heavier than 12 kg. High-risk breeds included Dachshunds, Pugs, Boston Terriers, Miniature Pinschers, and Chihuahuas. Overall, the increased incidence of adverse events in small dogs and their relationship to

multiple dosing suggests that veterinarians should look carefully at the practice of giving the same dose of vaccine to all dogs irrespective of their size.

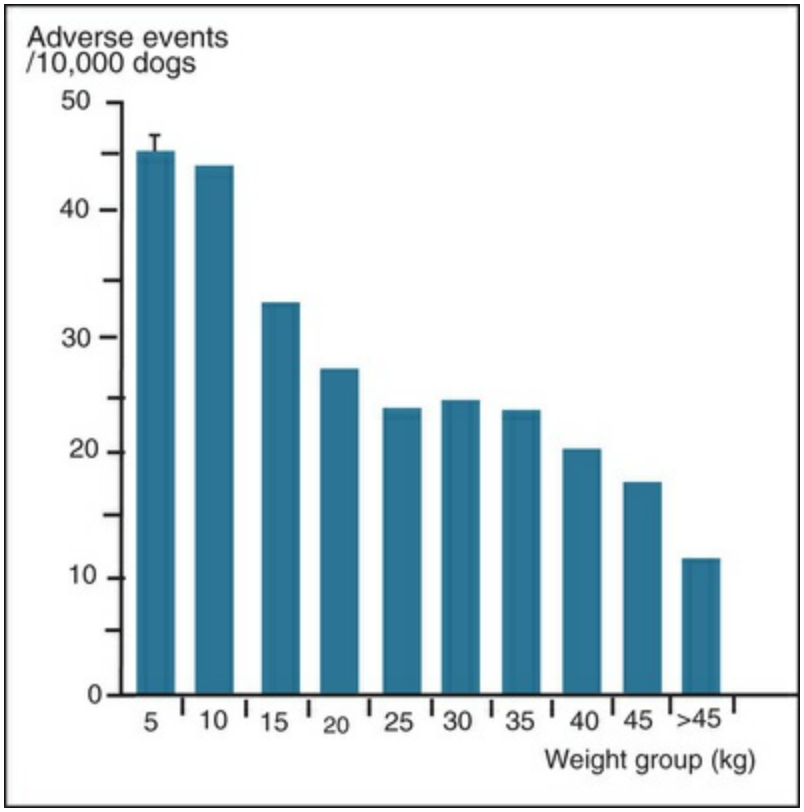


FIG. 25.5 Vaccine-associated adverse events are much more likely to occur in small rather than in large dogs. Mean ± SEM vaccine-associated adverse event rates by 5-kg weight groups in 1,226,159 dogs vaccinated at 360 veterinary hospitals from January 1, 2002 to December 31, 2003. These adverse events were diagnosed within 3 days of vaccine administration. (From Moore GE, Guptill LP, Ward MP, et al: Adverse events diagnosed within three days of vaccine administration in dogs, *J Am Vet Med Assoc* 227:1102-1108, 2005.)

A similar study examined the prevalence of vaccine-associated adverse events following the administration of 1,258,712 doses of vaccine to 496,189 cats. The investigators reported 2560 adverse events (51.6/10,000 cats vaccinated). The risk was greatest for cats 1 year old. For unknown reasons, risk was greater in neutered than in sexually intact cats. Lethargy was the most commonly reported event (Fig. 25.6). The number of adverse events increased significantly when multiple vaccines were given during a single visit.

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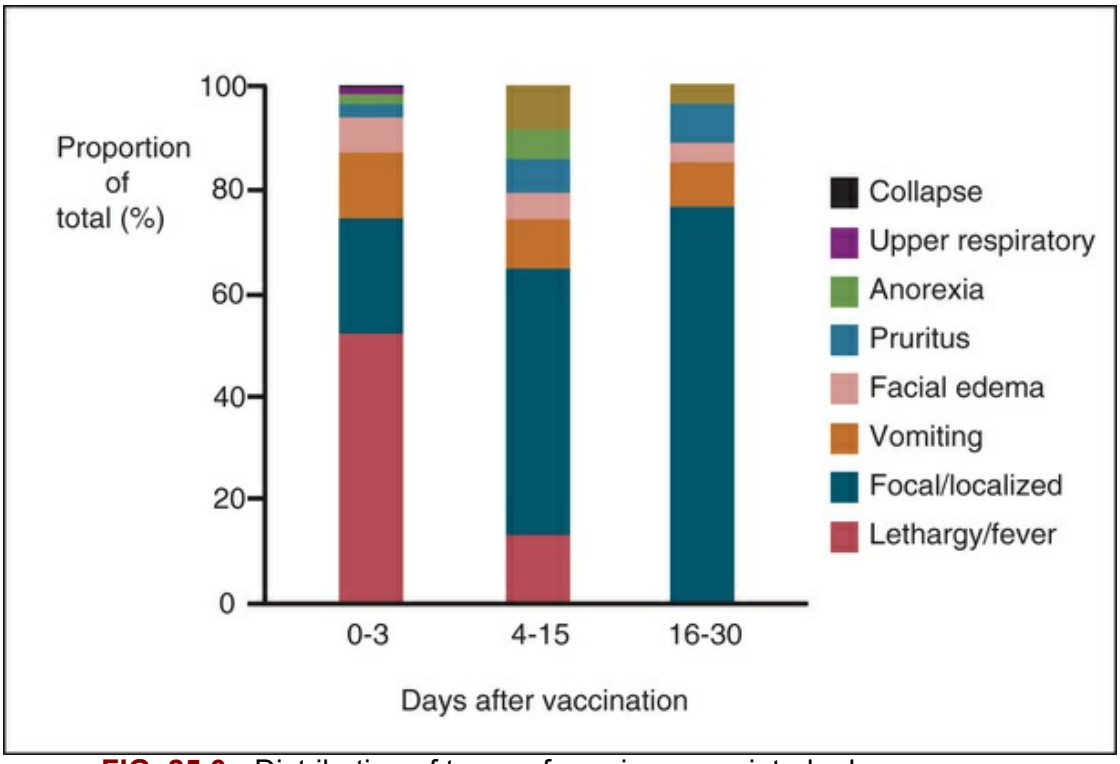


FIG. 25.6 Distribution of types of vaccine-associated adverse events diagnosed during various periods after vaccination in 496,189 cats administered one or more vaccines from January 1, 2002 to December 31, 2004. (It is highly likely that many of the events reported later than 16 days postvaccination are unrelated to the procedure.) (From Moore GE, DeSantis-Kerr AC, Guptill LF, et al: Adverse events after vaccine administration in cats: 2,560 cases (2002-2005), *J Am Vet Med Assoc* 231: 94-100, 2007.)

Identification of an adverse event is based on the clinical judgment of the attending veterinarian and is subject to bias. Standard case definitions of a vaccine-associated adverse event are not yet available. On the other hand, the significance of such bias is reduced by the use of such a large database.

In a report from Japan, 359 dogs showed an adverse event out of 57,300 vaccinated (62.7/10,000 doses). Of these 351 dogs, 41 had anaphylaxis, 244 had dermatological signs, and 160 had gastrointestinal signs. About half the anaphylaxis events occurred within 5 minutes of vaccination. Additional analysis of these anaphylaxis cases reported 87% collapse, 77% cyanosis, and both in 71% of affected dogs. These are higher rates than reported elsewhere. This adverse event rate (62.7/10,000 vaccinated dogs) was higher than the USA (38.2/10,000).

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"Normal" Toxicity

Vaccines commonly elicit transient inflammatory reactions, and some degree of inflammation is required for the efficient induction of protective immune responses. This may cause pain. Thus the sting produced by some vaccines may present problems not only to the animal being vaccinated but also, if the animal reacts violently, to the vaccinator. More commonly, swellings may develop at the reaction site. These may be firm or edematous and may be warm to the touch. They appear about 1 day after vaccination and can last for about a week. Unless an injection-site abscess develops, these swellings leave little trace. Vaccines containing killed Gram-negative organisms may be intrinsically toxic owing to the presence of endotoxins that can cause cytokine release, leading to shock, fever, and leukopenia. Although such a reaction is usually only a temporary inconvenience to male animals, it may be sufficient to provoke abortion in pregnant females. Thus it may be prudent to avoid vaccinating pregnant animals unless the risks of not giving the vaccine are considered to be too great. Vaccination with either immune-stimulating complex (ISCOM) vaccines or live recombinant vectored vaccines against influenza and tetanus may induce an acute-phase response in horses.

Inappropriate Responses

Vaccines may cause rare but serious allergic reactions. For example, allergic responses may occur when an animal produces immunoglobulin E (IgE) in response not only to the immunizing antigen but also to other antigens found in vaccines, such as egg antigens or antigens from tissue culture cells. All forms of hypersensitivity are more commonly associated with multiple injections of antigens and therefore tend to be associated with the use of killed vaccines. It is important to emphasize that a type I hypersensitivity reaction is an immediate response to an antigen and occurs within a few minutes or hours after exposure to an antigen. Reactions occurring more than 2 or 3 hours after administration of a vaccine are likely not type I hypersensitivity reactions.

Type III hypersensitivity reactions are also potential hazards.

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These may cause intense local inflammation, or they may present as a generalized vascular disturbance such as purpura. A type III reaction can occur in the eyes of dogs vaccinated against infectious canine hepatitis (see Fig. 27.8). Some rabies vaccines may induce a local complement-mediated vasculitis leading to ischemic dermatitis and local alopecia. This type of reaction is most often seen in small dogs such as Dachshunds, Miniature Poodles, Bichon Frises, and Terriers.

Type IV hypersensitivity reactions may occur in response to vaccination, but a more common reaction is granuloma formation at the site of inoculation. This may be a response to depot adjuvants containing alum or oil. Vaccines containing a water-in-oil adjuvant produce larger and more persistent lesions at injection sites than vaccines containing alum and aluminum hydroxide. These lesions can be granulomas or sterile abscesses. If the skin is dirty at the injection site, these abscesses may become infected. For this reason, it is inappropriate to administer vaccines intramuscularly to animals used for human consumption.

Postvaccinal canine distemper virus encephalitis is a rare complication that may develop after administration of a modified live canine distemper vaccine. The affected animal may show aggression, incoordination, and seizures or other neurological signs. The pathogenesis of this condition is unknown, but it may be due to residual virulence, increased susceptibility, or triggering of a latent paramyxovirus by the vaccine.

Errors in Manufacture or Administration

Some problems associated with vaccine use may be due to poor production or administration. Thus some modified live vaccines may retain the ability to cause disease. For example, some modified live herpes vaccines or calicivirus vaccines given intranasally may spread to the oropharynx and result in persistent infection. Indeed, such a virus vaccine may infect (and protect) other animals in contact. Even if these vaccines do not cause overt disease, they may reduce the rate of growth of farm animals with significant economic consequences.

Some vaccines may trigger a mild immunosuppression. For

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example, some modified live parvovirus vaccines may cause a transient decrease in lymphocyte responses to mitogens or even a lymphopenia in some puppies, although not all strains of canine parvovirus 2 are immunosuppressive. Some polyvalent canine viral vaccines can cause a transient drop in absolute lymphocyte numbers and their responses to mitogens (see Fig. 40.1). This occurs even though the individual components of these vaccines may not have this effect. Several vaccine combinations may result in transient immunosuppression between 5 and 11 days after vaccination. For example, a combination of canine adenovirus type 1 or type 2 with canine distemper virus suppresses lymphocyte responses to mitogens. This T cell suppression may be accompanied by simultaneous enhancement of B cell responses and raised immunoglobulin levels. Rather than being a pure immunosuppressive effect, it may simply reflect a transient change in the Th1/Th2 balance.

Live bluetongue vaccine has been reported to cause malformations in the offspring of ewes vaccinated while pregnant. The stress from this type of vaccination may also be sufficient to reactivate latent infections; for example, activation of equine herpesvirus has been demonstrated following vaccination against African horse sickness. Mucosal disease may develop in calves vaccinated against bovine virus diarrhea.

Vaccine-Associated Autoimmune Disease

It is widely believed that the prevalence of autoimmune disease in domestic pets, especially dogs, has risen in recent years. Some investigators have attributed this rise to excessive use of potent vaccines. This link is by no means proven; nevertheless, there is limited evidence that supports an association between vaccination and autoimmunity. A retrospective analysis of the history of dogs presenting with immune-mediated hemolytic anemia (IMHA) (Chapter 37) showed that 15 of 70 dogs with IMHA had been vaccinated within the previous month, compared with a randomly selected control group in which none had been vaccinated. Dogs with IMHA that developed within a month of vaccination differed in some clinical features from dogs with IMHA unassociated with

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prior vaccination. Epidemiological studies using very large databases tend to confirm this effect, in that they show an approximately three-fold increase in diagnoses of autoimmune thrombocytopenia, and a two-fold increase in diagnoses of IMHA in dogs in the 30 days following vaccination compared with other time periods. The overall prevalence of these diseases, however, is low, and they can be diagnosed at times not temporally associated with vaccination. Vaccination may therefore serve as a trigger for these diseases in some dogs, but other, undefined, stimuli must also exist.

Vaccine-associated neonatal pancytopenia of calves is a lethal hemorrhagic disease that results from ingestion of maternal colostrum containing anti-BoLA antibodies. Its pathogenesis is discussed in detail in Chapter 31.

Contaminating thyroglobulin found in some vaccines (usually from the presence of fetal bovine serum) may lead to the production of antithyroid antibodies in vaccinated dogs. Lymphocytic thyroiditis has been found in 40% of Beagles on necropsy, but there was no association detected between vaccination and the development of this thyroiditis.

It is well recognized that Guillain-Barré syndrome, an autoimmune neurological disease of humans, can be triggered by administration of some vaccines such as influenza vaccine. At least one case has been reported in a dog following vaccination with a polyvalent distemper-hepatitis-parvovirus vaccine (Chapter 37). In some animals, the administration of potent, adjuvanted vaccines may stimulate the transient production of autoantibodies to connective tissue components such as fibronectin and laminin.

Vaccine-Induced Osteodystrophy

Vaccination of some Weimaraner puppies may lead to the development of a severe hypertrophic osteodystrophy. The disease appears within 10 days of administration of MLV canine distemper vaccine. Systemic signs include anorexia, depression, fever, and gastrointestinal, nervous, and respiratory symptoms in addition to symmetrical metaphyseal lesions with painful swollen metaphyses. Radiological examination shows radiolucent zones in the

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metaphyses, flared diaphyses, and formation of new periosteal bone. Hind and fore limbs are equally affected. It is possible that the condition is triggered in genetically susceptible animals by the vaccine. The disease responds well to corticosteroid therapy. In many cases, these dogs show a preexisting immune dysfunction with low concentrations of one or more immunoglobulin classes, recurrent infections, and inflammatory disease (Chapter 39). It has been suggested that Weimaraners are especially susceptible to this condition and that they therefore receive only killed virus vaccines.

A mild transient polyarthritis has been reported in some dogs following vaccination. The dogs show a sudden onset of lameness with swollen and painful joints within 2 weeks of vaccination. The dogs recover within 2 days. No specific breed or vaccine has been associated with this problem. Vaccination against calicivirus has been associated with polyarthritis and a postvaccination limping syndrome in cats.

Injection Site–Associated Sarcomas

The development of tumors (sarcomas) at sites of vaccine injection is discussed in detail in Chapter 35.

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Adverse Effect Principles

In determining whether a vaccine causes an adverse effect, the following three principles should apply. First, is the effect consistent? The clinical responses should be the same if the vaccine is given to a different group of animals, by different investigators, and irrespective of the method of investigation. Second, is the effect specific? The association should be distinctive and the adverse event linked specifically to the vaccine concerned. It is important to remember that an adverse event may be caused by vaccine adjuvants and components other than the major antigens. Finally, there must be a temporal relationship. Administration of the vaccine should precede the earliest manifestations of the event or a clear exacerbation of a continuing condition.

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Production, Presentation, and Control of Vaccines

The production of veterinary vaccines is controlled by the Animal and Plant Health Inspection Service of the United States Department of Agriculture; by the Canadian Centre for Veterinary Biologics of the Canadian Food Inspection Agency; and by the Veterinary Medicines Directorate in the United Kingdom; as well as by appropriate government agencies in other countries. In general, regulatory authorities have the right to license establishments where vaccines are produced and to inspect these premises to ensure that the facilities are appropriate and that the methods employed are satisfactory. All vaccines must be checked for safety and potency. Safety tests include confirmation of the identity of the organism used and of the freedom of the vaccine from extraneous organisms (i.e., purity), as well as tests for toxicity and sterility. Because the living organisms or antigens found in vaccines normally die or degrade over a period of time, it is necessary to ensure that they will be effective even after storage. It is usual, therefore, to use an antigen in generous excess of the dose required to protect animals under laboratory conditions, and potency is tested both before and after accelerated aging. Vaccines that contain killed organisms, although much more stable than living ones, also contain an excess of antigens for the same reason. Vaccines approved for licensing on the basis of challenge exposure studies must usually show evidence of protection in 80% of vaccinated animals, whereas at least 80% of the unvaccinated controls must develop evidence of disease after challenge exposure (the 80 : 80 efficacy guideline). The route and dose of administration indicated on the vaccine label should be scrupulously heeded since these were probably the only route and dose tested for safety and efficacy during the licensing process. Vaccines usually have a designated shelf-life, and although properly stored vaccines may still be potent after the expiration of this shelf-life, this should never be assumed. Correct storage and handling are essential. All expired vaccines should be discarded. Adverse reactions should always be reported

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to the appropriate licensing authorities as well as to the vaccine manufacturer. Because MLV vaccines carry with them the risks for residual virulence and for contamination with other agents, certain countries will not approve their use.

Inactivated vaccines are commonly available in liquid form and usually contain suspended adjuvant. These should not be frozen, and they should be shaken well before use. The presence of preservatives such as phenol or merthiolate will not control massive bacterial contamination, and multidose containers should be discarded after partial use. Many vaccines containing MLV are susceptible to heat inactivation but are much more resistant if lyophilized. Remember, however, that intense sunlight and heat can destroy even lyophilized vaccines. They store well but should be kept cool and away from light and should only be reconstituted with the fluid provided by the manufacturer.

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26

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Immunity to Bacteria and Fungi

CHAPTER OUTLINE

Innate Immunity, 286
Adaptive Immunity, 287
Immunity to Toxigenic Bacteria, 287
Immunity to Invasive Bacteria, 287
Heat-Shock Protein Response, 287
Immunity to Intracellular Bacteria, 288
Modification of Bacterial Disease by Immune Responses, 288
Evasion of the Immune Response, 289
Evasion of Innate Immunity, 289
Evasion of Adaptive Immunity, 292
Some Antibacterial Vaccines, 293
Toxoids, 293
Bacterins, 293
Living Bacterial Vaccines, 294
Adverse Consequences of the Immune Responses, 294
Serology of Bacterial Infections, 294
Immunity to Fungal Infections, 295

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LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Explain the role of innate immunity in combating bacterial invasion.
- Explain how antibodies can neutralize bacterial toxins.
- Describe how antibodies alone or antibodies and complement will opsonize bacteria or kill them directly through the terminal complement complex.
- Explain why T cell–mediated activation of macrophages is required to kill intracellular bacteria.
- Understand why, under some circumstances, especially in mycobacterial disease, a type 2 response rather than the required type 1 response may lead to severe disease and death.
- Summarize the multiple mechanisms by which bacteria resist immune destruction.
- Explain why cell-mediated immune responses are usually required to protect against fungal infections.
- Define leukotoxin, bacterin, heat shock proteins, and toxoid.

Although animals live in environments densely populated with bacteria, most of these neither invade animal tissues nor cause disease. This is unsurprising for several reasons. First, the combined efforts of the innate and adaptive immune systems are sufficient to prevent invasion. Second, even organisms that successfully invade the animal body gain very little by harming their host. On the contrary, illness or death of the host animal may well reduce the survival of the bacteria and is therefore normally avoided. Indeed, as discussed in Chapter 21, the bacterial microbiota are essential for the animal's well-being since they protect against other invaders, assist in the digestion of foods such as celluloses, and promote the development of the immune system. Nevertheless, many commensal bacteria are also pathobionts. For example, *Clostridium tetani* and *C. perfringens* are commonly found among the intestinal microbiota of horses, and *Bordetella bronchiseptica* is found in the nasopharynx of healthy swine. Bacterial disease is not, therefore, an inevitable consequence of the presence of pathogenic organisms on

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the body surface. The development of disease is related to many other factors, including the response of the host, the presence of damaged tissues, the location of the bacteria, and their disease-producing power (or virulence).

The ability of many bacteria to survive within an animal also depends on virulence factors. Many of these virulence factors are encoded on mobile genetic elements that can be transmitted between species (e.g., plasmids). These virulence factors permit the bacteria to adapt to a specific environment and promote their transmission between hosts. Depending on their niche within the body, bacteria can use virulence factors to penetrate surface epithelia, to bind to cell surfaces, to acquire iron, to evade immune responses, to hide within cells, and to promote transmission to another host. Some of these strategies result in damage to host tissues and must be blocked by the immune system. It should also be pointed out that many bacteria are unable to invade and cause disease in healthy normal hosts but will take the opportunity offered by immunosuppression or other weaknesses in host defenses to invade the body.

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Innate Immunity

Antimicrobial immunity consists of an early innate response followed by a sustained adaptive response. Recognition of invading bacteria through toll-like receptors (TLRs) and other pattern-recognition receptors (PRRs) induces cytokine release, complement activation, inflammation, and phagocytosis. If this is insufficient to eliminate the invaders, adaptive immune mechanisms take over. Thus dendritic cells and macrophages ingest invading bacteria and initiate adaptive immunity by secreting cytokines and triggering both T and B cell responses. The importance of these innate defenses is emphasized by the observation that the resistance of chickens to *Salmonella enterica* Typhimurium appears to be linked to allelic variations in TLR4, and the resistance of foals to *Rhodococcus equi* is linked to TLR2. TLRs are responsible in large part for the initial recognition of invading bacteria. Binding of microbial pathogen-associated molecular patterns (PAMPs) to TLRs triggers a signal cascade that activates genes encoding proteins that are critical in host defense.

The production of cytokines by horse neutrophils following exposure to *R. equi* provides an example of these responses. Thus after exposure to *R. equi,* neutrophils express increased amounts of interleukin-23 (IL-23). This IL-23 promotes Th17 cell differentiation. Driven by transforming growth factor-β (TGF-β) and IL-6, the Th17 cells then promote inflammation. Not only do these Th17 cells produce IL-17 but also IL-6, GM-CSF, G-CSF, chemokines, and metalloproteases. They thus trigger inflammation and coordinate early neutrophil recruitment to infection sites. Th17 cells confer protection against extracellular bacteria and fungi, especially at epithelial surfaces. Type I interferons are also produced in response to bacterial PAMPs. IFN-α/β boosts macrophage responses enhancing their production of IFN-γ, nitric oxide, and TNF-α.

Natural killer (NK) cells play a protective role in some bacterial, protozoan, and fungal infections. For example, some bacteria may activate NK cells by upregulating expression of NKG2D ligands on cells. Activated NK cells produce a large amount of IFN-γ that in turn activates both macrophages and dendritic cells (Chapter 19).

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Although many bacteria are destroyed by phagocytosis, others are killed when free in the circulation. Bacteria can be destroyed by complement acting through the alternate or lectin pathways. Bacterial cell walls, lacking sialic acid, inactivate FH and stabilize the alternate C3 convertase (C3bBbP). As a result, these bacteria are either opsonized or lysed. Activation leads to production of terminal complement complexes (TCCs). These TCCs alone may be unable to insert themselves into the carbohydrates of the microbial cell wall. However, lysozyme in the blood may digest the cell wall and enable the TCCs to insert themselves into the lipid bilayer of the inner bacterial membrane.

Antimicrobial peptides are critical for the defense against bacteria such as the mycobacteria (Box 26.1). Pulmonary collectins and surfactant proteins play an important role against *Mycobacterium avium* infections in the lung. They probably act by binding to, and agglutinating and opsonizing the bacteria. Suppression of bacterial growth by withholding iron is discussed in Chapter 7.

Box 26.1

Vitamin D and Immunity

When an intracellular bacterium such as *Mycobacterium tuberculosis* interacts with TLR1 or TLR2 on the surface of macrophages, it upregulates many different genes and enhances their antimicrobial activity. In mice, this is mainly mediated by nitric oxide. In humans, however, nitric oxide is not elevated, and other mechanisms are involved (Fig. 26.1). One gene activated by TLR1/2 signaling in humans is that encoding the vitamin D receptor. This receptor is therefore upregulated on activated macrophages. Binding of vitamin D to its receptor upregulates expression of the gene for the antibacterial peptide cathelicidin. The cathelicidin, in turn, can kill intracellular *M. tuberculosis*. It is no coincidence, therefore, that resistance to tuberculosis is directly related to serum vitamin D levels and that humans with a deficiency of vitamin D show significantly decreased resistance to this infection. It is of interest to recall that sanatorium treatment of tuberculosis classically involved exposure to fresh air and sunlight, a procedure that would be expected to increase vitamin D levels in human

patients. Conversely, mice are nocturnal mammals that would not be expected to have high vitamin D levels and must rely on other pathways.

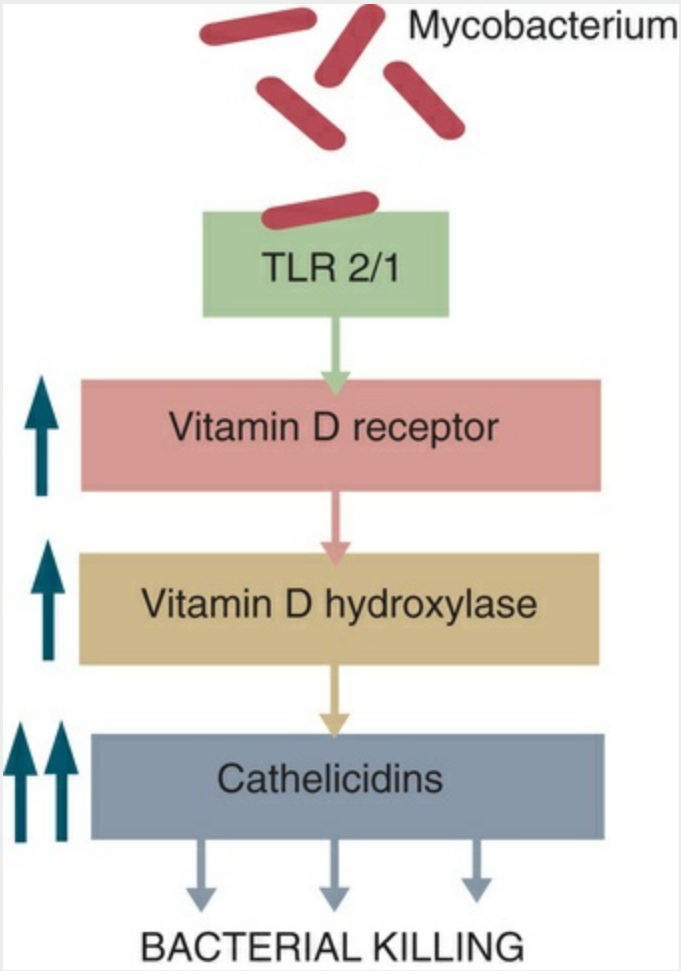


FIG. 26.1 Immunity to tuberculosis is governed in many species by the availability of vitamin D. The vitamin D receptor is upregulated on activated macrophages. Binding of vitamin D to this receptor upregulates vitamin D hydroxylase, which in turn increases production of the antibacterial cathelicidins and enhances disease resistance.

When activated, platelets release antibacterial peptides and reactive oxygen species (ROS). They also bind to neutrophils to form aggregates and enhance neutrophil activities. Horse platelets exposed to bacterial lipopolysaccharide from *Escherichia coli* and lipoteichoic acid from *Staphylococcus aureus* are activated and can inhibit *E. coli* growth as a result of the release of ROS.

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Adaptive Immunity

There are five mechanisms by which the adaptive immune responses combat bacterial infections (Fig. 26.2): (1) neutralization of toxins or enzymes by antibody; (2) killing of bacteria by the classical complement pathway; (3) opsonization of bacteria by antibodies and complement, resulting in their phagocytosis and destruction; (4) destruction of intracellular bacteria by activated macrophages; and (5) direct killing of bacteria by cytotoxic T cells. The relative importance of each of these processes depends on the species of bacteria involved and on the mechanisms by which they cause disease.

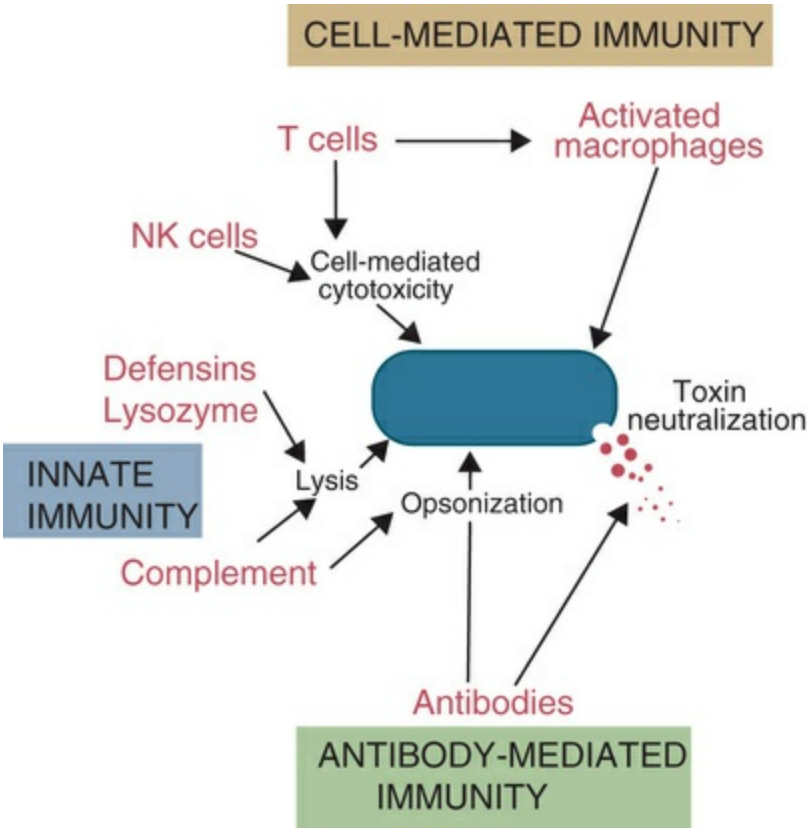


FIG. 26.2 The mechanisms by which the immune responses can protect the body against bacterial invasion.

Immunity to Toxigenic Bacteria

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In disease caused by toxigenic bacteria such as clostridia or *Bacillus anthracis,* the immune response must not only eliminate the invading bacteria but also neutralize their toxins. Destruction of the bacteria, however, may be difficult if they are embedded in a mass of necrotic tissue, and toxin neutralization is a priority. Neutralization occurs when antibody prevents the toxin from binding to its receptors on a target cell. The neutralization process therefore involves competition between receptors and antibodies for the toxin molecule. Once a toxin has bound to its cell receptors, antibodies are relatively ineffective in reversing this combination.

Immunity to Invasive Bacteria

Protection against invasive bacteria is usually mediated by antibodies directed against their surface antigens. Efficient phagocytosis requires that the bacteria be coated with opsonins that can be recognized by phagocytic cells. These opsonins include antibodies and C3b in addition to the innate opsonins such as mannose-binding lectin. Antibodies not only are effective opsonins in their own right but also increase the binding of C3b by activating the classical complement pathway. Antibodies directed against capsular (K) antigens may neutralize the antiphagocytic properties of bacterial capsules, thus permitting their destruction. In bacteria lacking capsules, antibodies directed against O antigens act as opsonins. Protection also results when antibodies are produced against the *E. coli* pilus antigens F4 (K88) and F5 (K99). The antibodies may interfere with the expression of pili. Once the adherence pili are suppressed, these strains of *E. coli* cannot bind to the intestinal wall and thus are no longer pathogenic.

The importance of bacterial capsules in pathogenesis is seen in anthrax. *B. anthracis* possesses both a capsule and an exotoxin. Antitoxic immunity is protective but slow to develop. In addition, toxin production tends to be prolonged since the organism is encapsulated and phagocytic cells have difficulty eliminating it. As a result, death is usually inevitable in unvaccinated animals. The vaccine commonly employed against animal anthrax contains an unencapsulated but toxigenic strain of *B. anthracis.* Given in the form of spores that can germinate, the unencapsulated bacteria are

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eliminated by phagocytic cells before dangerous amounts of toxin are synthesized but not before antitoxic immunity is established.

Molecule for molecule, immunoglobulin M (IgM) is about 500 to 1000 times more efficient than IgG in opsonization and about 100 times more potent than IgG in sensitizing bacteria for complement-mediated lysis. During a primary immune response, therefore, the quantitative deficiency of IgM is compensated for by its quality, ensuring early and efficient protection.

Many antibodies have antimicrobial activities. Antibodies against *E. coli* may be bacteriostatic since they interfere with production of the iron-binding protein enterochelin and thus prevent bacterial iron scavenging. IgM and IgG antibodies against *Borrelia burgdorferi* damage surface proteins on the bacteria and are bactericidal in the absence of complement. Some antibodies are able to generate oxidants and may kill bacteria directly.

Heat-Shock Protein Response

When bacteria are stressed, they produce many new proteins. These stressors include heat, starvation, and exposure to oxidants; toxins such as heavy metals; protein synthesis inhibitors; and viral infections. The heat-shock proteins (HSPs) are the best understood of these new proteins. Low levels of HSPs are present in bacteria at normal temperatures. Mild stress such as a low-grade fever induces HSP production. For example, HSP levels climb from 1.5% to 15% of the total protein in stressed *E. coli.* There are three major bacterial HSPs: HSP-90, -70, and -60. (The number refers to their molecular weight.) When a bacterium is phagocytosed and exposed to the neutrophil respiratory burst, the resulting stress triggers the production of bacterial HSP. As a result, HSP 60 is the dominant antigen in infections caused by mycobacteria, *Coxiella burnetii,* legionella, treponema, and *Borrelia* species. These HSPs are highly antigenic for several reasons. First, they are produced in abundance within the infected host; second, they are readily processed by antigen-presenting cells; and third, the immune system may possess unusually large numbers of cells capable of responding to HSPs. In addition, some γ/δ T cells may preferentially recognize bacterial HSPs. Thus anti-HSP responses may induce significant protection against many bacterial pathogens.

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Immunity to Intracellular Bacteria

As discussed in Chapter 18, some bacteria such as *Brucella abortus, Mycobacterium tuberculosis, Campylobacter jejuni, R. equi, Listeria monocytogenes, Corynebacterium pseudotuberculosis, Coxiella burnetii,* and some serotypes of *Salmonella enterica* can grow readily inside resting macrophages. In addition, *L. monocytogenes* can travel from cell to cell without exposure to the extracellular fluid through cytoskeletal membrane protrusions.

Autophagy, as described in Chapter 5, is a key process in the destruction of intracellular bacteria. The same cellular machinery used to destroy unwanted organelles can be employed to eliminate intracellular organisms. Thus bacteria that escape into the cytosol may be surrounded by an autophagosome-like structure and subsequently destroyed by lysosomal enzymes. Autophagy (or more correctly, xenophagy) may also play a key role in delivering microbial antigens to major histocompatibility complex (MHC) molecules. That said, *B. abortus* can employ autophagy pathways to spread from cell to cell. The bacterium selectively suppresses some of the components of autophagosome formation so that it is not killed.

Protection against intracellular bacteria is mediated by cell-mediated mechanisms. Early in the immune response, activated M1 macrophages and cDC1 dendritic cells secreting IL-12 and TNF-α are most important (Chapter 18). Although macrophages from unimmunized animals cannot usually destroy these bacteria, this ability is acquired about 10 days after infection. Later in the response, IFN-γ from Th1 cells and the activities of cytotoxic $CD8^+$ T cells become critical. IFN-γ, especially in association with TNF-α, greatly enhances the production of cytokines such as TNF-α, IL-6, IL-1β, and IL-12, enzymes such as indoleamine 2,3-dioxygenase (IDO) and nitric oxide synthase 2 (NOS2), and the release of reactive oxygen and nitrogen species. Th17 cells may act with Th1 cells to promote protection against *Brucella.* M1 activation has been shown to be important in resistance to *L. monocytogenes, S. enterica* Typhi and Typhimurium, *R. equi,* mycobacteria, and chlamydia. For example, IFN-γ and TNF-α produced by primed T cells generate M1 macrophages, acidify their phagosomes, and kill mycobacteria. Uncontrolled M1 activation by bacteria such as streptococci and *E.*

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coli, however, can contribute to disease by inducing sepsis, tissue damage, and organ failure. The response of these activated macrophages tends to be nonspecific, particularly in listerial infections, and M1 macrophages are able to destroy many normally resistant bacteria. Thus an animal recovering from an infection with *L. monocytogenes* develops increased resistance to *M. tuberculosis.* The development of M1 macrophages often coincides with the appearance of delayed (type IV) hypersensitivity responses to intradermally administered antigen (Chapter 33). *M. tuberculosis* can persist, in activated as well as resting macrophages. However, this persistence requires a bacterial dormant stage when its replication and many metabolic processes are suspended.

NK and $CD8^+$ T cells also participate in immunity to intracellular bacteria such as *L. monocytogenes.* These cytotoxic cells bind to target cells and use perforins to generate pores that allow the delivery of lytic granule contents into the infected cells. Granulysin then penetrates the bacterial cell wall and so permits granzymes to enter the bacteria. These granzymes generate ROS that disrupt key enzyme pathways. For good measure, the granzymes may also trigger apoptosis in the target cell that further limits bacterial spreading. *R. equi*–infected macrophages can also be recognized and killed by cytotoxic T cells in a MHC class I unrestricted manner.

It has been observed that protective immunity against intracellular bacteria cannot be induced by vaccines containing killed bacteria. Only vaccines containing living bacteria are protective. This is because of the differential activation of helper T cell populations by live and dead bacteria. Infection of mice with live *B. abortus* stimulates Th1 cells to secrete IFN-γ; a type 1 response. Conversely, immunization of these mice with purified *Brucella* proteins induces Th2 cells to secrete IL-4; a type 2 response. Likewise, live but not dead *L. monocytogenes* or *B. abortus* organisms induce macrophage production of TNF-α. Killed *Brucella* organisms stimulate IL-1 production to a greater extent than live bacteria. Resistance to these intracellular bacteria is generally short lived, persisting for only as long as viable bacteria remain in the body. (Tuberculosis is an exception, where memory is prolonged.)

If, in a bacterial disease, it is observed that dead vaccines do not

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give good protection, that serum cannot confer protection, that antibody levels do not relate to resistance, and that delayed hypersensitivity reactions can be elicited to the bacterial antigens, then type 1 responses probably play an important role in resistance, and the use of vaccines containing living bacteria should be contemplated.

Modification of Bacterial Disease by Immune Responses

The immune response influences the course and severity of an infection. At best, it will result in microbial destruction and a cure. In the absence of a cure, however, the infection may be profoundly modified. Much depends on whether a cell-mediated or antibody response is generated. Thus the type of helper T cells induced during infection affect the course of disease. As described in Chapter 18, type 1 cell-mediated responses are required to control intracellular bacteria since only activated macrophages can prevent their growth. Macrophage activation requires that Th1 cells produce IFN-γ. Once activated, the M1 cells may localize or cure these infections. If an animal mounts an inappropriate Th2 response so that M2 but not M1 macrophages are generated, chronic progressive disease may result. This is seen in Johne's disease of sheep. Some animals develop multibacillary (MB) disease, in which their intestinal lesions contain enormous numbers of bacteria (Fig. 26.3) and little histological evidence of a cell-mediated response. Their granulomas lack organization, with large numbers of bacteria-laden macrophages intermixed with lymphocytes. In contrast, other sheep may develop paucibacillary (PB) disease, in which the lesions contain very few bacteria but large numbers of lymphocytes. These are organized nodular lesions with epithelioid cells and multinucleated giant cells at the center surrounded by fibrous connective tissue. The two forms of the disease are associated with differential expression of cytokine and chemokine receptors. Thus animals with the PB disease have increased numbers of $CD25^+$ T cells that produce more IL-2 and much more IFN-γ than sheep with the MB form of the disease (Fig. 26.4). In contrast, sheep with the MB disease have higher antibody levels

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and a lack of cellular immune responses. Thus sheep with PB lesions mount a type 1 response, whereas those with MB disease mount a type 2 response.

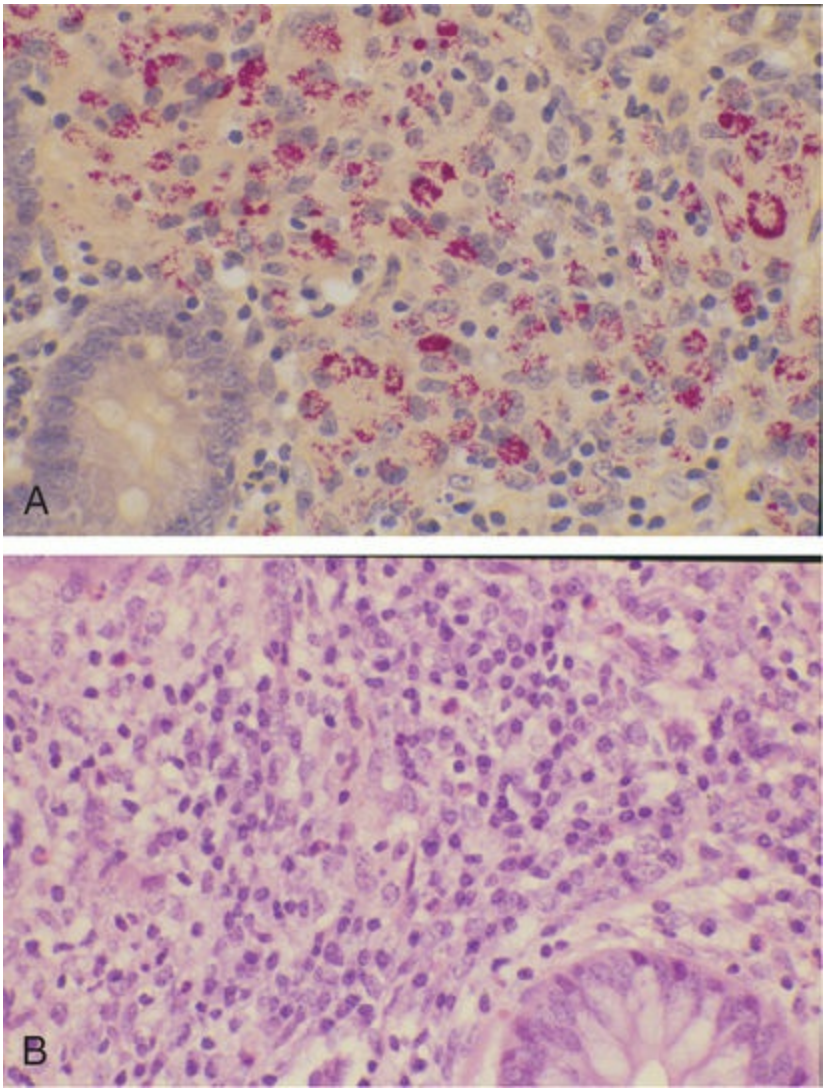


FIG. 26.3 The two forms of Johne's disease in sheep. **A,** Section of terminal ileum from a case of multibacillary Johne's disease, showing abundant acid-fast organisms within large infiltrating macrophages. **B,** Section of terminal ileum from a case of paucibacillary Johne's disease, showing very few acid-fast bacteria and a significant lymphocyte infiltration. Ziehl-Nielsen stain. (Courtesy Dr. C.J. Clarke.)

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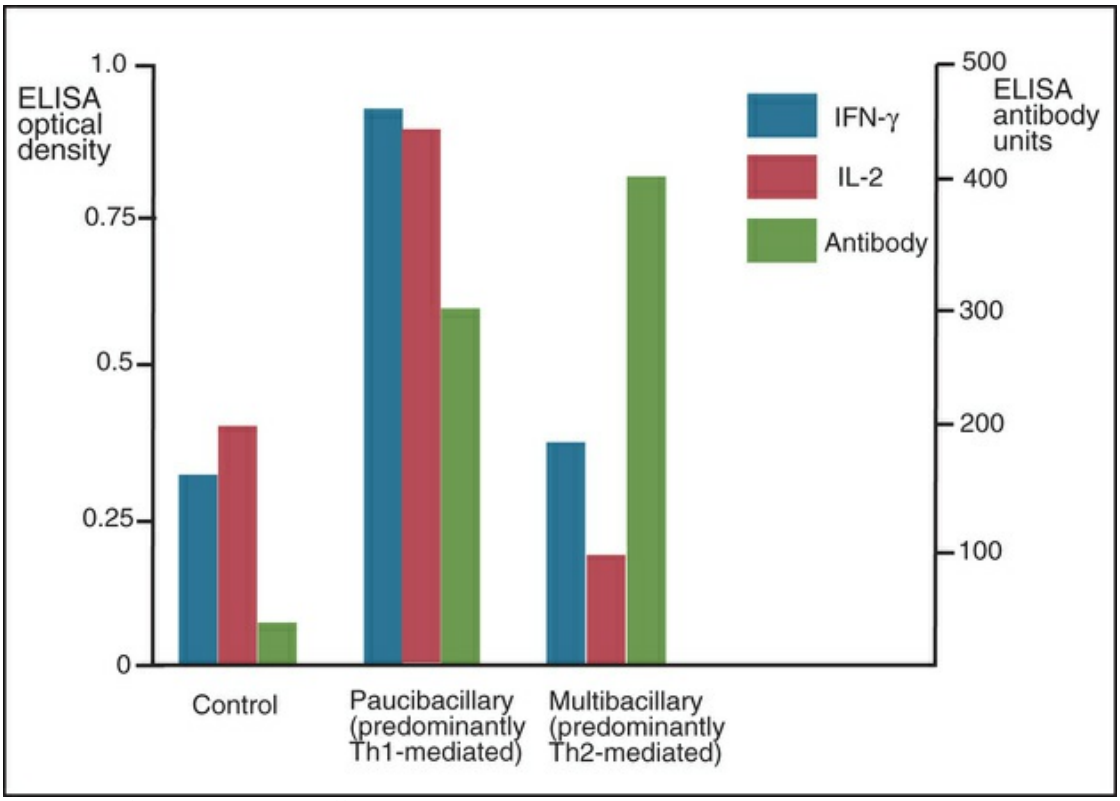


FIG. 26.4 The differences in peripheral blood lymphocyte IL-2, IFN-γ, and antibody production between sheep with the paucibacillary (PB) form and sheep with the multibacillary (MB) form of Johne's disease. Note that there is a marked tendency for the T cells from animals with the PB form of the disease to produce more Th1 cytokines than those with the MB form. Despite this, animals with the latter form appear to produce more antibodies. (From data provided by C. Clarke and C. Burrells.)

In bovine *M. bovis* infections, $WC1^+$ γ/δ T cells accumulate within tuberculous lesions and play a role in granuloma development. They also affect the levels of IgG2 antibodies, the increase in IFN-γ, and the decrease in IL-4 levels so they shape the character of the antimycobacterial response.

IL-4 is a key cytokine that triggers Th2 responses while suppressing Th1 responses. In addition to normal IL-4, cattle produce two variants, called IL-4δ2 and IL-4δ3, by alternative splicing of pre–messenger RNA. These splice variants may bind and block IL-4 receptors and regulate its activity. As a result, they affect the resistance of cattle to tuberculosis. Animals showing significant resistance to tuberculosis produced high levels of IL-4δ3 compared to susceptible cattle.

The specific helper T cell subset involved in immunity to tuberculosis can change once the response is established. Time-

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based studies have shown that immune responses to bacterial infections may swing between Th1 and Th2 responses, perhaps several times, before a final response is established. This final response may well be a Th1 or Th2 response, or even some intermediate point in the Th1 to Th2 spectrum. This variability appears to be a common feature of chronic infections such as tuberculosis.

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Evasion of the Immune Response

The outcome of any infection depends upon the continuously evolving battle between the host and the microbe. To survive within an animal, bacteria must evade or inhibit the immune defenses. The complex interrelationships between bacteria and their animal hosts, described in Chapter 21, include the role of commensal bacteria on mucosal surfaces in regulating the growth and development of the immune system. These bacteria generally do not aggressively seek to invade the body, so equilibrium can be achieved. Nevertheless, an invading microorganism becomes a pathogen because it can invade the body, evade the immune defenses, and survive at least for a time within its host. Pathogenic bacteria, like all organisms, try to avoid destruction. They have evolved many different mechanisms to overcome host innate and adaptive immune responses.

Evasion of Innate Immunity

The key to successful microbial invasion, at least initially, is evading innate immunity. Bacteria employ diverse mechanisms to prevent or at least delay an unpleasant fate. Only some selected examples can be mentioned here.

For example, some bacteria interfere with TLR signaling pathways and inflammasome activation (Fig. 26.5). The methods used include the production of modified PAMPs that will not trigger TLRs, masking of PAMPs, blockage of TLR signaling pathways, destruction of signaling molecules, destruction of NF-κB, and misdirection of signaling pathways toward antiinflammatory pathways. Thus *M. tuberculosis* uses a masking lipid to cover its PRRs. *Leptospira* have lipopolysaccharides that are recognized by TLR2, but not by TLR4. *Campylobacter jejuni* makes a form of flagellin that is not recognized by TLR5. *Yersinia pestis* reduces acetylation of lipid A so that it cannot be recognized by TLR4. Bacteria also differ in the amount of CpG dinucleotides in their DNA, and so differ in their ability to trigger TLR9. Potent stimulators of TLR9 include *M. tuberculosis* and *Pseudomonas*

aeruginosa. Weak TLR9 stimulators include *C. jejuni* and *S. epidermidis.*

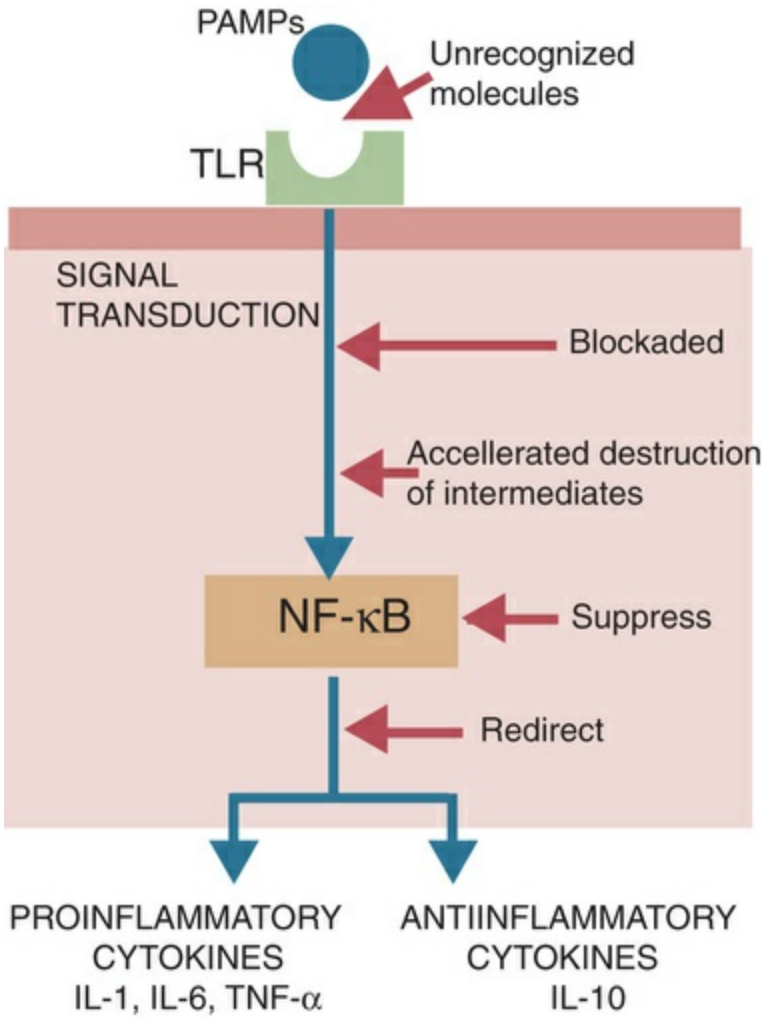


FIG. 26.5 Bacteria can interfere with TLR signaling pathways in many different ways and at many different positions as described in the text. This can include redirecting the signaling pathways from proinflammatory to antiinflammatory pathways.

Many bacteria interfere with intracellular signaling pathways. *Brucella* synthesize a protein called TcpB that closely resembles the mammalian toll/IL-1 receptor. As a result, it accelerates degradation of an adaptor protein and blocks the TLR signaling pathway. *P. aeruginosa* secretes a protein that impairs the regulation of NF-κB. The MAP kinase pathway (Chapter 8) can be inhibited by anthrax, Shigella and Yersinia. Misdirection of signaling pathways occurs when products from Candida, Yersinia, or Mycobacteria trigger signaling through TLR2, leading to production of IL-10.

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Another useful skill for a bacterium to possess is the ability to resist antibacterial peptides. For example, staphylokinase from *S. aureus* can bind and neutralize defensins. Another staphylococcal enzyme, aureolysin, destroys cathelicidins. *Salmonella* and *S. aureus* can produce proteins that change the negative charge and fluidity of the bacterial outer membrane resulting in decreased defensin binding. *Klebsiella pneumoniae* capsular polysaccharide blocks β-defensin expression by airway epithelial cells. Proteases from *Bacillus anthracis* can destroy defensins and cathelicidins.

Many bacteria can block phagocytosis (Fig. 26.6). For example, *S. aureus* expresses protein A. Protein A attaches to IgG Fc regions and so prevents antibodies from binding to Fc receptors on phagocytic cells or activating the classical complement pathway. Encapsulated bacteria such as pneumococci possess a hydrophilic capsule that cells cannot bind. Many bacteria can evade opsonization by complement. Thus the M protein of streptococci binds fibrinogen and masks C3b-binding sites. It also binds factor H, and so inactivates bound C3b. *S. aureus* produces a protein that blocks C3 convertases. Other bacteria produce proteases that can destroy complement components. *S. enterica* Typhimurium has a gene called *Rck* that confers resistance to complement-mediated lysis by preventing insertion of the terminal complement complex into the bacterial outer membrane.

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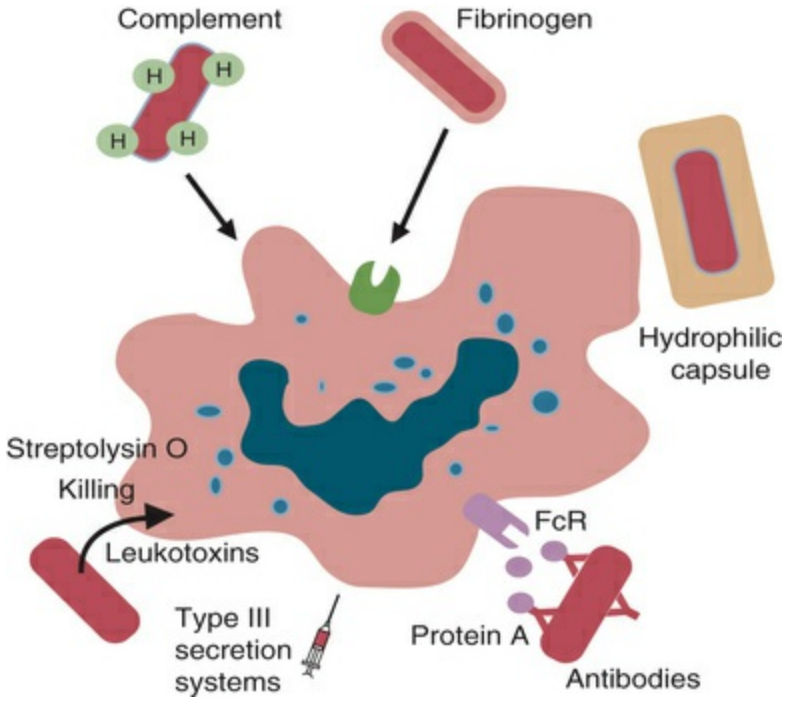


FIG. 26.6 Some of the many different mechanisms by which bacteria can avoid being killed by phagocytic cells such as neutrophils.

Strains of *Streptococcus pneumonia* growing in suspension readily activate C3 leading to deposition of C3b on their surface and promoting their opsonization. This does not occur on pneumococci growing within biofilms where binding of C-reactive protein and C1q is reduced. Conversely, recruitment of FH, the regulator of the alternative complement pathway is enhanced in biofilms. Thus biofilm formation is an efficient method of evading both the classical and alternative complement pathways. *Staphylococcus aureus* and *P. aeruginosa* can also evade phagocytosis by switching from planktonic growth to biofilm production.

Bacteria can, of course, avoid being eaten simply by killing phagocytic cells. Thus *Streptococcus canis* produces streptolysin O that lyses neutrophil cell membranes. Several Gram-negative bacteria of veterinary importance, such as *Mannheimia hemolytica* and *Fusobacterium necrophorum,* secrete leukotoxins that kill leukocytes, especially granulocytes. The most important leukotoxins are the RTX ("repeats in toxin") proteins. *M. hemolytica* secretes an RTX toxin that kills ruminant neutrophils, alveolar macrophages, and lymphocytes. This leukotoxin binds to CD18 as well as lipid rafts on leukocytes and induces their apoptosis.

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Moraxella bovis also secretes a leukotoxin for bovine neutrophils. *Actinobacillus pleuropneumoniae* secretes a toxin that kills porcine macrophages. *Mycoplasma mycoides* can kill bovine T cells. Other bacteria trigger lymphocyte death by apoptosis. These include *B. anthracis,* Streptococci, Shigella, *L. monocytogenes, S. aureus,* and Yersinia. Leukotoxins are a relatively crude method of assassination. Some bacteria use much more sophisticated methods such as injecting toxins into their targets. Gram-negative bacteria such as *Salmonella, Pseudomonas,* and *E. coli* have developed an elaborate needle complex, a type III secretion system, to convey effector molecules directly into the cytosol of effector cells. These injection systems are turned on when a bacterium is ingested by a cell and exposed to a low pH within the phagosome. Once the needle complex enters the cytosol and detects its neutral pH, injection of effector molecules occurs. These molecules activate guanosine triphosphatases and disrupt intracellular signaling pathways. At high concentrations, they produce transmembrane pores and kill the cells.

Although killing leukocytes is an effective way to avoid being eaten, other bacteria are content to simply prevent intracellular destruction. Some bacteria generate a resistant cell wall to protect themselves against lysosomal enzymes. For example, the cell wall waxes of *Corynebacterium pseudotuberculosis* make it resistant to lysosomal enzymes. *S. aureus* uses a cell wall peptidoglycan that is completely resistant to lysozyme. Some bacteria produce antioxidants that neutralize the products of the respiratory burst. For example, the carotenoid pigments responsible for the yellow color of *S. aureus* can quench singlet oxygen. *Salmonella enterica* Typhimurium can prevent assembly of the NADPH-oxidase complex and down-regulate host NOS2 activity. *Pasteurella multocida* and *Histophilus somni* are also able to inhibit the respiratory burst. Anthrax toxins LF and EF also inhibit NAPDH oxidase activity. *Staphylococcus aureus* produces catalase that inactivates hydrogen peroxide and the free radicals produced during the respiratory burst. It also produces lactate dehydrogenase that also helps it to resist NO-mediated oxidation. Some bacteria can defend themselves against hypochlorite through the actions of a molecular chaperone called Hsp33. This protein unfolds on

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exposure to HOCl and then binds and protects essential proteins against bleach-induced aggregation.

Bacteria such as enteropathogenic *E. coli, Yersinia pestis, M. tuberculosis,* and *P. aeruginosa* secrete molecules that can reduce their death by neutrophils. For example, *E. coli* produces lysozyme inhibitors. Other bacteria ensure that they are never exposed to these enzymes by interfering with phagosomal maturation (Fig. 26.7). Mycobacteria, *Aspergillus flavus, B. abortus,* and *Chlamydophila psittaci* can establish themselves within vacuoles that exclude proteases and oxidants by blocking lysosome-phagosome fusion. In the case of *M. tuberculosis,* the bacterium enters the macrophage through cholesterol-enriched membrane microdomains that are coated on the cytosolic side with a protein (tryptophan-aspartate-containing coat protein, or TACO) that prevents phagosome maturation. Thus lysosomes cannot fuse with the phagosome. They remain distributed within the cytosol, and the bacteria continue to survive and grow. Mycobacteria also prevent acidification of phagosomes by preventing recruitment of the proton pump adenosine triphosphatase from the vacuolar membrane so that lysosomal cathepsins remain inactive. Another mechanism used by bacteria to avoid destruction is simply to escape from the phagosome by migrating into the cytosol surrounded by a coat of polymerized actin. This method is employed by mycobacteria and by *L. monocytogenes.* Listeria secretes listeriolysin O that destroys cell membranes and so permits the organism to enter the cytosol (Table 26.1).

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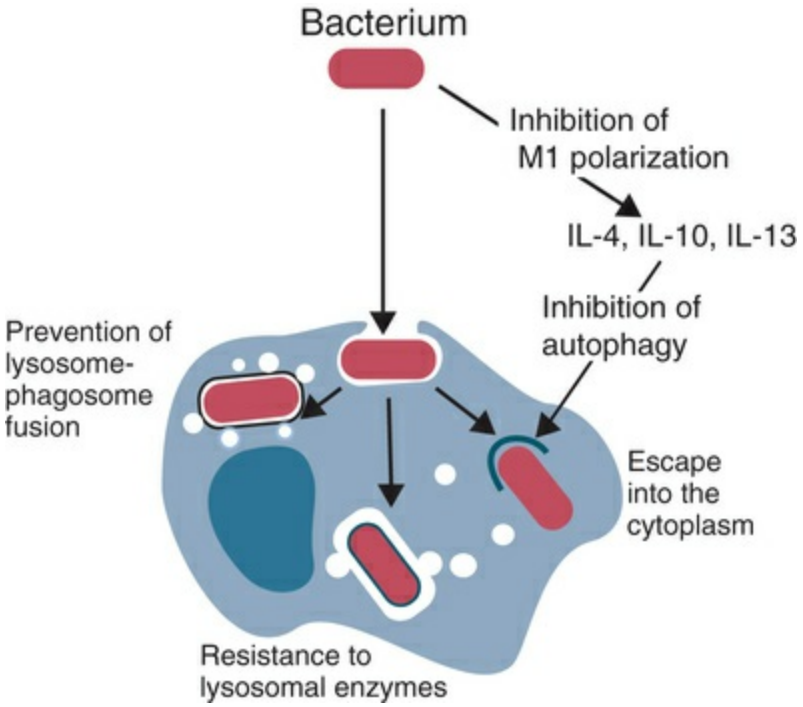


FIG. 26.7 The mechanisms by which intracellular bacteria can evade intracellular destruction.

TABLE 26.1

Facultative Intracellular Bacteria and Their Mechanisms of Survival

Organism	Method of Intracellular Survival
Brucella abortus	Reduced PAMP expression Prevents phagosome maturation Suppression of autophagy
Corynebacterium pseudotuberculosis	Resistant cell wall
Listeria monocytogenes	Neutralizes respiratory burst Escapes into the cytosol
Mycobacterium tuberculosis	Lipid cell wall Prevents phagosome maturation Suppresses antigen presentation Detoxifies oxidants
Salmonella enterica	Prevents phagosome maturation Modifies endosomal trafficking Detoxifies oxidants Downregulates NOS2 and NOX
Rhodococcus equi	Survives in phagosomes

NOS2, Nitric oxide synthase; *NOX,* NADPH-oxidase; *PAMP,* pathogen-associated molecular pattern.

Even extracellular killing can be inhibited. Neutrophils and macrophages can release intranuclear DNA and associated chromatin proteins, leading to the formation of NETs and METs (see Fig. 5.11). This extracellular DNA is studded with antimicrobial

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proteins, including granule components that can kill extracellular bacteria. However, bacteria such as the pneumococci and *S. aureus* can secrete endonucleases that degrade these DNA NETs. Additionally, the released phosphonucleotides can be dephosphorylated by another staphylococcal enzyme and generate deoxyadenosine that then triggers apoptosis and eliminates any nearby macrophages!

Metabolic privation is the process by which animal hosts seek to sequester essential nutrients thus preventing microbial growth. Tryptophan is one such nutrient. It is stored within macrophages and converted into IDO. This tryptophan depletion requires bacteria to activate tryptophan synthesis if they are to survive. Macrophages also sequester iron as described in Chapter 7. As a result pathogens must produce their own siderophores to capture any available iron. For example, pathogenic strains of *Streptococcus equi* produce equibactin, an iron-binding siderophore. Less pathogenic persistent strains do not.

Evasion of Adaptive Immunity

Bacteria also seek to avoid or modify adaptive immune responses—a more difficult task.

The body will not respond effectively to organisms it cannot detect or to organisms that it encounters for the first time. *Campylobacter fetus* subspecies *venerealis,* an organism that normally colonizes the genital tracts of cattle, prevents effective immune elimination by changing its surface coat. The destruction of most of these bacteria by a local immune response leaves a remnant population that possesses new and different antigens. This population multiplies but is largely eliminated in turn by a second immune response, leaving organisms of a third antigenic type. This process of cyclical antigenic variation may be repeated for a long time, resulting in a persistent infection. *Anaplasma marginale,* a bacterium that lives within bovine red cells, also shows sequential antigenic variation. As a result, the number of anaplasma in blood cycles at 6- to 8-week intervals. The number of bacteria gradually increases and then falls rapidly as a result of an antibody response. This is followed by development of a new antigenic variant that

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repeats the cycle. *A. marginale* is transmitted by ticks, so successful spread depends on maintenance of a high bacteremia.

Some bacteria secrete proteases that can destroy immunoglobulins or cytokines. For example, proteases specific for IgA are produced by *Neisseria gonorrhoeae, Haemophilus influenzae,* and *S. pneumoniae.* These organisms can thus prevent opsonization and Fc receptor–mediated phagocytosis. *Mannheimia hemolytica* secretes a protease specific for bovine IgG1. *P. aeruginosa* secretes a protease that destroys IL-2. *B. abortus* produces a B cell mitogen that stimulates the cells to secrete IL-10. This causes transient immunosuppression and permits the bacterium to successfully establish a chronic infection.

Pathogenic mycobacteria have evolved to survive within host macrophages, and *Mycobacterium avium paratuberculosis* (MAP), the cause of Johne's disease, is highly adept at such survival. MAP interacts with receptors on macrophages to initiate cell signaling and phagocytosis. Thus MAP crosses the intestinal epithelial barrier by triggering IL-1β production. This recruits macrophages to the apical side of the epithelium and facilitates entry into its macrophage habitat. MAP also traffic easily through M cells where they can be picked up by dendritic cells or macrophages and carried to the mesenteric lymph node. Mannosylated lipoarabinomannan (Man-lam) is a major component of the MAP cell wall. Man-lam binds to TLR2, triggering transcription of IL-10. The IL-10 suppresses production of proinflammatory cytokines and attenuates phagosome acidification and phagolysosome fusion. Thus Man-lam appears to be largely responsible for suppressing the inflammatory and antimicrobial responses against MAP. IDO is also generated in MAP infections and may down-regulate the host's immune responses. Antigen stimulation of mesenteric lymph node cells from cows severely infected with MAP reveals upregulation of not only IL-10 but also IFN-γ, IL-13, IL-17A and TNF-α. This suggests that cytokine synthesis is severely dysregulated. MAP also blocks macrophage activation by gamma interferon. Macrophage activation is required in order to kill the intracellular invader and if activation does not happen then the bacterium can persist. The interferon blockage results from inhibition of the JAK-STAT pathway (Chapter 8). MAP-infected cells cannot phosphorylate key

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peptides in the pathway.

Avirulent mycobacteria are taken up by macrophages and induce their apoptosis. This produces a "cellular corpse" with an impermeable envelope that prevents the bacteria from escaping. Thus the mycobacteria are killed only when the apoptotic cell is removed. Virulent mycobacteria, in contrast, cause necrosis when taken up by macrophages. This produces a dead cell with a permeable cell membrane that enables the bacteria to escape and spread.

It has long been recognized that *M. tuberculosis* survives within macrophages by blocking fusion of the phagosome with lysosomes. IFN-γ can overcome this maturation block by triggering autophagy. Thus a new autophagosome forms around the blocked phagosome, and this then fuses with lysosomes, permitting the killing of the mycobacteria. Conversely, Th2 cytokines such as IL-4 and IL-13 inhibit autophagy and permit mycobacterial survival. Several other intracellular bacteria are eliminated by autophagy. These include *Streptococcus pyogenes* and *Salmonella enterica* Typhimurium. Other organisms such as *Listeria* and *Shigella* have evolved mechanisms to avoid autophagy and survive within autophagosomes.

Bacteria may interfere with macrophage polarization to promote their own survival. Some *Salmonella* and mycobacteria can neutralize M1-related effectors, or inhibit M1 cytokine secretion or expression. *S. enterica* Dublin suppresses IL-18, and *Brucella suis* inhibits TNF-α production. Proteins from *M. tuberculosis* can inhibit activation of NF-κB. Responding mycobacteria may induce the synthesis of IL-6, IL-10, and TGF-β and hence prolong their own survival. IL-10 is especially effective in inhibiting macrophage activation, suppressing oxidant production, and reducing MHC class II expression.

The evolution of bacterial diseases into persistent chronic infections is associated with a tendency to M2 polarization mediated by IL-10. This occurs in chronic brucellosis, Q fever, and tuberculosis. Some pathogens, such as *Yersinia enterocolitica* and *Coxiella burnetii,* actually stimulate M2 polarization.

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Some Antibacterial Vaccines

Toxoids

The immunoprophylaxis of tetanus is restricted to toxin neutralization. Tetanus toxoid in an aluminum hydroxide suspension is given for routine prophylaxis, and a single injection will induce protective immunity in 10 to 14 days. Conventional immunological wisdom would suggest that the previous use of tetanus immune globulin should interfere with the immune response to toxoid and must therefore be avoided. This is not a problem in practice, however, and both may be successfully administered simultaneously (at different sites) without problems. This may be because of the relatively small amount of immune globulin usually needed to protect animals.

Some veterinary vaccines combine both toxoid and killed bacteria in a single dose by the simple expedient of adding formaldehyde to a whole culture. These products, sometimes called anacultures, are used to vaccinate against *Clostridium haemolyticum* and *C. perfringens*. Trypsinization of the anaculture may make it more immunogenic. Toxoids, usually incorporated with an alum adjuvant, are available for most clostridial diseases and for infections caused by toxigenic staphylococci.

Bacterins

Vaccines containing killed bacteria are called bacterins. It is usual to kill the bacteria with formaldehyde and to add alum or aluminum hydroxide adjuvants. As with other dead vaccines, the immunity produced by bacterins is relatively short lived, usually lasting no longer than 1 year and sometimes considerably less.

Bacterins may be improved by adding purified immunogenic antigens to the killed bacteria. *E. coli* bacterins against enteric colibacillosis may be enriched and made much more effective by the addition of K88 or K99 pilus antigens. Antibodies to these antigens block *E. coli* binding to the intestinal wall and thus contribute to protection. Similarly, *Mannheimia* bacterins enriched

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with the leukotoxoid show greater efficacy than conventional bacterins.

One problem encountered, especially when using coliform and *Campylobacter* vaccines, is strain specificity. Several different strains of each organism commonly occur, and successful vaccination requires immunization with appropriate strains. This is sometimes not possible if a commercial vaccine must be employed. One method of overcoming this difficulty is to use autogenous vaccines. These are vaccines that contain organisms obtained either from infected animals on the farm where the disease problem is occurring or from the infected animal itself. These can be very successful if carefully prepared since the vaccine will contain all the antigens required for protection in that specific location. As an alternative to the use of autogenous vaccines, some manufacturers produce polyvalent vaccines containing a mixture of antigenic types. For example, leptospirosis vaccines commonly contain up to five different serovars.

An alternative approach to the development of vaccines against Gram-negative bacteria is the use of common core antigens. As pointed out in Chapter 2, the outer layer of the Gram-negative bacterial cell wall consists of lipopolysaccharide. This lipopolysaccharide consists of a variable oligosaccharide (O antigen) bound to a highly conserved core polysaccharide and lipid A. The O antigen varies greatly among Gram-negative bacteria so that an immune response against one O antigen confers no immunity against bacteria expressing other O antigens. In contrast, the underlying core polysaccharide is similar between Gram-negative bacteria of different species and genera. Thus an immune response directed against this common core structure has the potential to protect against a wide variety of different Gram-negative bacteria.

Mutant strains of *E. coli* (J5) and *S. enterica* Minnesota and Typhimurium (Re) have been used as sources of core antigen. J5 is a rough mutant that is deficient in uridine diphosphate galactose 4-epimerase. As a result, the organism makes an incomplete oligosaccharide side chain, having lost most of the outer lipopolysaccharide structure (see Fig. 2.2). Immunization with J5 thus provides protection against *E. coli, K. pneumoniae, A.*

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pleuropneumoniae, and *H. influenzae* (type B). J5 has been reported to protect calves against organisms such as *S. enterica* Typhimurium and *E. coli* and pigs against *A. pleuropneumoniae*.

Living Bacterial Vaccines

Successful living bacterial vaccines include strains 19 and RB51 of *B. abortus*. Another successful living vaccine is that employed for the prevention of anthrax. Older anthrax vaccines used Pasteur's technique of culturing the bacteria at a relatively high temperature (42° to 43° C) to reduce their virulence. The anthrax vaccines currently available for animals contain unencapsulated mutants that remain capable of forming spores. The vaccine is prepared as a spore suspension and is administered with saponin.

A rough strain of *S. enterica* Dublin (strain 51) is used in Europe to give good protection to calves when administered at 2 to 4 weeks of age. As discussed earlier, immunity to salmonellosis involves macrophage activation and is thus relatively nonspecific. For this reason, strain 51 may also give good protection against *S. enterica* Typhimurium.

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Adverse Consequences of the Immune Responses

Although immune responses are beneficial in that they eliminate invading bacteria, this is not always the case. The immune responses can influence the course of a bacterial disease without producing a cure and in some situations may increase its severity. The adverse consequences of the immune responses correspond in their mechanisms to the hypersensitivity types described in Chapters 29 to 33. For example, a local allergic reaction is sometimes seen in sheep vaccinated against foot rot by means of *Dichelobacter nodosus* vaccine, but in this case it is believed that the hypersensitivity may assist in preventing reinfection.

Type II (cytotoxic) reactions may account for the anemia that occurs in animals with salmonellosis. In these infections, bacterial lipopolysaccharides from disrupted bacteria are adsorbed onto erythrocytes. The subsequent immune response against the bacterium and its products therefore results in red cell destruction.

Type III (immune-complex) reactions may contribute to the development of arthritis in *Erysipelothrix rhusiopathiae* infections in pigs or to the development of intestinal lesions in Johne's disease due to MAP. In the former case, bacterial antigen tends to localize in joints, where local immune-complex formation then results in inflammation and arthritis. Passively administered antiserum may therefore exacerbate the arthritis in these infected animals. In Johne's disease, type I or type III reactions occurring in the intestinal mucosa may increase the outflow of fluid and diarrhea. It is clear, however, that the intestinal lesions in this disease are etiologically complex since diarrhea can be transferred to normal calves by either plasma or leukocytes, and antihistamine drugs may reduce the diarrhea. Type III hypersensitivity reactions are involved in purpura hemorrhagica of horses, in which immune-complex lesions result from *Streptococcus equi* infection (Chapter 32).

Although cell-mediated (type IV) immune responses are manifestly beneficial, they do contribute to the development of granulomatous lesions in some chronic infections.

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Serology of Bacterial Infections

Bacterial infections may be diagnosed by detecting specific antibodies in serum. Thus the agglutination test is widely employed to detect bacterial infections, particularly those involving Gram-negative bacteria such as *Brucella* and *Salmonella*. The usual procedure in bacterial agglutination tests is to titrate serum (antibody) against a standard suspension of antigen. Bacteria are not, of course, antigenically homogeneous but rather are covered by a mosaic of many different antigens. Thus motile bacteria will have flagellar (H) antigens, and agglutination by antiflagellar antibodies will produce fluffy cotton-like floccules as the flagella stick together, leaving the bacterial bodies only loosely agglutinated. Agglutination of the somatic (O) antigens results in tight clumping of the bacterial bodies so that the agglutination is finely granular in character. Many bacteria possess several O and H antigens, as well as capsular (K) and pilus (F) antigens. By using a set of specific antisera, it is possible to characterize the antigenic structure of an organism and consequently to classify it. It is on this basis, for instance, that the 2400 or so different serovars of *S. enterica* are classified.

Flagella (H) antigens are destroyed by heating, whereas O antigens are heat resistant and therefore remain intact on heat-killed bacteria. K antigens vary in their heat stability: the L antigen of *E. coli*, which is a capsular antigen, is heat labile, whereas another K antigen, antigen A, is heat stable. *S. enterica* Typhi possesses an antigen called Vi that, although heat stable, is removed from the bacterial cells by heating. The presence of K or Vi antigens on an organism may render them O-inagglutinable and thus complicate agglutination tests. It should also be pointed out that rough forms of bacteria do not form stable suspensions and therefore cannot be typed by means of agglutination tests.

Bacterial agglutination tests may be performed by mixing drops of reagents on glass slides or by titrating the reagents in tubes or wells in plastic plates. Tube agglutination tests are commonly used for such diseases as salmonellosis, brucellosis, tularemia, and campylobacteriosis. Slide agglutination tests are commonly used as

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screening tests. These include the *Brucella*-buffered antigen tests, in which killed, stained *Brucella* organisms are suspended in an acid buffer (pH 3.6). The dye used, either the red dye rose-bengal or a mixture of crystal violet and brilliant green, enables the test to be easily read. At this low pH, nonspecific agglutination by IgM antibodies is eliminated. The *Brucella*-buffered plate agglutination test has a specificity of as high as 99% and a sensitivity of 95%. The efficient and widespread use of these tests has eliminated bovine brucellosis from so many countries.

S. enterica Pullorum infection in poultry can be diagnosed by a slide agglutination test, in which killed bacteria stained with gentian violet are mixed with whole chicken blood. Agglutination is readily seen if antibodies are present. Leptospirosis is diagnosed by a microscopic agglutination test, in which mixtures of living organisms and test serum are examined under the microscope for agglutination. This technique preferentially detects IgM antibodies and is thus an excellent test for detecting recent outbreaks as well as for distinguishing between infected and vaccinated animals.

It is not mandatory that serum be used as the source of antibody for diagnostic tests. The presence of antibodies in body fluids other than serum, such as milk whey, vaginal mucus, or nasal washings, may be of more significance, especially if the infection is of a local or superficial nature. One such example is the milk ring test used to detect the presence of antibodies to *B. abortus* in milk (Fig. 26.8). Fresh milk is shaken with bacteria stained with hematoxylin or triphenyl tetrazolium and is allowed to stand. If antibodies, especially IgM or IgA, are present, the bacteria will clump and adhere to the fat globules of the milk and rise to the surface with the cream. If antibodies are absent, the stained bacteria will remain dispersed in the milk, and the cream, on rising, will remain white.

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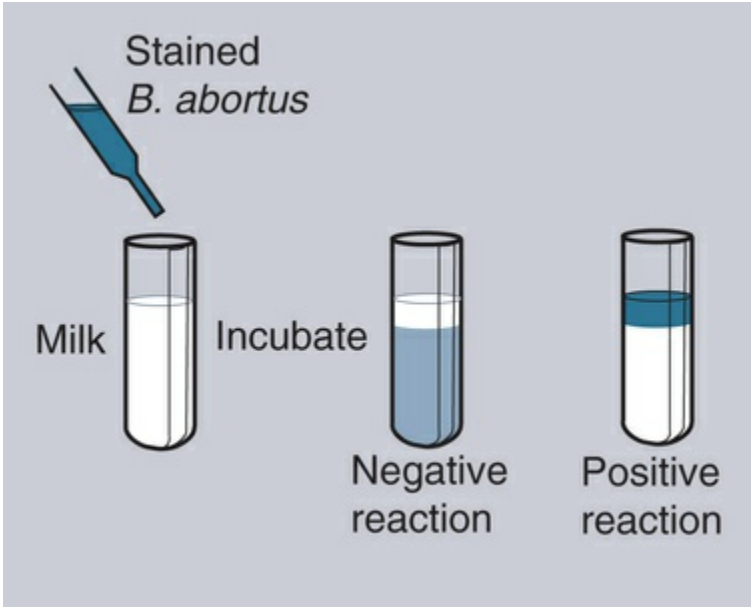


FIG. 26.8 The milk ring test. Stained *Brucella* remain suspended in the milk in a negative test but in the presence of antibodies, they bind to the fat droplets and so rise with the cream.

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Immunity to Fungal Infections

Fungal infections are of three major types (Fig. 26.9). The first are primary infections by fungi that affect the skin or other surfaces, such as *Microsporum* or *Candida* species, and cause diseases such as ringworm or thrush. The second type includes primary infections by dimorphic fungi that mainly cause respiratory infections, for example *Histoplasma capsulatum, Blastomyces dermatitidis,* and *Coccidioides immitis.* The third type consists of secondary infections by opportunistic fungi in immunodeficient animals, such as the Mucorales *(Rhizopus, Mucor,* and *Absidia)* and *Pneumocystis.* The body uses both innate and adaptive immune mechanisms to defend itself against primary infections. Thus innate immune mechanisms against invasive fungi such as *Candida* or *Aspergillus* species include activation of the alternate complement pathway, resulting in attraction of neutrophils and attempts by these neutrophils to destroy the invading hyphae or pseudohyphae. Neutrophils are also activated by the IL-23/IL-17 axis during fungal infections.

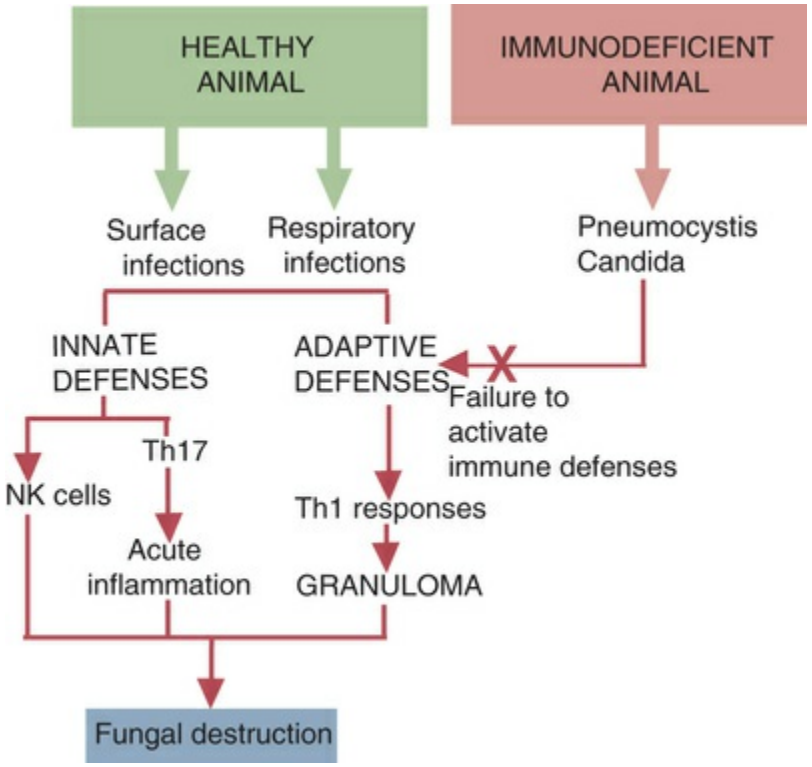


FIG. 26.9 The mechanisms of antifungal immunity.

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Immunodeficient or immunosuppressed animals are especially susceptible to fungal diseases.

Fungal PAMPs acting either through the toll- or NOD-like receptors or through cell-surface C-type lectins such as the dectins, mannose-binding lectins, and scavenger receptors such as DC-SIGN (CD209, Chapter 2) play a key role in early innate defenses. Thus Dectin-1 (CD369) binds to fungal β-glucans and triggers IL-22 and IL-23 synthesis. Both contribute to antiaspergillus resistance. IL-23 activates Th17 cells. The IL-17 produced by these cells then activates both neutrophils and endothelial cells and promotes acute inflammation. Dectin-3 associates with dectin-1, while Dectin-2 and -3 form a heterodimeric PRR on macrophages that can recognize α-mannans in *Candida* hyphae. DC-SIGN binds mannose. It is of interest to note that culturing T cells and monocytes in the presence of *Candida* hyphae promotes the generation of Th17 cells. In contrast, culture in the presence of the *Candida* yeast form promotes production of IL-12 and a Th1 response. Because of their size, neutrophils cannot totally ingest invading fungi. Nevertheless, by releasing enzymes and oxidants into the tissue fluid, neutrophils may severely damage fungal hyphae. Small fungal fragments or spores may be ingested and destroyed by macrophages or by NK cells.

Once established, fungal infections are mainly destroyed by Th1-mediated mechanisms. Thus some species of *Aspergillus* are facultative intracellular parasites, and chronic or progressive fungal diseases are commonly associated with defects in the T cell system. Th1 cells function in fungal infections by activating macrophages and by promoting epidermal growth and keratinization. Some T and NK cells can exert a direct cytotoxic effect on yeasts such as *Cryptococcus neoformans* and *Candida albicans.* It is not uncommon for recovered animals to develop a type IV hypersensitivity to fungal antigens. The critical importance of adaptive immunity to fungi is seen in the way that fungal infections, such as those caused by *Pneumocystis,* develop in immunosuppressed animals such as dogs with canine distemper. Although defense against *Pneumocystis* is critically dependent upon $CD4^+$ T cell function, it is also dependent on Th17 cells. Depletion of IL-17 or IL-23 increases the severity of *Pneumocystis* pneumonia.

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27

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Immunity to Viruses

CHAPTER OUTLINE

Virus Structure and Antigens, 298
Pathogenesis of Virus Infections, 298
Innate Immunity, 299
Pattern-Recognition Receptors, **299**
Interferons, 299
Antiviral Activities, 299
RNA Interference, 301
Adaptive Immunity, 301
Antibody-Mediated Immunity, 301
Cell-Mediated Immunity, 302
Evasion of the Immune Response by Viruses, 302
Negative Cytokine Regulation, 302
Interference With Antigen Processing Pathways, 303
Evasion of Natural Killer Cells, 303
Alterations in the B Cell System, 303
Alterations in the T Cell System, 304
Viral Evasion Through Latency, 305
Inhibition of Apoptosis, 305

Adverse Consequences of Immunity to Viruses, 305
Antibody-Dependent Enhancement, 305
Some Selected Virus Diseases, 306
Feline Infectious Peritonitis, 306
Aleutian Disease of Mink, 307
Equine Infectious Anemia, 308
Porcine Respiratory and Reproductive Syndrome, 308
Some Antiviral Vaccines, 308
Serology of Viral Diseases, 309
Tests to Detect and Identify Viruses, 309
Tests to Detect and Identify Antiviral Antibodies, 309

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Describe how viral nucleic acids are detected by TLRs and other PRRs, and as a result, are triggered to produce antiviral type I interferons.
- Summarize the antiviral mechanisms of interferons.
- List the different types of interferons and their biological significance.
- Explain why antibodies are effective against extracellular viruses.
- Describe why T cell–mediated responses are primarily responsible for antiviral immunity.
- Explain how viruses employ a wide variety of methods to evade immune destruction.
- Explain how some viruses employ sequential antigenic variation and describe its consequences.
- Discuss how significant damage and disease may be caused by the immune

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response to some viruses.

- Define, interference, virokines, antigenic shift and drift, and virion.
- Understand the diversity of pathogenic mechanisms induced by viruses
- Describe how viral infections may be diagnosed using serologic tests.
- Understand the key features of antiviral vaccines.

Since viruses are obligate intracellular organisms, their very existence is threatened if they are destroyed by the immune system or by the death of their host. Because of this, both viruses and their hosts have been subjected to rigorous selection and adaptation. Viruses are selected for their ability to evade the host's immune responses, while at the same time animals are selected for resistance to virus-induced disease. Viruses that are eliminated before they replicate cannot spread. Hosts eliminated by viruses can no longer serve as hosts. An “oversuccessful” virus will reduce the availability of susceptible hosts, whereas a very successful host will be the largest target for the next generation of viruses. As a result, there can never be a “solution” to the problem of viruses.

For example, in infections where virus-host adaptation is poor, diseases tend to be lethal. Rabies is an excellent example of this. The virus is inevitably lethal in dogs, cats, horses, and cattle because they are unnatural hosts. On the other hand, in its natural hosts, especially bats and skunks, rabies virus persists and may be shed in saliva for a long period without causing disease. From the virus's “point of view,” infection of dogs, cattle, or horses is unprofitable since those animals almost never transmit rabies to skunks. Other diseases of this type include feline panleukopenia, canine parvovirus-2, and the virulent forms of Newcastle disease. Vaccination is relatively successful in controlling these infections since the virus has not adapted to the host's defenses.

When the virus and its host are more adapted, although disease may be severe, mortality may not be high, and the virus may be persistent. In this type of disease, further attacks may occur as a result of infection by variants of the same virus. Examples of this type of virus infection include foot-and-mouth disease and influenza. Vaccination against diseases of this type is complicated

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by the diversity of these viruses.

Even more adapted viruses can result in persistent infection because the immune system is unable to eliminate them. Diseases of this type include the lentivirus infections, equine infectious anemia, and maedi-visna of sheep. Vaccination against these diseases is essentially unsuccessful. As their adaptation increases, viruses may cause latent infections and relatively mild, non-lethal disease. Some herpesvirus infections also fall into this category. The most extreme examples of virus adaptation are those in which the viral nucleic acid becomes stably integrated into the host genome. These endogenous viruses are common in the genomes of domestic mammals.

In studying the nature of the host responses to viruses, it is important to recognize that this continuing selective pressure on both host and virus exists and profoundly influences the outcome of all viral infections.

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Virus Structure and Antigens

Virus particles, called virions, consist of a nucleic acid core surrounded by a layer of proteins (see Figs. 9.2 and 40.2). This protein layer, called the capsid, is made up of subcomponents called capsomeres. The virions may also be covered by a lipid envelope. The complexity of viruses varies. Some, such as poxviruses, are complex, whereas others, such as foot-and-mouth disease virus, are relatively simple. Antibodies can be produced against all these viral proteins. Antibodies against the nucleoprotein components are not usually protective, but they may be useful for serologic diagnosis.

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Pathogenesis of Virus Infections

Adsorption, the first step in the invasion of a cell by a virus, occurs when a virus binds to cell surface receptors. These receptors are not there for the convenience of viruses but have some other physiological function. Thus rabies virus binds to the receptor for acetylcholine, a neurotransmitter. The Epstein-Barr virus (the cause of infectious mononucleosis) binds to a receptor for C3. Rhinoviruses that cause the common cold bind to cell surface integrins. The chemokine receptor CCR5 is also the receptor used by West Nile virus. The nature, number, and distribution of cell receptors determine the host range and tissue tropism of a virus. Once bound, the virion is taken into the cell through endocytosis or by fusion with the plasma membrane. Once inside a cell, the capsid is dismantled so that its nucleic acid is released into the cell cytoplasm, a process called uncoating. Once the virus genome is uncoated, replication begins (Fig. 27.1). Host cell DNA transcription is usually inhibited so that only viral genetic information is processed. If the virus, for example, a herpesvirus, contains DNA, this viral DNA is replicated. The new viral DNA is then transcribed into viral messenger RNA (mRNA), and this mRNA is translated into new capsid proteins. These new proteins are then assembled into virions. The host cell also replicates the viral nucleic acid so that large quantities of viral DNA are produced. The viral DNA is packaged inside the new capsids so that complete virions are formed. If the virus is un-enveloped, the infected cells rupture, and the virions are released into the environment. If the virions are enveloped, they leave the cell by budding through the cell surface. The cell membrane that encloses them serves as the new envelope. The released virions may then spread to nearby cells and invade them in turn.

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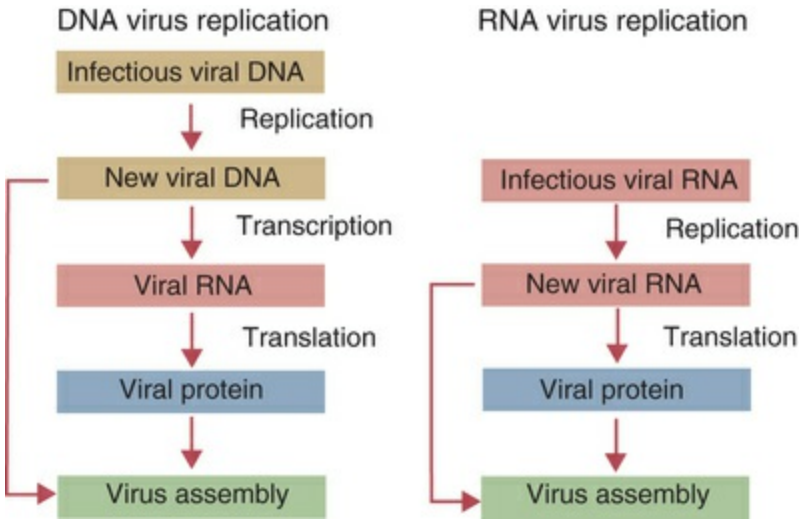


FIG. 27.1 The mechanisms of replication of DNA and RNA viruses.

If a virus contains RNA rather than DNA, its replication takes a slightly different course. For most RNA viruses, such as Newcastle disease or foot-and-mouth disease virus (FMDV), viral DNA is not used. Thus in FMDV infection, the viral single-stranded RNA (the "plus strand") is used as a template to synthesize a complementary "minus strand" of RNA. These minus strands are then used to generate new plus strands that can be translated into viral proteins. Some viruses contain double-stranded RNA (dsRNA) and use only one of the strands generated during replication. In other RNA viruses, the infecting virus RNA may be complementary to the newly synthesized viral RNA that will translate into viral proteins.

A different replication mechanism is employed in the case of some RNA tumor viruses and immunodeficiency viruses (Fig. 27.2). These are called retroviruses since their RNA is first reversely transcribed into DNA by a reverse transcriptase. The new viral DNA is then integrated into the host cell genome as a provirus. This proviral DNA can then be transcribed into RNA, as well as being able to copy itself. The proteins and RNA can then be packaged into a complete new virion.

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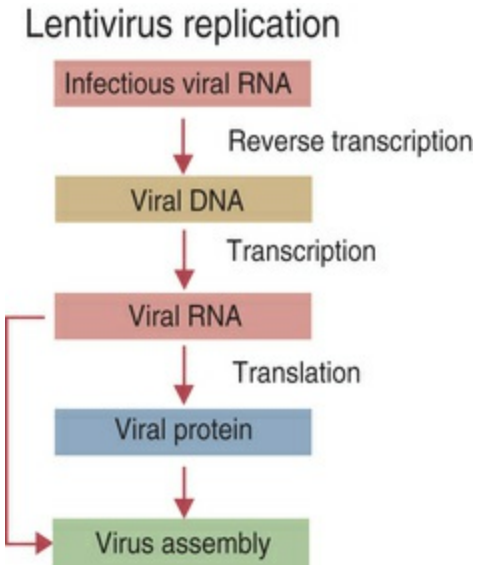


FIG. 27.2 The mechanism of replication of the retroviruses.

Changes in virus-infected cells may be minimal, perhaps only detectable by the expression of new proteins on the cell surface. Sometimes, however, the changes may be extensive and result in either cell death or malignant transformation.

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Innate Immunity

Rapid, powerful innate immune responses limit many viral infections. Interferons are especially important in this process. Lysozyme can destroy some viruses, as can many intestinal enzymes and bile. C-type lectins bind to viral glycoproteins and block virus interaction with host cells. For example, conglutinin, mannose binding lectin and the surfactant proteins A and D can all inactivate influenza viruses. Defensins from leukocytes and mucosal epithelial cells play a dual role in antiviral defenses since they can act both on the virus and in the host cell. Thus defensins can inactivate enveloped virions by disrupting their envelopes or by interacting with their glycoproteins. Some defensins can block intracellular signaling pathways in infected cells and interfere with transcription of viral RNA. Finally, cells invaded by viruses may undergo premature apoptosis, preventing successful viral invasion and replication.

Pattern-Recognition Receptors

Viruses, unlike bacteria and fungi, do not contain easily recognizable microbe-specific structures since they are constructed from host-derived components. For this reason, animal cells have evolved the ability to recognize their only virus-specific components, their nucleic acids. Three pattern-recognition receptor systems recognize viral nucleic acids. One system consists of RIG-like receptors found within the cytosol of all nucleated cells. These proteins detect viral dsRNA and then signal through several adaptor proteins to activate the interferon-β (IFN-β) gene. The second system is mediated by toll-like receptors (TLRs). TLR3 recognizes dsRNA. TLR7 and TLR8 recognize single-stranded RNA viruses such as vesicular stomatitis and influenza viruses. TLR9 detects unmethylated CpG motifs in DNA. These motifs are common in both DNA viruses and bacteria. Mice deficient in either TLR7 or TLR9 or their adaptor protein MyD88 have a reduced ability to defend themselves against viruses. Plasmacytoid dendritic cells (pDCs) use a specialized signaling pathway that links TLR7

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and TLR9 to the production of very large amounts of type I interferons. The third PRR system uses receptors with nucleotide-binding oligomerization (NOD)-like domains.

Interferons

Interferons protect cells against viral, bacterial, and protozoan invasion. They are glycoproteins of 20 to 34 kDa classified into three types: I, II, and III. Type I interferons include multiple forms of IFN-α and IFN-β, as well as single gene products such as IFN-ω, -δ, -ε, -ν, -τ, -κ, and -ζ. There are 18 isoforms of IFN-α in humans, 12 in pigs and cattle, 4 in horses, and 2 in dogs. IFN-α is produced in large quantities by pDCs and in much smaller amounts by lymphocytes, monocytes, and macrophages. IFN-β can be produced by almost any virus-infected cell. (There are five isoforms in cattle and pigs and one in dogs and humans.) IFN-ω is produced by lymphocytes, monocytes, and human, horse, pig, rabbit, and dog trophoblast cells (eight functional genes in pigs, one in humans, two in horses, 15–20 in cattle, 13 in cats, and none in dogs or mice). Another type I interferon, IFN-τ, is found in the ruminant trophoblast (3–5 genes). IFN-δ is found in the placental tissues of pigs, sheep, and horses (two in horses). IFN-δ is only distantly related to the other type I interferons. IFN-κ is produced by keratinocytes. Bovine IFN-κ has been characterized and acts through JAK/STAT pathways in a manner similar to the other type I interferons (Chapter 8). IFN-ζ is found in mice where it is also called limitin. IFN-ε is a member of the type I family whose expression is limited to reproductive and brain tissues. It plays a role in protecting the female reproductive tract. In most cases, these molecules act on virus-infected cells to inhibit viral growth. The trophoblast interferons also regulate the maternal immune response to the fetus (see Fig. 34.8).

There is only one type II interferon, IFN-γ, produced by antigen-stimulated Th1 cells. It is also produced in the pig trophoblast.

Four type III interferons have been identified, IFN-λ1, -2, and -3 (also known as interleukin-29 [IL-29], IL-28A, and IL-28B) and IFN-λ4. They are mainly restricted to epithelial cells such as those on mucosal surfaces. (Pigs lack IFN-λ2.) They signal through a unique

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receptor complex consisting of IL-10Rβ and IL-28Rα. While structurally unrelated to type I interferons, they induce similar intracellular signals and a similar gene expression profile. Their effects are most apparent at intestinal and respiratory epithelia and at the blood-brain barrier (Box 27.1).

Box 27.1

Measuring Interferons

Interferons may be assayed by measuring their antiviral effects. For example, serum samples to be tested for interferon activity are added to fibroblast cultures at various dilutions and incubated for 18 to 24 hours. The fibroblast monolayers are then washed, and a standard quantity of bovine vesicular stomatitis virus is added to each culture. After 48 hours of incubation, the monolayers are stained, and virus plaques are seen as cleared areas in the monolayer. The presence of interferons in the test serum will reduce the number of plaques formed. This is called a plaque reduction assay.

Antiviral Activities

The two major type I interferons (IFN-α and IFN-β) are produced within a few hours of viral invasion, and high concentrations are achieved long before adaptive immunity develops. For example, in cattle infected with bovine herpesvirus-1 (BHV-1), peak interferon levels in serum are reached 2 days later and then decline, but they are still detectable by 7 days (Fig. 27.3). In contrast, antibodies are not usually detectable in serum until 5 to 6 days after the onset of a virus infection.

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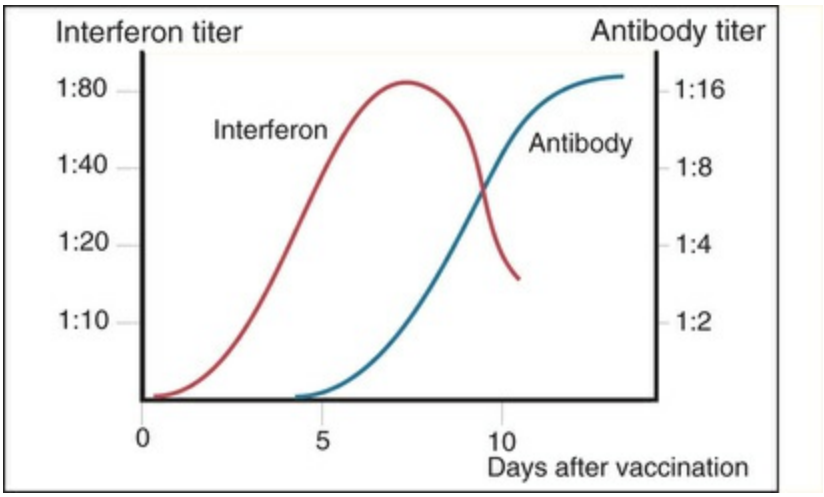


FIG. 27.3 The sequential production of interferon and antibody following intranasal vaccination of calves with infectious bovine rhinotracheitis vaccine. (From data kindly provided by Dr. M. Savan.)

IFN-α and IFN-β are produced when viral nucleic acids bind TLRs-7 and -9 or RIG-1. Both bind to receptors on nearby cells and activate JAK/STAT signaling pathways (Fig. 27.4; see also Fig. 8.8). These pathways turn on at least 300 genes, many of which encode antiviral proteins. The result is the development of an "antiviral state" within a few minutes that peaks by 5 to 8 hours.

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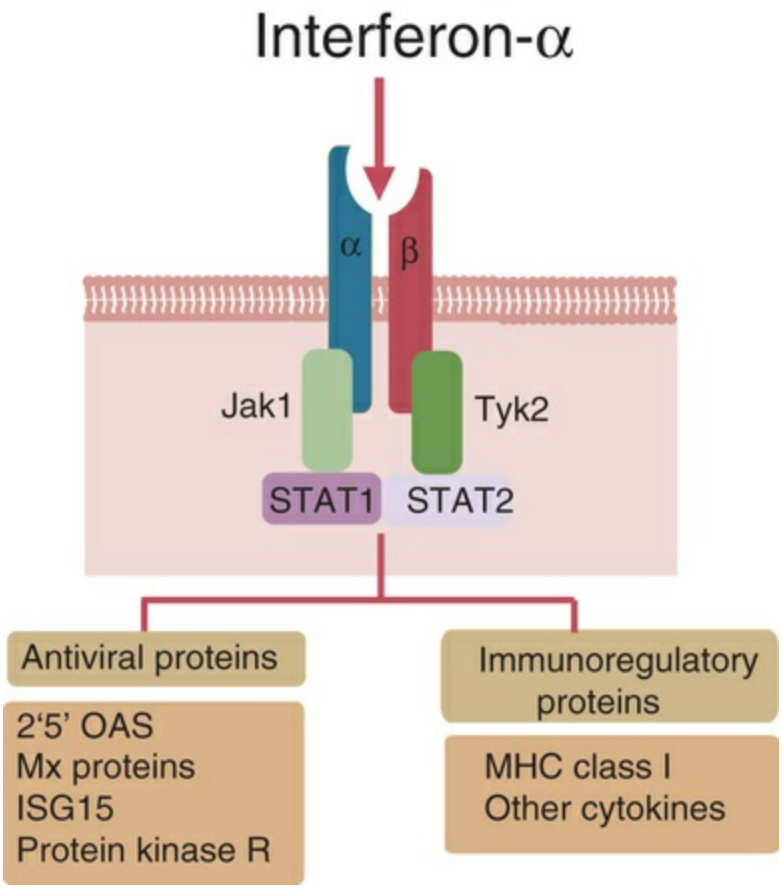


FIG. 27.4 The receptor for the type I interferons (IFNAR). Ligand binding triggers the JAK-STAT transduction pathway and eventually activates both antiviral and immunoregulatory pathways.

IFN-stimulated genes act through many different pathways and have diverse effects on viruses. Some broadly inhibit viral growth while others target specific viruses. They may target different stages of viral replication such as viral entry, envelope uncoating, genome replication, protein assembly, or viral release. The existence of diverse IFN-α isoforms, even though they signal through a common receptor, suggests that they have different functional roles. Interferons also target cells to promote viral clearance or induce apoptosis. These include increased neutrophil survival, activation of macrophages, and regulation of natural killer (NK) cells, DCs, B cells, $CD8^+$T cells, and Th1 cells. They are, in effect, broad-spectrum antivirals.

Here are six of the most important pathways.

- *The 2′5′ A pathway:* Type I interferons upregulate transcription of the genes coding for 2′5′-oligoadenylate synthetases (2′5′-OAS). These enzymes are then activated by exposure to long dsRNA

from viruses in the cytoplasm. They act on adenosine triphosphate (ATP) to form 2′5′ adenylate oligomers. These oligomers bind and activate a ribonuclease called RNAase L (Fig. 27.5). RNAase L degrades viral RNA and so inhibits viral growth.

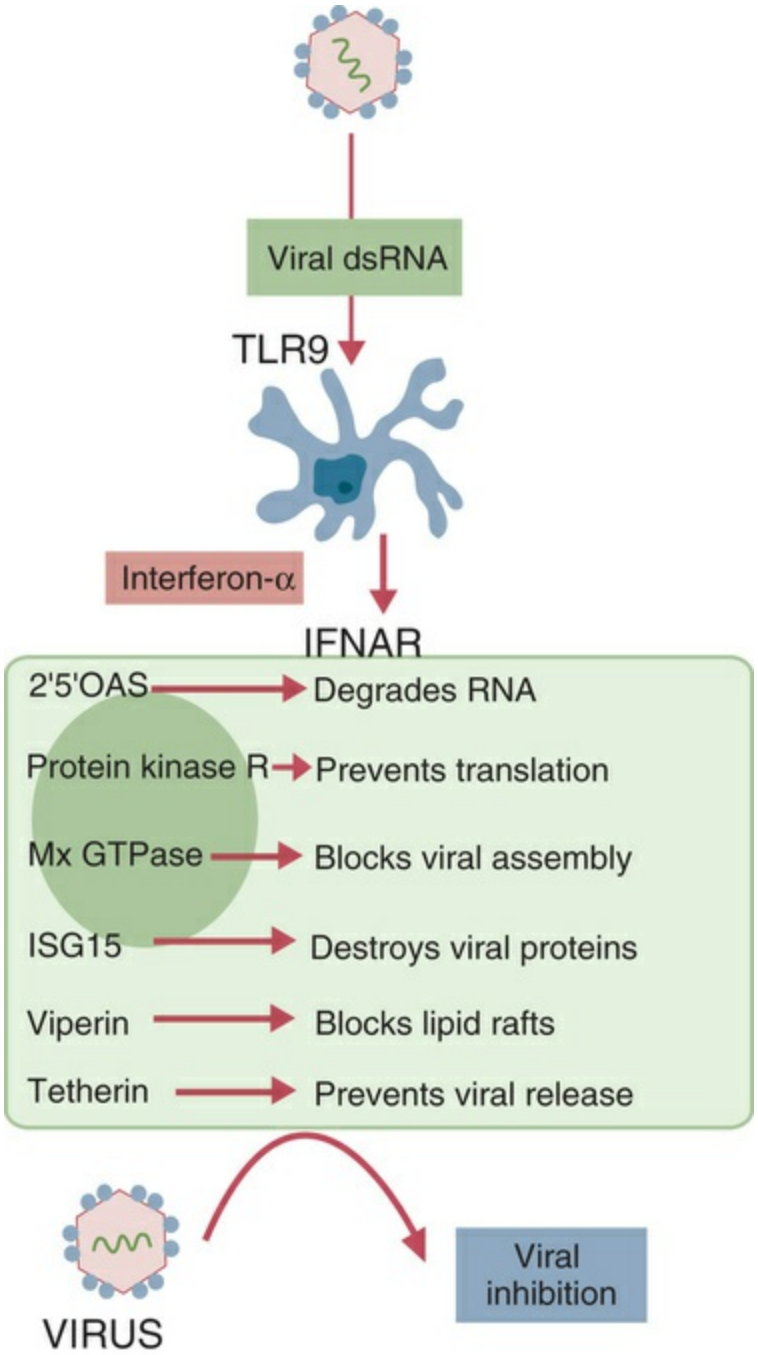


FIG. 27.5 Some of the mechanisms by which the interferons can exert their antiviral activities. IFNAR: Interferon alpha/beta receptor. OAS: Oligoadenylate synthetase. ISG: Interferon-stimulated genes.

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- *The Mx guanosine triphosphatase (GTPase) pathway:* Mx proteins are large interferon-induced GTPases that accumulate as oligomers on intracellular membranes. Following viral infection, Mx monomers are released. These bind and trap viral nucleocapsids and other essential viral components and so block the assembly of new viruses. Mx proteins are expressed in many different cell types such as hepatocytes, endothelial cells, and immune cells. They inhibit a wide range of RNA viruses, including the influenza viruses.
- *The protein kinase R (PKR) pathway:* PKR is induced by type I interferons. The inactive kinase accumulates in the cell nucleus and cytoplasm, where it is activated by viral RNA. Activated PKR regulates several cell signaling pathways and phosphorylates an initiation factor called eIF2α, which then prevents translation initiation by viral mRNA.
- *The ISG15 pathway: ISG15* codes for a ubiquitin-like protein that binds to many different proteins and enhances their destruction. It is not known how this results in increased antiviral resistance and reduced viral replication.
- *The viperin pathway:* Viperin is a protein that has direct antiviral activity. It is induced by all three classes of interferon, double-stranded DNA and RNA, and by many different viruses. It appears to act on cellular lipids and interferes with lipid raft formation at different stages in the viral life cycle, depending upon the virus.
- *Tetherin.* This is an interferon-stimulated gene that encodes a small membrane protein. It physically crosslinks (tethers!) virions to the plasma membrane and thus inhibits the release of enveloped viruses from the cell surface.

The ability of cells to produce interferons varies. Virus-infected leukocytes, especially pDCs, produce large amounts of IFN-α; almost any virus-infected cell can produce IFN-β; and antigen-stimulated T cells are the major source of IFN-γ (Chapter 18).

NK cells can kill virus-infected cells (Chapter 19). NK cell cytotoxicity is stimulated by type I interferons and, as a result, is important early in a virus infection. Indeed, NK cells provide a first line of defense against many viruses. NK cells also produce large

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amounts of IFN-γ and perforins, and these too have direct antiviral effects. NK cells may therefore reduce the severity of viral infections long before the development of adaptive immunity and the appearance of specific cytotoxic T cells.

IFN-α not only activates NK cells but most other immune cell populations as well. Thus it stimulates the differentiation of monocytes into dendritic cells, as well as the maturation and activity of dendritic cells. IFN-α stimulates memory T cell proliferation, activates naïve T cells in chronic viral diseases, and enhances antigen-specific T cell priming. It promotes B cell functions such as IgG production and MHC expression.

RNA Interference

RNA interference (RNAi) is an innate antiviral pathway that is important in plants and many invertebrates (Chapter 43).It has recently been recognized in mammals. Viral double-stranded RNA (dsRNA) is broken up by an intracellular nuclease called DICER into small interfering RNAs (siRNAs). These siRNAs are loaded into an RNA-induced silencing complex (RISC) that then binds to the viral RNA and destroys it, thus preventing viral growth. These siRNAs have been detected in a mouse germ cell line. Conversely, they are absent from somatic cells from adult mice. Embryonic cell lines cannot produce type I interferons but adult somatic cells can. It has been suggested therefore that germ cells and embryos rely on RNA interference but as they develop, they switch to an interferon response.

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Adaptive Immunity

Antibody-Mediated Immunity

Virus proteins are antigenic, and it is against these that antiviral antibody responses are largely mounted (Fig. 27.6). Antibodies can prevent cell invasion by blocking the adsorption of virions to target cells, by stimulating virus phagocytosis, by triggering complement-mediated virolysis, or by causing viral clumping and thus reducing the number of infectious units available for cell invasion. Binding of antibodies alone does not destroy viruses since the splitting of virus-antibody complexes releases infectious virions.

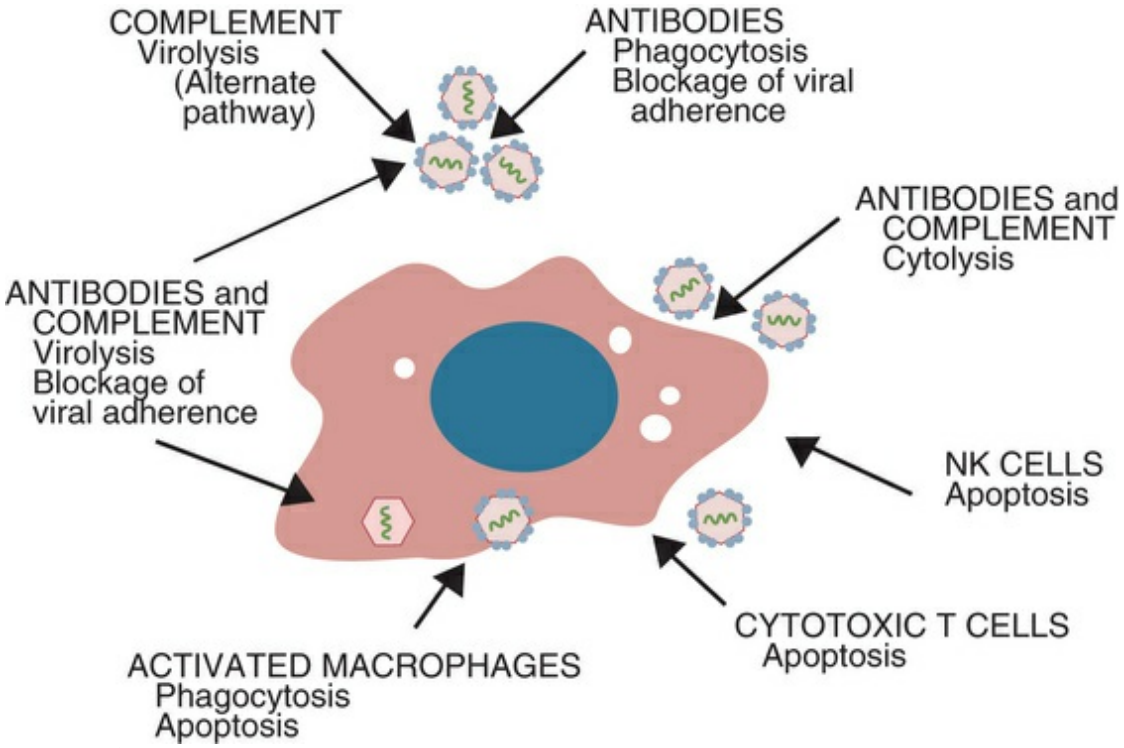


FIG. 27.6 The ways in which the immune system can protect the body against viruses.

Antibodies are not only directed against virion proteins but are also against viral proteins expressed by infected cells. As a result, these infected cells are also destroyed. Antibody-mediated destruction of virus-infected cells occurs in Newcastle disease, rabies, bovine virus diarrhea, infectious bronchitis of birds, and

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feline leukemia. Antibodies may kill infected cells using complement or antibody-dependent cell-mediated cytotoxicity (ADCC). The cytotoxic cells include lymphocytes, macrophages, and neutrophils with Fc receptors to bind antibody-coated target cells.

Virus-neutralizing antibodies include IgG and IgM in serum and IgA in secretions. As in antibacterial immunity, IgG is quantitatively the most significant immunoglobulin, whereas IgM is qualitatively superior.

Although most viruses infect cells by binding directly to target cell receptors, some use an intermediate molecule. For instance, some antibody-coated viruses bind to cells through Fc receptors. This triggers viral endocytosis and enhances virus infection. Complement may enhance virus infections in a similar fashion. Examples of virus infections that are enhanced by antibodies include feline infectious peritonitis, Aleutian disease of mink, African swine fever, and human immunodeficiency virus.

Cell-Mediated Immunity

Although antibodies and complement can neutralize free virions and destroy virus-infected cells, cell-mediated immune responses are much more important in controlling virus diseases. This is readily seen in immunodeficient humans (Chapter 39). Those who cannot mount an antibody-mediated response suffer from overwhelming bacterial infections but tend to recover from the common viral diseases. In contrast, humans with T cell deficiencies are commonly resistant to bacterial infection but highly susceptible to viruses.

Viral antigens may be expressed on infected cells long before progeny viruses are produced. When this endogenous antigen is presented by MHC class I molecules to $CD8^+$ T cells, the infected cells are recognized as foreign and killed. Viruses require host cells in which to replicate. Elimination of infected cells prevents viral spread. Although antibodies and complement or ADCC can assist, T cell–mediated cytotoxicity is usually the major protective mechanism. Cytotoxic T cells recognize peptide-MHC complexes and kill the presenting cells. Type I interferons can sensitize virus-

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infected cells to this cytotoxic effect. Under some circumstances, cytotoxic T cells may kill intracellular viruses without killing the infected cells. This effect is mediated by T cell–derived IFN-γ and tumor necrosis factor-α (TNF-α). These cytokines activate two virucidal pathways. One pathway eliminates viral nucleocapsid particles, including their contained genomes. The second pathway destabilizes viral RNA.

Some viral antigens may act as superantigens by binding directly to TCR Vβ chains. For example, rabies virus nucleocapsid binds to mouse Vβ8 T cells. By stimulating helper T cell activity, rabies viruses can switch on Th2 cells. This in turn can result in an enhanced immune response to rabies viruses, as well as a polyclonal B cell response.

Macrophages develop antiviral activity following activation. Viruses are readily endocytosed by macrophages and are usually destroyed. If the viruses are noncytopathic and can grow inside macrophages, a persistent infection may result. Under these circumstances, the macrophages must be activated to eliminate the virus. Thus macrophage activation mediated by IFN-γ is a feature of some virus diseases (Chapter 18). For example, macrophages from birds immunized against fowlpox show an enhanced antiviral effect against Newcastle disease virus and will prevent the intracellular growth of *Salmonella gallinarum,* a feature that is not a property of normal macrophages.

The duration of immunological memory to viruses is highly variable. Antiviral antibodies may persist for many years in the absence of the virus. On the other hand, cytotoxic T cells die soon after virus elimination while memory T cells can persist for many years (Chapter 18).

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Evasion of the Immune Response by Viruses

As discussed earlier, during the millions of years they have coexisted with animals, viruses have evolved numerous methods of evading host immune responses (Fig. 27.7).

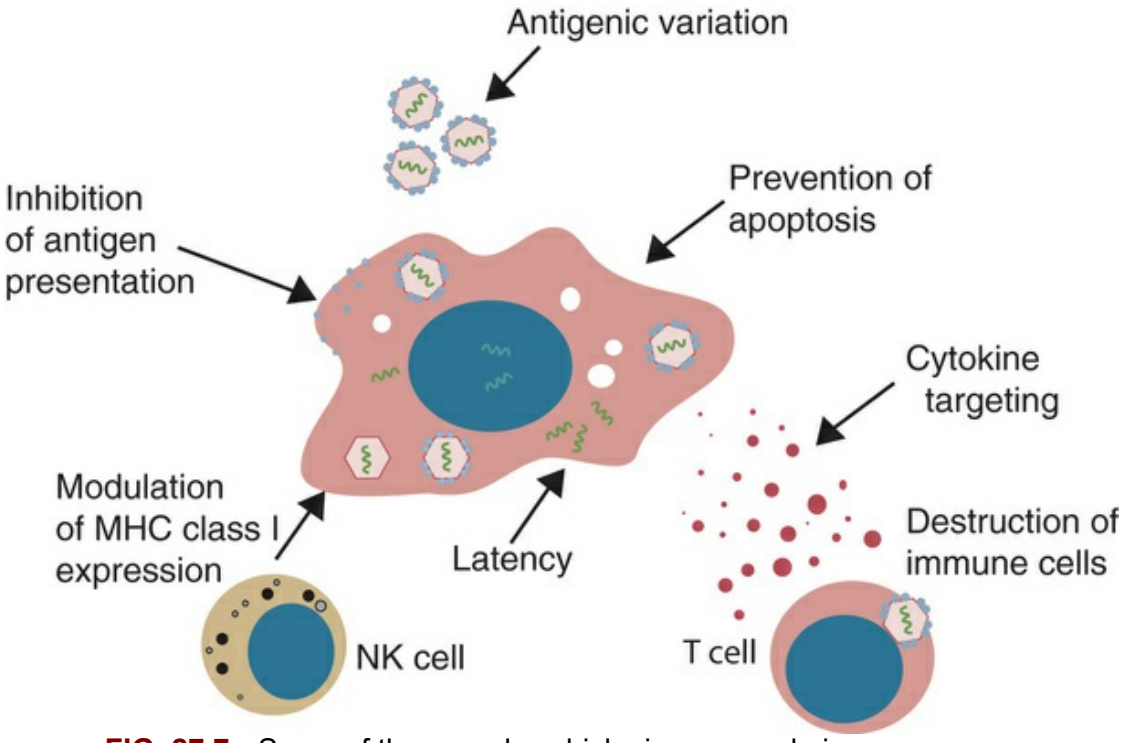


FIG. 27.7 Some of the ways by which viruses evade immune destruction.

RNA viruses have a very small genome with little room to spare for genes dedicated to suppressing immunity. As a result, RNA viruses tend to rely on antigenic variation as their principal mechanism of immune evasion. On the other hand, DNA viruses have a larger genome and can afford to devote many different genes to immune evasion. In large DNA viruses such as the poxviruses and herpesviruses, as much as 50% of the total genome may be devoted to immunoregulatory genes.

Negative Cytokine Regulation

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Viruses can block interferon activity. Methods range from blocking interferon receptor signal transduction to synthesizing soluble interferon receptors. Some viruses inhibit IFN-γ production by blocking the activities of IL-18 and IL-12, both of which are required for its production. Myxoma and poxviruses produce a protein related to the IFN-γR. By binding free IFN-γ, this prevents it binding to cell receptors. Equine herpesvirus-1 suppresses IFN-β production, and as a result, expression of viperin was also suppressed. Interestingly, it does not suppress IFN-α production.

Some viruses make viral versions of cytokines and chemokines and their receptors. These have been called virokines or immunoevasins. For example, equine herpesvirus makes CCR3, the receptor for CCL11. Marek's disease virus makes a protein related to CXCL8. Poxviruses make a version of the immunosuppressive cytokine IL-10. Cowpox virus also makes an IL-1β-binding protein that reduces its availability.

Interference With Antigen Processing Pathways

Many viruses interfere with the expression of MHC class I molecules and so inhibit antigen presentation. They use many different suppressive techniques, including reducing transcription of MHC genes, blocking transporter protein function and the transfer of peptides into the endoplasmic reticulum, inhibiting proteasomal degradation of viral proteins, inhibiting the intracellular transport of MHC class I α chains, preventing delivery of the loaded MHC to the cell surface, and ubiquinating and hence destroying MHC molecules. Thus bovine herpesvirus-1 suppresses the expression of MHC class I molecules by interfering with transporter protein functions and downregulating the expression of mRNA for MHC class I molecules. Other viruses may cause MHC class I molecules to be retained within a cell; they may prevent peptide binding to transporter proteins, prevent proteasomal degradation, redirect MHC molecules to lysosomes for degradation, or even encode inhibitors that block caspase activity. Influenza A viruses can block macrophage differentiation into dendritic cells. Other viruses may downregulate the expression of co-stimulating

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molecules such as ICAM-1, CD4, and CD28.

Evasion of Natural Killer Cells

NK cells kill virus-infected cells at an early stage of infection before T and B cells are fully activated. Cytotoxic T cells kill targets that express foreign antigens on MHC class I molecules. NK cells kill targets that fail to express these MHC class I molecules. For a virus to survive, it must induce the selective downregulation of some MHC class I molecules, enabling the virus-infected cells to evade destruction by T cells while at the same time preventing NK cell activation. Some viruses may decrease the expression of the stress-related protein MIC-B and so inhibit NK cell mediated cytotoxicity.

Alterations in the B Cell System

One of the simplest mechanisms of viral immune evasion involves antigenic variation of RNA viruses. Rapidly occurring point mutations accompanied by poor editing functions of RNA polymerases permit the generation of closely related but distinct viruses. The most significant examples of this occur among the influenza A viruses and the lentiviruses.

Influenza A viruses express envelope proteins called hemagglutinins and neuraminidases. There are at least 18 different hemagglutinins and nine neuraminidases found among the type A influenza viruses; they are identified according to a standard nomenclature system. The hemagglutinin of the swine influenza virus is called H1, and its neuraminidase is called N1. The two subtypes of the equine influenza viruses are typified by A/equine/Prague/56, which has H7 and N7, and A/equine/Hong Kong/92, which has H3 and N8. Highly pathogenic bird flu virus strains include H7N9 and H5N1. H3N8 and H3N2 strains are circulating in dogs in the United States (Table 27.1).

TABLE 27.1

Examples of Influenza A Virus Strains and Their Antigenic Structure

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Species	Virus Strain	Antigenic Structure
Human	A/New Caledonia/20/99*	H1N1
	A/California/7/09 (swine flu)	H1N1
	A/Perth/16/09	H3N2
Canine	A/Canine/Florida/04	H3N8
	A/Canine/Beijing/359/09	H3N2
Equine	A/Equine/Prague/1/56	H7N7
	A/Equine/Miami/1/63	H3N8
	A/Equine/South Africa/4/03	H3N8
	A/Equine/Richmond/1/07	H3N8
Swine	A/Swine/Iowa/15/30	H1N1
Avian	A/Fowl Plague/Dutch/27	H7N7
	A/Duck/England/56	H11N7
	A/Turkey/Ontario/6118/68	H8N4
	A/Chicken/Hong Kong/258/97	H5N1
	A/Chicken/Shantou/4231/03	H5N1
	A/Chicken/Jiangsu/60457/16	H7N9

*The first number is the isolate number; the second is the year of isolation.

As influenza viruses spread through a population, they undergo mutation and gradually change the structure of their hemagglutinins and neuraminidases. These changes alter the antigenicity of the virus. This gradual change is called antigenic drift, and it permits the virus to persist in a population for many years. In addition, influenza viruses sporadically undergo a sudden, major genetic change in which a new strain develops whose hemagglutinins show no apparent relationship to the hemagglutinins of prior strains. Such a major change, called an antigenic shift, is not due to mutation but results from recombination between two virus strains. This occurs readily since the influenza virus has a segmented genome. It is the development of these naturally recombinant influenza viruses with a completely new antigenic structure that accounts for the periodic pandemics of influenza in humans and poultry. In horses and pigs, in contrast, the rapid turnover of the population and the constant production of large numbers of susceptible young animals ensure the persistence of influenza viruses without the necessity for extensive antigenic drift. As a result, the antigenic structure of equine and swine influenza viruses has changed only slowly since they were first described. For example, the H3N8 equine influenza virus strains changed very little between 1963 and 1988. In 1989 they split into two clades. These two clades, one European and one American, differ in the structure of their hemagglutinin. Viruses of both clades can circulate in horse populations at the same time. Examples of the European clade include A/Italy/99 and A/Richmond/07. The American clade includes A/Ohio/03 and A/South Africa/03. The two

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clades are sufficiently antigenically different as to require both in vaccines.

A second form of immune evasion by viruses is seen in caprine arthritis-encephalitis (CAE), Aleutian disease of mink, and African swine fever. Although infected animals respond to these viruses, their antibodies are incapable of virus neutralization. Thus parvovirus-antibody complexes from Aleutian disease-infected mink are fully infectious. Goats with CAE make large amounts of anti-envelope antibodies, but they develop negligible levels of neutralizing antibodies. In this case, goats fail to recognize and respond to the virus-neutralizing epitopes. If rabbits are immunized with CAE virus, they can readily produce virus-neutralizing antibodies; even goats will produce these antibodies if immunized with large amounts of an adjuvanted viral antigen. The antibodies produced in these hyperimmunized goats are very specific and will react only with the immunizing strain of the virus. Notwithstanding the absence of neutralizing antibodies, other antibodies can bind to CAE virions, and the opsonized virions are endocytosed by macrophages. Unfortunately, this virus grows within macrophages so that opsonizing antibodies merely speed up virus replication, an example of antibody-mediated enhancement. Attempts to vaccinate goats against CAE lead only to more severe disease.

A third mechanism by which viruses can evade destruction by antibodies is seen in yet another lentiviral infection, maedi-visna, in sheep. (Maedi is a chronic pneumonia; visna is a chronic neurological disease caused by the same virus.) In maedi-visna infections, neutralizing antibodies are produced slowly. These neutralizing antibodies are unable to reduce the viral burden in infected sheep, and cyclical relapses do not occur. The antibodies have a low affinity for viral epitopes and take at least 20 minutes to bind to the virus and 30 minutes to neutralize it. In contrast, it takes only 2 minutes for this virus to infect a cell. Thus the virus can spread between cells much faster than it can be neutralized. The maedi-visna virus also invades monocytes and macrophages. In most of these cells, the replication of the virus stops after its RNA has been reversely transcribed into proviral DNA. As a result, the cells are persistently infected by the virus without expressing viral

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antigens. The virus may therefore be disseminated without provoking immunological attack. Maedi-visna is associated with extensive infiltration of the lungs, mammary gland, and central nervous system with T cells and macrophages. This virus does not infect lymphocytes so there is no immunosuppression due to a loss of $CD4^+$ T cells. Immunosuppression reduces the severity of the lesions, whereas immunization against the virus increases their severity. It is suggested that virus-infected macrophages stimulate the T cells to release cytokines. These cytokines delay the maturation of monocytes and restrict virus replication. They also enhance macrophage MHC class II expression and trigger T cell proliferation and chronic lymphoid hyperplasia.

Alterations in the T Cell System

Viruses may, of course, use the cells of the immune system as their hosts. Viruses like HIV, feline immunodeficiency virus (FIV), canine distemper (CDV), and feline leukemia virus (FeLV) infect lymphocytes and either kill them or otherwise impair their ability to function normally (Chapter 40). Glucocorticoids are profoundly suppressive for T cells and T cell responses. Influenza virus triggers a generalized stress response leading to a sustained increase in serum glucocorticoid levels and resulting in immunosuppression.

Viral Evasion Through Latency

If viruses cause a state of reversible nonproductive infection, this is called latency. It is a consistent feature of the herpesviruses. During latency, viruses express only the absolute minimum number of genes. Because they do not express viral antigens, they are not detected by the immune system and may remain in that state for many years.

In contrast to the short-lived immune response against bacteria, antiviral immunity is, in many cases, very long-lasting. The reasons for this are unclear, but they are often related to virus persistence within cells, perhaps in a slowly replicating or a non-replicating form as typified by latent herpesviruses. It is usually difficult to isolate viruses from an animal that has recovered from a

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herpesvirus infection. Some time later, however, especially when the individual is stressed, the herpesvirus may reappear and may even cause disease. During the latent period, when it is present in the host but cannot be reisolated, the virus nucleic acid persists in host cells, but its transcription is blocked, and viral proteins are not made. The persistent virus may periodically boost the immune response of the infected animal and in this way generate long-lasting immunity to superinfection. The immune responses in these cases, although unable to eliminate viruses, may prevent the development of clinical disease and therefore serve a protective role. Immunosuppression or stress may permit disease to occur in persistently infected animals. The association between stress and the development of some virus diseases is well recognized. It is likely that the increased steroid production in stressful situations may be sufficiently immunosuppressive to permit activation of latent viruses or infection by exogenous ones.

Sometimes viruses may interact with bacteria to overcome the immune system. For example, *Mannheimia hemolytica* and bovine herpesvirus (BHV-1), acting together, cause severe respiratory disease in cattle. BHV-1 infection increases expression of the β_2-integrin LFA-1 on lung neutrophils. The leukotoxin of *M. hemolytica* binds to this integrin and then kills the neutrophils, permitting growth of the invading bacteria.

Inhibition of Apoptosis

Apoptosis may be considered a protective response since viruses also die when a cell dies. This is especially significant if a cell dies before viruses are released. It is therefore to a virus's advantage to delay apoptosis until progeny viruses can be released. Thus cowpox and some herpesviruses (including equine herpesvirus-2) encode apoptosis inhibitors in their genomes. Viruses may also benefit by killing cells of the immune system. Rapidly dividing lymphocytes are susceptible to death signals. For example, lymphoid cell apoptosis is a feature of canine distemper, a disease characterized by severe immunosuppression (Chapter 40).

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Adverse Consequences of Immunity to Viruses

The immune response to viruses can, on occasion, be a disadvantage. Indeed, there are many virus diseases in which disease results from inappropriate or excessive immune responses. For example, bovine respiratory syncytial virus (RSV) induces a type 2 response in infected cattle with production of IL-4 and specific IgE antibodies in the lungs. This may result in a type I hypersensitivity reaction since there is a direct correlation between lung IgE levels and the severity of clinical disease.

The destruction of virus-infected cells by antibody is classified as a type II hypersensitivity reaction (Chapter 31) and, although normally beneficial, may exacerbate virus diseases. Thus viruses are removed at the cost of cellular destruction. The severity and significance of this destruction depend on how widespread the infection becomes. In some diseases in which the virus causes little cell destruction, most of the tissue damage may result from immunological attack. A good example of this is seen in distemper encephalitis, in which neurons are demyelinated as a result of an antiviral immune response. Macrophages in these brain lesions ingest immune-complexes and infected cells leading to the release of oxidants and other toxic products. These toxic products damage nearby cells, especially oligodendroglia, causing demyelination.

Type III (immune-complex) lesions (Chapter 32) are associated with viral diseases, especially those in which viremia is prolonged. For example, a membranoproliferative glomerulonephritis resulting from the deposition of immune-complexes is a common complication of equine infectious anemia, Aleutian disease of mink, feline leukemia, chronic hog cholera, bovine virus diarrhea-mucosal disease, canine adenovirus infections, and feline infectious peritonitis. A generalized vasculitis due to deposition of immune-complexes throughout the vascular system is seen in equine infectious anemia, Aleutian disease of mink, malignant catarrhal fever, and possibly equine viral arteritis.

In dogs infected with canine adenovirus-1 (infectious canine

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hepatitis), an immune-complex–mediated uveitis and a focal glomerulonephritis both develop. The uveitis, commonly called "blue-eye," is seen both in dogs with natural infections and in those vaccinated with live attenuated adenovirus vaccine (Fig. 27.8). The uveitis results from the formation of virus-antibody complexes in the anterior chamber of the eye and in the cornea with complement activation and consequent neutrophil accumulation. The neutrophils release enzymes and oxidants that damage corneal epithelial cells, leading to edema and opacity. The condition resolves spontaneously in about 90% of affected dogs.



FIG. 27.8 A case of blue-eye in a coyote puppy. This is a type III hypersensitivity reaction to canine adenovirus 1 (ICH) occurring in the cornea. (Courtesy Dr. G.J. Costanzo.)

In Borna disease, a lethal viral encephalitis, the noncytopathic virus does not kill neurons. Infected animals do, however, mount a strong Th1 response that results in neuronal destruction by T cells. The development of bornaviral disease in both birds and mammals can be prevented therefore by treatment with immunosuppressive drugs such as cyclosporine.

Antibody-Dependent Enhancement

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There are many examples of virus infections in which the presence of antibodies results in increased susceptibility or severity of infection. Several mechanisms are involved in this, and many are poorly understood. One important mechanism observed in cats with feline coronavirus or FIV is antibody-dependent enhancement. This can involve, as described previously, antibody coating of virions so that their entry into cells through Fcγ receptors is enhanced. This FcR-mediated entry may be less effective than other routes in triggering interferon production. Some experimental FIV, simian immunodeficiency virus (SIV), and equine infectious anemia virus vaccines may increase disease susceptibility by a similar mechanism. FIV envelope-specific antibodies appear especially effective in enhancing infection.

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Some Selected Virus Diseases

Viruses, if they are to survive, must evade the immune responses. They use many different mechanisms including, but not limited to severe immunosuppression. These may result in significant immunopathology.

Feline Infectious Peritonitis

Feline infectious peritonitis (FIP) is a fatal granulomatous disease of wild and domestic cats caused by feline enteric coronavirus (FECV). There are two distinct genotypes of feline enteric coronavirus, avirulent and virulent. The avirulent genotype prefers to replicate within intestinal epithelial cells, whereas the virulent genotype prefers to replicate within macrophages. Macrophages also spread the virus throughout the body. FIP presents in two major forms: (1) an effusive ("wet") form with peritonitis or pleuritis characterized by the presence of large amounts of proteinaceous fluid in the body cavities and associated with a vasculitis, and (2) a noneffusive ("dry") form characterized by multiple small granulomas on the surface of the major abdominal organs. Both forms of the disease are uniformly lethal, with affected cats dying between 1 week and 6 months.

The pathogenesis of FIP differs between the two forms of the disease. After invading a cat, the virus first replicates in intestinal epithelial cells. The virus shed by epithelial cells is then spread by monocytes and taken up by phagocytic cells in the target tissues. These target tissues include the serosa of the peritoneum and the pleura, as well as the meninges and the uveal tract. The course of the infection then depends on the nature of the immune response to the virus—a phenomenon also seen in several bacterial diseases (Chapter 26). Immunity to FIP virus is entirely cell mediated, and a Th1 response is protective. A cat that mounts a good Th1 response will become immune, regardless of the amount of antibodies it makes. Some cats, however, mount a Th2 response to the viral spike proteins. In these animals, antibodies enhance virus uptake by macrophages, in which they then replicate. Virus-laden

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macrophages accumulate around the blood vessels of the omentum and serosa (Fig. 27.9). These macrophages are activated in that they are strongly positive for CD18 and produce TNF-α and IL-1β. Endothelial cells upregulate MHC class II expression. These antibodies also generate immune-complexes that are deposited in the serosa, causing pleuritis or peritonitis, and in glomeruli, leading to glomerulonephritis. The serosal vasculitis is responsible for the effusion of fibrin-rich fluid into the serosal cavities. This massive production of immune-complexes may also be responsible for the disseminated intravascular coagulation seen in these cats. IL-1 and IL-6 are found in unusually high concentrations in the peritoneal fluid from cats with effusive FIP. Cats with preexisting high levels of antibodies against FECV develop effusive FIP rapidly on challenge. Administering antiserum to FECV before FIP challenge may also enhance the peritonitis. It has been suggested that FECV-infected cats do not develop FIP since they avoid excessive macrophage activation by upregulating IL-10.

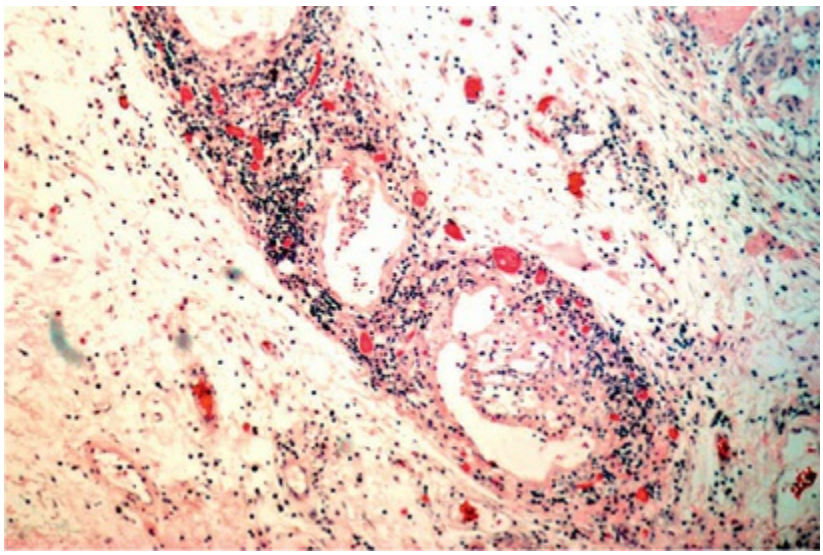


FIG. 27.9 Granulomatous vasculitis of serosal blood vessels in a cat with feline infectious peritonitis. Note the marked cellular infiltration of the vessel adventitia and media. This lesion may be due to the deposition of virus-antibody complexes in the vessel walls. (Courtesy Dr. R.C. Weiss.)

A modified live intranasal vaccine is available against FIP. The vaccine contains a temperature-sensitive mutant virus that replicates in the upper respiratory tract and induces a local IgA

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response in the mucosa. This local mucosal response should prevent coronavirus invasion without inducing high levels of serum antibodies. This vaccine, however, will only be effective if administered before coronavirus exposure. In highly endemic situations in which kittens are infected at a young age, vaccination at 16 weeks of age may be too late to prevent infection.

Aleutian Disease of Mink

Although immune-complex–mediated lesions are usually only of passing interest in many infectious diseases, they generate the major pathological lesions in Aleutian disease of mink. Aleutian disease due to a parvovirus infection that was first recognized in mink with the Aleutian coat color. Although all strains of mink are susceptible to this virus, Aleutian mink are genetically predisposed to the development of severe lesions since they are affected by the Chédiak-Higashi syndrome (Chapter 39). Persistently infected mink develop a slowly progressive lymphoproliferative disease with a plasmacytosis that has been compared to a myeloma since it results in a polyclonal or monoclonal gammopathy (Fig. 27.10). They also develop immune-complex–mediated lesions (Chapter 32), including glomerulonephritis and arteritis. They make autoantibodies to their own immunoglobulins (rheumatoid factors) and to DNA (antinuclear antibodies). Their serum IgG concentration increases, sometimes to very high levels. On occasion, these elevated immunoglobulins are monoclonal in origin. They are directed against the Aleutian disease parvovirus. The virus transforms B cells so that they proliferate and differentiate excessively.

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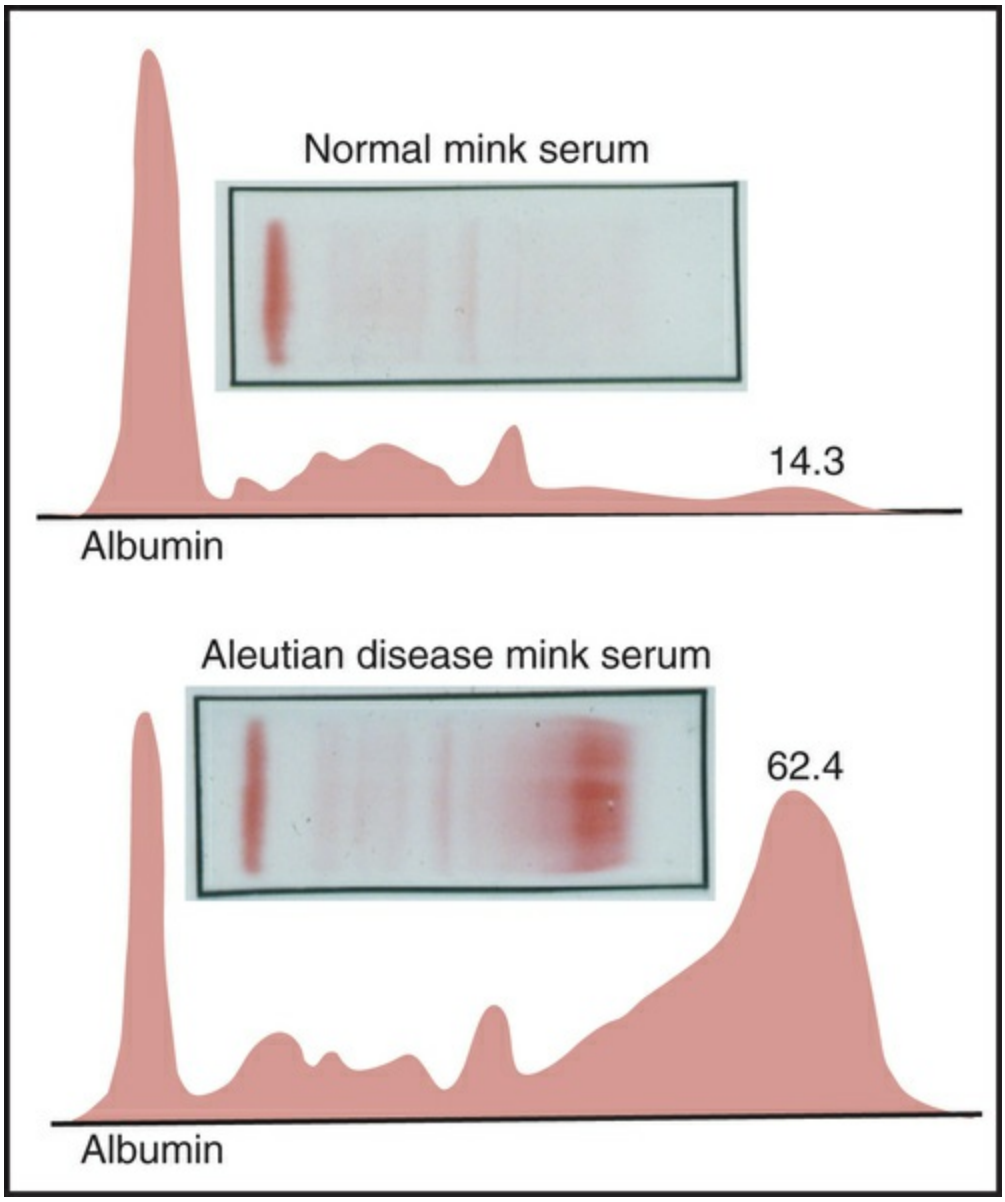


FIG. 27.10 The serum protein electrophoretic patterns seen in normal and Aleutian disease-infected mink. The serum of the infected animal shows a polyclonal gammopathy, so the γ-globulins account for 62.4% of the serum proteins in contrast to the normal level of 14.3%. (Courtesy Dr. S.H. An.)

The immune-complex–mediated lesions of Aleutian disease include an arteritis, in which IgG, C3, and viral antigen are found within vessel walls; and a glomerulonephritis, in which immune-complexes are deposited in the kidneys. In addition, infected mink are anemic because their red cells are coated with antiviral antibodies. The red cells of infected animals adsorb virus-antibody complexes from plasma. These coated red cells are then removed from the circulation by macrophages. As might be predicted, the use of immunosuppressive agents such as cyclophosphamide or

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azathioprine in infected mink prevents the development of many of these lesions and prolongs survival, whereas experimental vaccination with inactivated Aleutian disease virus increases the severity of infections.

Equine Infectious Anemia

This disease is caused by the equine infectious anemia virus (EIAV). The disease may be acute, chronic or inapparent. Waves of viremia are associated with clinical disease. Following recovery from the first attack of disease characterized by anemia, fever, thrombocytopenia, weight loss, and depression, horses may remain healthy for weeks or months. However, three or four relapses at 2- to 8-week intervals may occur before the horse either develops a chronic wasting disease or becomes clinically normal. Each episode of disease tends to be milder than the previous one. The fevers are lower and the anemia less severe. EIAV, like other lentiviruses, undergoes random mutation, and new, antigenically different variants are produced. The elimination of each variant is determined initially by the appearance of both $CD4^+$ and $CD8^+$ MHC-restricted cytotoxic T cells. Later, as new variants are produced, the infected horse makes neutralizing antibodies to that variant, and as a result, the viremia ends. Variants of EIAV, however, appear rapidly and randomly. The appearance of a new non-neutralizable variant leads to a viremic wave and clinical relapse. After the virus has undergone several antigenic variations and the horse has responded to them all, the neutralizing antibody spectrum of the horse's serum becomes very broad, and viremia drops to a low level. Large amounts of tissues may then have to be examined to isolate the virus.

In addition to evading the immune response through antigenic variation, EIAV triggers immune-mediated tissue damage. The red cells of viremic horses adsorb circulating the virus onto their surface. Antibodies and complement then bind to the virus, as a result of which the red cells are cleared from the circulation more rapidly than normal. Infected horses may also develop a glomerulonephritis as a result of immune-complex deposition on glomerular basement membranes. Horses infected with EIAV have

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unusually low levels of IgG3.

Porcine Respiratory and Reproductive Syndrome

Porcine respiratory and reproductive syndrome virus (PRRSV) is a single-stranded, positive-sense RNA virus belonging to the family Arteriviridae. It causes a syndrome characterized by reproductive failure, infertility, abortions, anorexia, and secondary pneumonia. When PRRSV invades the respiratory tract, it damages the mucociliary transport system, kills alveolar macrophages, and induces apoptosis of immune cells. Some PRRSV isolates downregulate TNF-α and IFN-γ while others induce Treg cells and IL-10, both of which can suppress the immune response within the lungs. Enhanced IL-10 secretion and suppressed TNF-α and IFN-γ production result in a Th1/Th2 imbalance. As a result of all this, PRRSV causes an increase in secondary pneumonia. When PRRSV infects neonatal piglets, it stimulates B cell activity. The piglets therefore present with polyclonal B cell activation, autoimmunity (antibodies specific for Golgi antigens and dsDNA), grossly enlarged lymph nodes, and hypergammaglobulinemia (a 100- to 1000-fold increase in IgG; a 10- to 100-fold increase in IgM and IgA). The immunoglobulins produced are not directed against PRRSV, and the B cell proliferative response is not purely polyclonal. The antibodies produced are derived from a limited number of dominant B cell clones and are not neutralizing. It is speculated that the virus produces some form of B cell superantigen. Affected pigs also show a decrease in $CD4^+$ T cells and an increase in $CD8^+$ cells after several weeks. Cell-mediated responses and virus-neutralizing antibodies to PRRSV do not develop for about 4 weeks as a result of the loss of $CD4^+$ cells. Because of this immunosuppression, PRRSV may cause persistent infections lasting for up to 6 months. The levels of IL-1, IL-6, TNF-α, and IFN-α are upregulated earlier and to a greater extent in pigs infected with a highly pathogenic strain of PRRSV than in pigs infected with less virulent strains. These cytokines are produced by alveolar macrophages and they may reduce adaptive immunity to this virus. When PRRSV infects mature dendritic cells it reduces the expression of CD80/86 and

MHC class II molecules and increases their production of IL-10.

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Some Antiviral Vaccines

Because of the lack of antiviral drugs, vaccination is the only effective method for the control of most viral diseases in domestic animals. As a result, the development of viral vaccines is, in many ways, more advanced than the development of their bacterial counterparts. It has, for example, proved relatively easy to attenuate many viruses so that effective vaccines containing modified live virus (MLV) are readily available.

As discussed in Chapter 25, MLV vaccines are usually good immunogens, but their use may involve certain risks. The most important problem encountered is residual virulence. One serious example of this was the development of clinical rabies in some dogs and cats following administration of older strains of MLV rabies vaccine. Some strains of infectious bovine rhinotracheitis and equine herpesvirus-1 vaccines may cause abortion when given to pregnant cows or mares, respectively, and MLV bluetongue vaccines may cause disease in fetal lambs if given to pregnant ewes (Chapter 23). More commonly, the residual virulence in these vaccines causes a mild disease. Thus intraocular or intranasal rhinotracheitis or calicivirus vaccines may cause a transient conjunctivitis or rhinitis in cats. MLV infectious bursal disease vaccines, some canine parvovirus-2 vaccines, and some bovine viral diarrhea vaccines can cause a mild immunosuppression (see Fig. 40.1).

Because of problems of this nature, attempts have been made to minimize residual virulence in vaccines. One method involves the use of temperature-sensitive (ts) mutants. Ts strains of BHV-1, for example, will grow only at temperatures a few degrees lower than normal body temperature. When this organism is administered intranasally, it is able to colonize the relatively cool nasal mucosa but is unable to invade the rest of the body. Thus the vaccine can stimulate local immunity without incurring the risk for a systemic invasion. (It also has the advantage that its activity is not blocked by maternal immunity.) Some vaccine viruses may persist in vaccinated animals and cause a prolonged carrier state. Although this is a problem largely associated with herpesviruses, concerns

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have been expressed that the widespread use of MLV vaccines may serve to seed viruses into animal populations and that untoward consequences may develop in the future. This is a threat not to be taken lightly.

An alternative approach to overcoming the problems caused by MLV involves the increasing use of inactivated and subunit vaccines. Excellent inactivated vaccines are available against diseases such as foot-and-mouth disease, equine herpesvirus-4 (rhinopneumonitis), pseudorabies, feline panleukopenia, feline herpes (rhinotracheitis), and rabies. At their best, these vaccines confer immunity comparable in strength and duration to that induced by MLV vaccines, with the assurance that they are free of residual virulence. Virus-like particles are synthetic structures that resemble viruses in structure and morphology and express viral antigens. In effect, they are viruses without a contained genome. They hold great potential for generating superior vaccines and are described in more detail in Chapter 24.

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Serology of Viral Diseases

Tests to Detect and Identify Viruses

Historically, serological tests were used to identify the presence of viruses within tissues The tests commonly employed for this purpose included fluorescent antibody tests, enzyme-linked immunosorbent assay (ELISA), hemagglutination inhibition, virus neutralization, complement fixation, and gel precipitation. The precise tests employed depended on the nature of the unknown virus. The development of the polymerase chain reaction (PCR) has made many of these techniques obsolete. Exquisitely sensitive, the PCR can be used to detect viral DNA. A reverse transcriptase PCR can be used to detect RNA viruses. The PCR is best suited for use in well-equipped laboratories. For animal-side testing or in situations in which the equipment is not available, a more suitable technique for the detection of viral antigen or antiviral antibodies is the membrane filter ELISA test or lateral chromatography tests (Chapter 42). These tests have the advantage that both positive and negative controls can be incorporated with the test serum in one well. In addition to serum, whole blood, plasma, or saliva may be employed as a source of antigen or antibody.

Tests to Detect and Identify Antiviral Antibodies

In general, the most widely employed techniques for detecting antibodies to viruses are hemagglutination inhibition, indirect ELISA, immunofluorescence, gel diffusion, Western blotting, complement fixation, and virus neutralization. The first four of these are technically simple and are thus preferred (Chapter 42). Complement fixation and virus neutralization tests are complex, restricting the circumstances in which they may be used. Virus neutralization tests are also extremely specific, which as discussed earlier tends to reduce their value as screening tests.

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28

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Immunity to Parasites

CHAPTER OUTLINE

Immunity to Protozoa, 311
- Innate Immunity, 311
- Adaptive Immunity, 311
- *Leishmaniasis, 313*
- Evasion of the Immune Response, 313
- Adverse Consequences, 314
- Vaccination, 315

Immunity to Helminths, 315
- Innate Immunity, 315
- Adaptive Immunity, 316
- *Immunity to Tissue Helminths, 316*
- *Eosinophils and Parasite Destruction, 316*
- *Immunity to Adult Helminths, 317*
- *Cell-Mediated Immunity, 319*
- Evasion of the Immune Response, 320
- Evasion of Innate Responses, 320
- Evasion of Adaptive Responses, 320
- Vaccination, 321

Immunity to Arthropods, 322
Demodectic Mange, 322
Flea-Bite Dermatitis, 322
Tick Infestation, 322
Hypoderma Infestation, 323

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Understand how parasites are able to evade their host's immune response for at least sufficient time for the parasite to reproduce.
- Explain how, in general, antibody-mediated immune responses protect against extracellular protozoa, whereas cell-mediated responses control intracellular protozoa.
- Explain how trypanosomes undergo sequential antigenic variation.
- Discuss the availability and effectiveness of vaccination against protozoan diseases.
- Describe the complex strategies employed by protozoan parasites to ensure their survival in the face of an animal's immune response.
- Explain how helminth parasites have a unique ability to trigger type 2 immune responses.
- Discuss the different forms of immune response required to combat intestinal versus tissue helminths.
- Explain how eosinophils are uniquely able to damage and kill helminths.
- Describe the mechanisms employed by helminths to evade their host's immune responses.
- Discuss the availability of vaccines against parasitic helminths.
- Discuss the role of type 2 responses in immunity to parasitic arthropods such as ticks and biting flies.

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Infectious diseases, as pointed out earlier, rarely result from the deliberate activities of a malicious microorganism. In most cases, disease occurs because of the host's reaction to the infection or because the invader inadvertently causes damage to its host. Well-adapted parasites do not make these mistakes. They exploit the host's resources without causing irreparable damage or triggering a destructive defensive response.

A consistent feature of all parasite infestations, however, is that they block or delay host defenses so that they may survive for sufficient time to reproduce. Some parasites may simply delay their destruction until they complete a single life cycle. Other well-adapted parasites may contrive to survive for the life of their host, protected from immunological attack by highly evolved evasive mechanisms (Fig. 28.1).

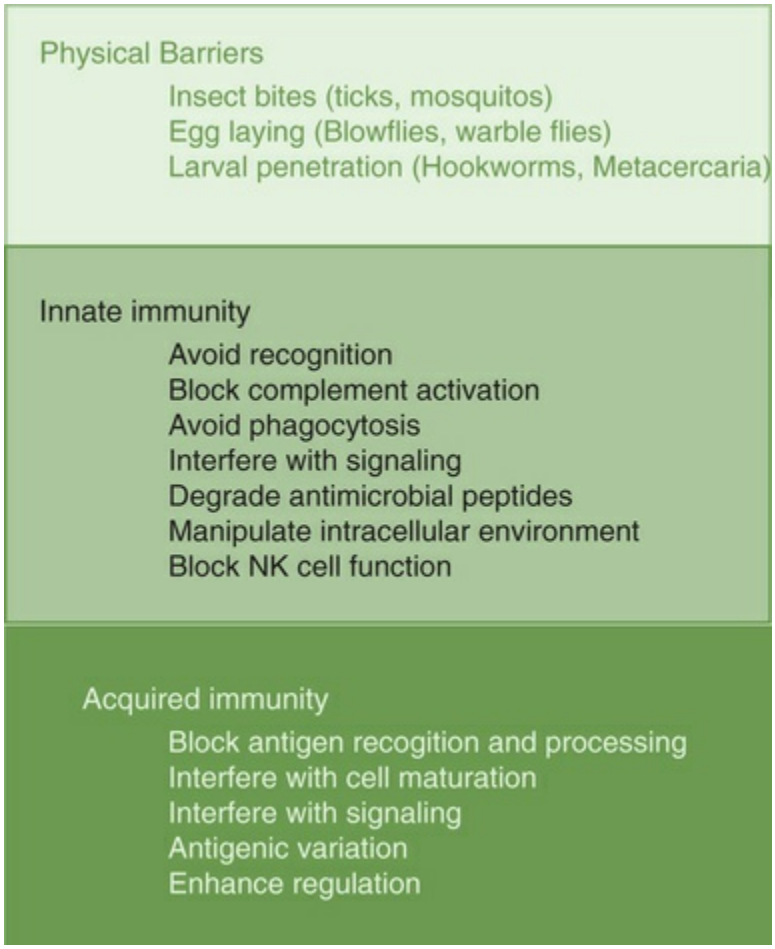


FIG. 28.1 Evasion of the immune response is critical to parasite survival. Some of the many ways by which parasites evade immune destruction or exclusion are shown.

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Ideally, a successful parasite will influence its host's immune responses, selectively suppressing these to permit parasite survival, while at the same time allowing other responses to proceed and thus minimizing immunosuppression. In addition, many parasites use their host's metabolic or control pathways for their own purposes. For example, epithelial growth factor and IFN-γ enhance the growth of *Trypanosoma brucei,* whereas IL-2 and GM-CSF promote the growth of *Leishmania amazonensis.* This sharing of cytokines by host and parasites reflects the long history of their association and their success in adapting to a parasitic lifestyle.

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Immunity to Protozoa

Innate Immunity

The mechanisms of innate resistance to protozoa are similar to those that prevent bacterial and viral invasion, although species and genetic influences may be of greater significance. For example, *T. brucei, T. congolense,* and *T. vivax* do not cause disease in the wild ungulates of East Africa but will kill domestic cattle, presumably as a result of lack of mutual adaptation. Similarly, the coccidia are extremely host specific; for example, *Toxoplasma gondii* tachyzoites can infect any species of mammal, but their coccidian stages affect only felids (e.g., cats).

Some breeds of African taurine cattle, most notably N'Dama, are resistant to trypanosomiasis. This "trypanotolerance" results from selection of the most resistant animals over many generations so that they develop a greater ability to resist the pathological effects of the parasite. The γ/δ T cells of N'Dama are much more responsive to trypanosome antigens than are the γ/δ T cells of susceptible cattle. Trypanotolerant animals produce more IL-4 and less IL-6 than susceptible animals. At the same time, they show neither the severe anemia nor the production loss seen in susceptible cattle. Trypanotolerant animals produce high levels of IgG against *T. congolense* cysteine protease. Since this enzyme contributes to the pathology of infection, these antibodies may partially account for their tolerance.

Adaptive Immunity

Like other invaders, protozoa stimulate both antibody- and cell-mediated immune responses. In general, antibodies control extracellular parasites in blood and tissue fluids, whereas cell-mediated responses are directed against intracellular protozoa.

Serum antibodies directed against protozoan surface antigens may opsonize, agglutinate, or immobilize them. Antibodies together with complement and cytotoxic cells may kill them, and some antibodies (called ablastins) may inhibit their division. In

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genital infections of humans, *Trichomonas vaginalis* stimulates a local IgE response. This allergic reaction increases vascular permeability, permitting IgG antibodies to reach the site of infection and immobilize and eliminate the organisms.

In babesiosis, the sporozoites invade red blood cells and the infected red cells incorporate *Babesia* antigens into their membranes. These antigens induce antibodies that opsonize the red cells and cause their removal by phagocytosis. Macrophages and cytotoxic lymphocytes recognize the *Babesia* antigen-antibody complexes on the surface of infected red cells and destroy them by antibody-dependent cell-mediated cytotoxicity.

Intracellular parasites use many strategies to invade cells and avoid destruction. Most gain entry by employing host-mediated processes such as phagocytosis. Apicomplexans such as *Toxoplasma* and *Cryptosporidium,* however, actively penetrate cells. Once inside, they persist within specially modified vacuoles. Protective immunity against apicomplexan protozoa, such as *Cryptosporidium, Eimeria, Neospora, Plasmodia,* and *Toxoplasma,* is generally mediated by Th1 responses. For example, *T. gondii* is an obligate intracellular parasite whose tachyzoites live within cells, especially macrophages (Fig. 28.2). They penetrate these cells by "gliding" through molecular junctions in the cell membrane and so do not trigger phagosome formation. *Toxoplasma* tachyzoites are therefore not destroyed since their "parasitophorous vacuoles" do not mature and fuse with lysosomes. *Toxoplasma* can thus persist inside cells in an environment free of antibodies, oxidants, or lysosomal enzymes. The parasites eventually produce perforin-like molecules that permeabilize the cell membrane and permit the tachyzoites to escape and invade other cells. Antibodies and complement can destroy extracellular *Toxoplasma* and prevent its spread between cells (Fig. 28.3). Antibodies, however, have little or no influence on the intracellular forms of the parasite. These intracellular organisms can only be destroyed by a Th1 response.

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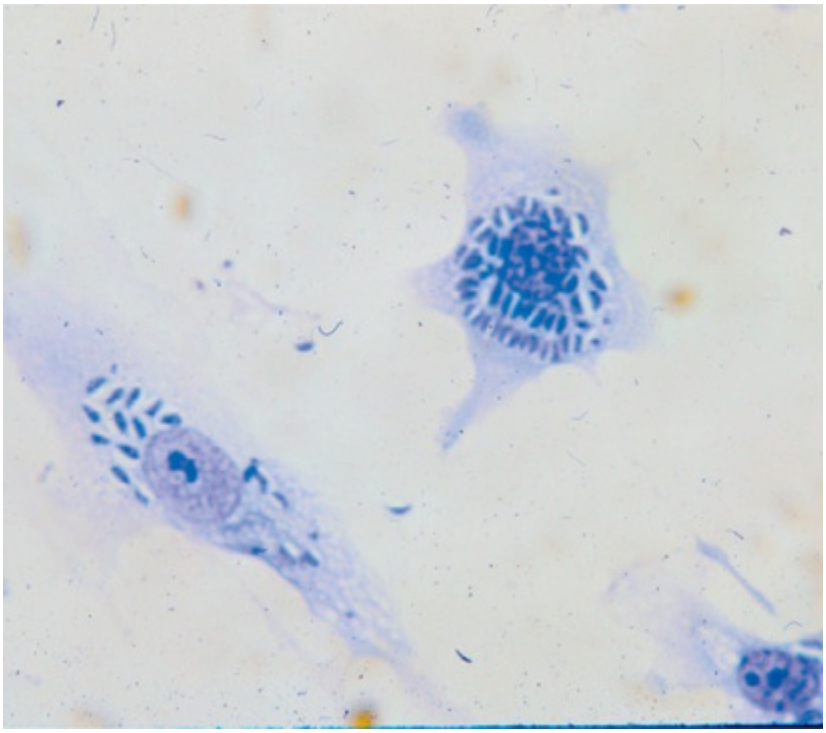


FIG. 28.2 Mouse macrophages containing healthy, growing tachyzoites of *Toxoplasma gondii.* After an immune response develops, these cells become activated and acquire the ability to destroy ingested tachyzoites. (Courtesy Dr. C.H. Lai.)

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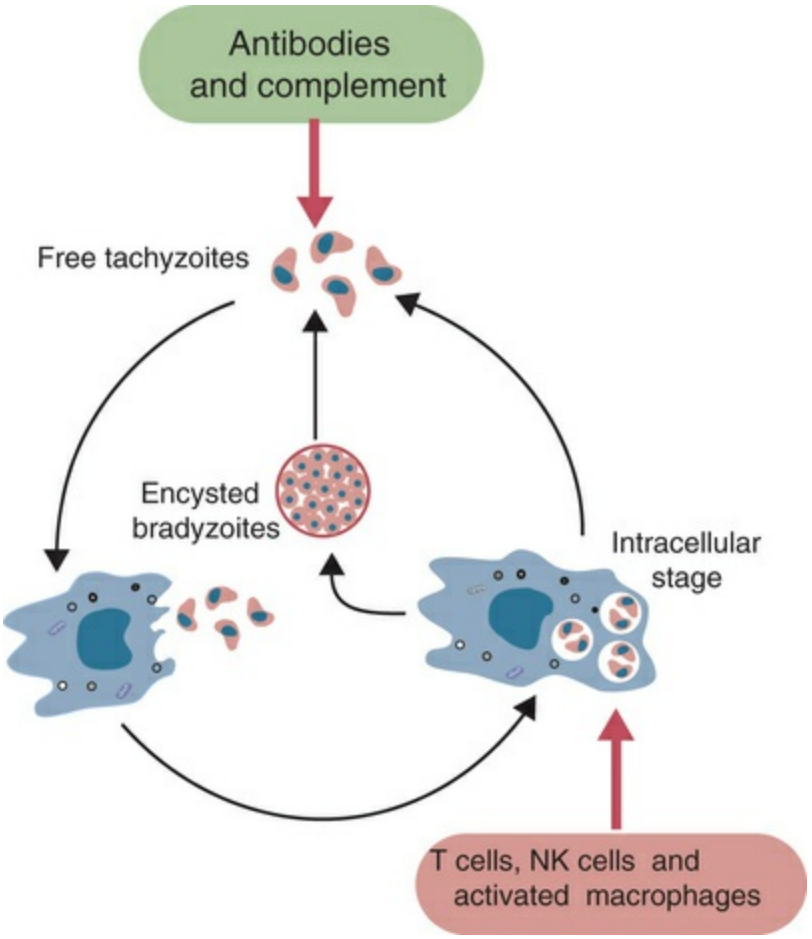


FIG. 28.3 The points in the life cycle of *Toxoplasma gondii* at which the immune system can exert a controlling influence.

A toxoplasma protein called profilin is a ligand for TLR11 in mice. Ligation of TLR11 on dendritic cells signals through the MyD88 pathway and stimulates IL-12 and IFN-γ production by natural killer (NK) and T cells. The IL-12 and IFN-γ in turn trigger a strong Th1 response. Activated Th1 cells secrete IFN-γ in response to *Toxoplasma* ribonucleoproteins. This IFN-γ activates macrophages, permitting lysosome-vacuole fusion, enhancing autophagy, and killing the intracellular organisms. Cytotoxic T cells can also destroy *Toxoplasma* tachyzoites and *Toxoplasma*-infected cells on contact. *Toxoplasma*-infected dendritic cells are attacked and killed by NK cells. The *T. gondii* tachyzoites, however, may convert into cysts containing bradyzoites. These cysts are weakly immunogenic and do not stimulate inflammation. It is possible that the cyst stage is not recognized as foreign so that *Toxoplasma* can persist indefinitely within tissues.

Th1-mediated macrophage activation is important in many

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protozoan diseases where the organisms are resistant to intracellular destruction. The most significant destructive molecules in these M1 cells are reactive nitrogen radicals. However, protozoa are also experts in surviving within macrophages; for example, *Leishmania, Toxoplasma,* and *Trypanosoma cruzi* can migrate into safe intracellular vacuoles by blocking phagosome maturation. *Leishmania* and *T. cruzi* can suppress the production of oxidants or cytokine production, while *Toxoplasma gondii* can promote macrophage apoptosis. *T. gondii* tachyzoites also inhibit inflammatory cytokine production by preventing nuclear translocation of NF-κB.

In *Theileria parva* infection of cattle (East Coast fever), sporozoites invade α/β and γ/δ T cells as well as B cells. These parasites activate NF-κB by continuously phosphorylating its inhibitor proteins Iκ-Bα and Iκ-Bβ (Chapter 8). The persistent NF-κB maintains the cell in an activated state, and prevents its apoptosis. These activated cells produce both IL-2 and IL-2R. As a result, a loop is established, by which infected cells secrete IL-2, which in turn stimulates their growth. As *Theileria* schizonts develop within lymphocytes, the infected cells enlarge and proliferate. The parasite divides with its host cell and its schizonts bind to the mitotic spindle. Thus they effectively infect both daughter cells leading to a rapid increase in parasitized cells, overwhelming infection, and death. Some animals, however, may recover from infection and become solidly immune. In these animals, $CD8^+$ T cells kill infected lymphocytes by recognizing parasite antigens in association with MHC class I. In susceptible animals, the parasites interfere with MHC class I expression.

Infection of chickens or mammals with *Eimeria* oocysts generally leads to strong, species-specific immunity that can prevent reinfection. The immune response inhibits the growth of trophozoites, the earliest invasive stage, within intestinal epithelial cells. This growth inhibition is reversible, since if the arrested stages are transferred to normal animals they can complete their development. Resistance to primary infection is mediated by multiple mechanisms that involve $CD4^+$ T cells, IL-12 and IFN-γ, macrophages, and NK cells. In contrast, resistance to secondary challenge by *Eimeria* is mediated by $CD8^+$ T cells. In chickens, IFN-

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γ, TNF-α, and TGF-β, as well as intraepithelial $CD8^+$ α/β T cells, are essential for anti-coccidial immunity.

For many years it was thought that a common feature of many protozoan infections was *premunition,* a term used to describe resistance that develops after the primary infection has become chronic and is only effective if the parasite persists in the host. It was believed, for example, that only cattle actually infected with *Babesia* were resistant to clinical disease. If all organisms were removed from an animal, resistance was believed to wane immediately. This is not entirely true. Cattle cured of *Babesia* infection by chemotherapy are resistant to challenge with the homologous strain of that organism for several years. Nevertheless, the presence of infection does appear to be mandatory for protection against heterologous strains. Babesiosis is also of interest since splenectomy of infected animals results in clinical disease. The spleen not only serves as a source of antibodies in this disease but also removes infected erythrocytes.

Leishmaniasis

The importance of immunity in determining the course and nature of a protozoan disease is well seen in canine leishmaniasis. This disease is caused by *Leishmania infantum* or its New World synonym *Leishmania chagasi* and transmitted by biting sandflies. When the promastigote forms of this parasite are injected by a sandfly into the skin of dogs, they are phagocytosed by neutrophils. When the neutrophils undergo apoptosis, the parasites are released and are then engulfed by macrophages and dendritic cells. In these cells, the organisms differentiate into amastigotes. They divide within the macrophages until the cells rupture, and neighboring cells then phagocytose the released organisms. Depending on the degree of host immunity, the parasites may be restricted to the skin (cutaneous disease); alternatively, infected dendritic cells may migrate to lymph nodes or enter the circulation and lodge in internal organs, leading to disseminated visceral disease. Although infection is widespread in endemic areas, most dogs are resistant to *Leishmania,* and only 10% to 15% develop visceral disease.

Macrophages are the main host cells for *Leishmania* but are also responsible for parasite killing. Parasites divide within the

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phagolysosomes of infected macrophages. Their resistance to intracellular destruction results from multiple mechanisms. A study of 245 macrophage genes showed that 37% were suppressed by *Leishmania* infection. *Leishmania* lipophosphoglycan delays phagosome maturation, so preventing the production of nitric oxide and inhibiting macrophage responses to cytokines. The parasite also reduces the antigen-presenting ability of macrophages by suppressing MHC class II expression. As a result of their persistence, the parasites trigger chronic inflammation. Initially characterized by granulocyte invasion, this is followed by macrophages, lymphocytes, and NK cells that collectively form granulomas.

Clinical leishmaniasis is directly linked to the immune response of the infected dog. In susceptible animals, the organisms may spread from the skin to the local lymph node, spleen, and bone marrow within a few hours. In resistant dogs, the parasites are restricted to the skin and draining lymph node so that the animals either remain healthy or develop a mild, self-limited disease. These resistant dogs mount a weak antibody response but a strong and effective Th1 response. They may have low antibody titers, but they produce IFN-γ in response to parasite antigens, generate type I granulomas, mount strong delayed hypersensitivity responses, and eventually destroy the parasites.

Resistance to *Leishmania* has a strong genetic component; for example, Ibizian Hounds appear to be resistant to this parasite. There is also an association between resistance and certain MHC class II alleles as well as some Slc11a1 (Nramp) alleles in dogs. Susceptible dogs, in contrast, mount a Th2 response characterized by high antibody levels but poor cell-mediated immunity. These differences have been attributed to the activities of IL-10 from Treg cells. In addition, the parasite may actively suppress transcription of the *IL-12* gene, ensuring that a Th2 response predominates.

Chronic, progressive disease develops in susceptible dogs. Parasite-laden macrophages accumulate, but the organism continues to multiply. These macrophages spread throughout the body, resulting in disseminated infection. Dogs develop severe generalized nodular dermatitis, granulomatous lymphadenitis, splenomegaly, and hepatomegaly. They show polyclonal

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(occasionally monoclonal) B cell activation involving all four IgG classes, as well as hypergammaglobulinemia, and they develop lesions associated with type II and type III hypersensitivity. Thus polyclonal immunoglobulin production can lead to development of an immune-mediated hemolytic anemia, thrombocytopenia, and the production of antinuclear antibodies. Glomerulonephritis, uveitis, and synovitis may result from chronic immune-complex deposition, leading to renal failure and death.

Evasion of the Immune Response

Despite their antigenicity, parasitic protozoa survive by using evasion mechanisms acquired over millions of years of evolution. For example, *Toxoplasma gondii* can avoid neutrophil attachment and phagocytosis. *Theileria parva* invades and destroys T cells. Other protozoa such as the African trypanosomes employ multiple suppressive mechanisms such as promoting the development of regulatory macrophages and Treg cells, reducing complement activation, depleting dendritic cells, or stimulating the B cell system to exhaustion. It is unsurprising that death in bovine trypanosomiasis is commonly due to bacterial pneumonia or sepsis as a result of this immunosuppression.

Parasite-induced immunosuppression may assist parasite survival. For example, *Babesia bovis* is immunosuppressive for cattle. As a result, infected cattle have more ticks than noninfected animals, and the efficiency of transmission of *B. bovis* is enhanced. It must be pointed out, however, that parasite-induced immunosuppression may kill the host as a result of secondary infection, so it is not always beneficial to the parasite. In addition to immunosuppression, protozoa have evolved two very effective evasive techniques. One involves becoming less antigenic, and the other involves the ability to alter surface antigens rapidly and repeatedly. An example of a nonantigenic organism is the encysted bradyzoite stage of *T. gondii,* which, as mentioned previously, does not appear to stimulate a host response. Some protozoa may mask themselves with host antigens. Examples of these include *Trypanosoma theileri* in cattle and *Trypanosoma lewisi* in rats, both non-pathogenic trypanosomes that survive in the bloodstream

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because they are masked by host serum proteins and are not recognized as foreign.

Many protozoa successfully employ repeated antigenic variation. If cattle are infected with the pathogenic trypanosomes *T. vivax*, *T. congolense*, or *T. brucei* and their parasitemia measured, it is found that periods of high parasitemia alternate regularly with periods of low or undetectable parasitemia (Fig. 28.4). Serum from infected animals contains antibodies against trypanosomes isolated prior to each bleeding but not against those that develop subsequently. Each period of high parasitemia corresponds to the expansion of a population of trypanosomes with a new surface glycoprotein antigen. The elimination of this population by antibodies leads to a rapid fall in parasitemia. Among the survivors, however, are parasites that express new surface glycoproteins and grow without hindrance. As a result, a fresh population arises to produce yet another period of high parasitemia (Fig. 28.5). This cyclical parasitemia, with each peak reflecting the appearance of a new population with new surface glycoproteins, can continue for many months.

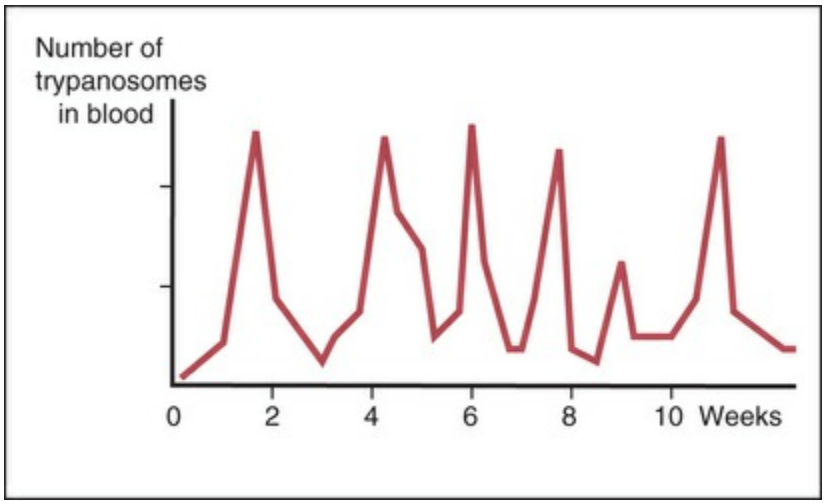


FIG. 28.4 The time course of *Trypanosoma congolense* parasitemia in an infected calf. Each parasitemic peak represents the development of a new, antigenically original population of organisms.

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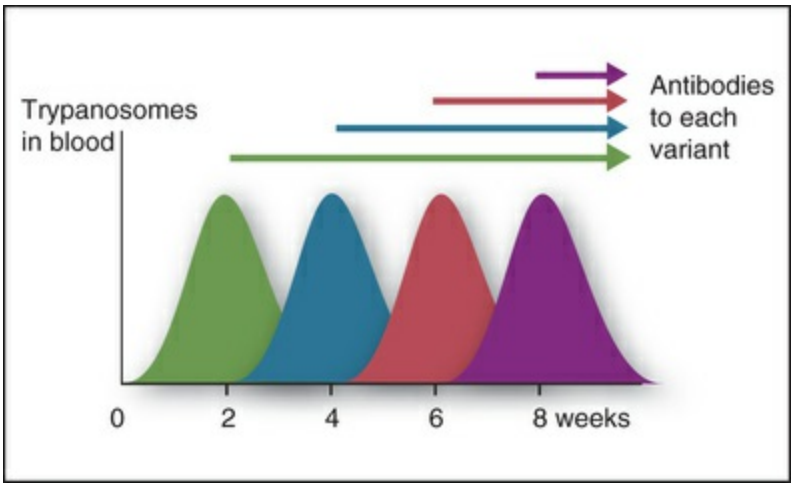


FIG. 28.5 A diagram showing how repeated antigenic variation accounts for the cyclical parasitemia observed in African trypanosomiasis. Each peak represents the growth and destruction of a new antigenic variant.

The major antigens of these trypanosomes are known as variant surface glycoproteins (VSGs). The VSGs form a thick coat on the surface of the trypanosome that hides other cell surface antigens. VSGs are therefore targeted by host antibodies. As previous VSGs are recognized by the immune system and the organisms destroyed, new VSGs emerge giving rise to waves of parasitemia. The VSGs produced early in trypanosome infections tend to develop in a predictable sequence. However, as the infection progresses, the production of VSGs becomes more random. When antigenic change occurs, the VSGs in the old coat are shed and replaced by a different VSG. These trypanosomes possess about 2000 *VSG* genes, with an additional 1600 silent genes, of which two-thirds are pseudogenes. Antigenic variation occurs as a result of repeated DNA breaking and repair, replacing an active *VSG* gene with one from the silent gene pool. Each parasite expresses one VSG at a time. Since only a small part of the tightly packed VSG is exposed to host antibodies, it is not even necessary for the complete molecule to change. Replacement of exposed epitopes by gene conversion is sufficient for effective variation (Chapter 17). Early in infections, complete *VSG* gene replacement occurs. Later on, partial replacement and point mutations can create new antigenic specificities. In some cases, the expressed *VSG* gene can be a mosaic derived from several archival pseudogenes. The potential for recombination-based variation is therefore absolutely enormous.

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Trypanosomiasis is not the only protozoan infection in which variation of surface antigens occurs. It has also been recorded in infections by *Babesia bovis,* the plasmodia, and the intestinal parasite *Giardia lamblia.*

Since parasitic protozoa must evade the immune responses, it is not surprising that they preferentially invade immunosuppressed individuals. Organisms that are normally well controlled by the immune response, such as *Toxoplasma gondii* or *Cryptosporidium bovis,* can grow and produce severe disease in immunosuppressed animals. For this reason, acute toxoplasmosis and cryptosporidiosis commonly occur in humans immunosuppressed for transplantation purposes or for cancer therapy and in AIDS patients.

Parasites often trigger a very robust T cell response. Unfortunately this may fail to eliminate the pathogen that then persists in the form of a chronic infection. Over time this can lead to T cell exhaustion. The T cells may be functionally impaired or even eliminated. These defects tend to follow a consistent pattern with an initial inability to produce IL-2, followed by loss of cytotoxicity and proliferative ability, followed by impaired TNF-α and IFN-γ production. T cell exhaustion and loss of function occurs in chronic protozoan diseases such as Toxoplasmosis and Leishmaniasis.

Adverse Consequences

The immune responses to protozoa may result in hypersensitivity reactions. Type I hypersensitivity is a feature of trichomoniasis and results in local irritation and inflammation in the genital tract. Type II cytotoxic reactions are of significance in babesiosis and trypanosomiasis, in which they contribute to the anemia. In babesiosis, red cells express parasite antigens on their surfaces and are thus recognized as foreign and eliminated by hemolysis and phagocytosis. In trypanosomiasis, either fragments of disrupted organisms or possibly preformed immune-complexes bind to red cells and provoke their elimination, causing anemia. Immune complex formation on circulating red cells is not the only problem of this type in trypanosomiasis. Excessive immune complex formation may lead to vasculitis and glomerulonephritis (type III hypersensitivity; see Chapter 32). Immune-complex lesions are a

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marked feature of visceral leishmaniasis, as described previously.

Trypanosome infections may trigger an enormous increase in IgM-secreting cells so that very high levels of polyclonal IgM develop in the blood of infected animals. Some of these antibodies are directed against autoantigens. These include rheumatoid factor-like molecules, antibodies against thymocytes, single-stranded DNA, red cells, and platelets. The mechanism of this polyclonal B cell activation is unknown.

It is probable that a type IV hypersensitivity reaction contributes to the inflammation that occurs when *Toxoplasma* cysts break down and release fresh tachyzoites. Extracts of *Toxoplasma gondii* (toxoplasmin), if administered intradermally to infected animals, will cause a delayed hypersensitivity response (Chapter 33).

Vaccination

Successful vaccination against protozoan infections is currently limited to coccidiosis, giardiasis, leishmaniasis, babesiosis, theileriosis and toxoplasmosis.

Several live coccidial vaccines are given to poultry. These vaccines typically contain multiple species and strains of coccidia. Some consist of virulent, drug-sensitive organisms administered repeatedly in very low doses (trickle infection). Other vaccines have been attenuated by repeated passage through eggs, or they have been selected for precocity. Precocious strains mature very rapidly and, as a result, have less time to replicate and are thus less virulent. All of these vaccines provide solid immunity to coccidia when applied carefully under good conditions. Nevertheless, the dose of coccidia vaccine must be carefully controlled, and the vaccines must be harvested from the feces of infected birds. Vaccinated birds shed oocysts that are transmitted to other birds. Because of regional strain variation, vaccination may not be effective in protecting against field strains in all locations.

A vaccine is available to protect dogs and cats against *Giardia duodenalis*. The vaccine contains disrupted cultured *Giardia* trophozoite extracts administered subcutaneously and protects experimentally challenged dogs and cats against infection and clinical disease.

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Several different vaccines are available against canine leishmaniasis. All are designed to stimulate T cell–mediated responses. One such vaccine consists of a *Leishmania* component called the fucose-mannose ligand adjuvanted with saponin. This vaccine may also serve as an immunotherapeutic agent, producing clinical improvement in dogs with disseminated disease. An alternative vaccine containing an excretory protein of *L. infantum* promastigotes with either a muramyl dipeptide or a saponin adjuvant also appears to be effective. Experimental vaccines, including killed vaccines and DNA vaccines, have shown encouraging results.

Many factors contribute to the resistance of animals against babesiosis, including genetic factors (Zebu cattle are more resistant to disease than European cattle) and age (cattle show a significant resistance to babesiosis in the first 6 months of life). Animals that recover from acute babesiosis are resistant to further clinical disease. It is therefore possible to infect young calves when they are still relatively insusceptible to disease, so that they become resistant to reinfection. The organisms employed for this procedure are first attenuated by repeated passage through splenectomized calves and then administered to recipient animals in whole blood. As might be anticipated, the side effects of this type of controlled infection may be severe, and chemotherapy may be required to control them. The transfer of blood from one calf to another may also trigger the production of antibodies against the foreign red cells. These antibodies complicate any attempts at blood transfusion in later life and may provoke hemolytic disease of the newborn (Chapter 31). In a slightly different approach, cattle can be made resistant to East Coast fever (*T. parva* infection) by infecting them with virulent sporozoites and then treating them with tetracycline.

Since a primary infection with *T. gondii* will confer strong protective immunity on an animal, protective immunization is a real possibility. A live *Toxoplasma* vaccine containing the S48 incomplete strain has been used successfully for the control of toxoplasmosis in sheep. The strain was developed by prolonged passage in laboratory mice and has lost the ability to develop bradyzoites or to initiate the sexual stages of the life cycle in cats. It produces protection against a severe challenge for at least 18

months. Unfortunately, the vaccine has a shelf life of only 7 to 10 days and it can infect people.

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Immunity to Helminths

Worm burdens tend to increase slowly over time and reach a peak before slowly declining. This decline may reflect protective immunity. The major defense against gastrointestinal helminths is a type 2 adaptive immune response. The success of this response depends upon the animal's age, its nutritional status, and its genetics and the site of infection, as well as the species of worm involved and whether the infection is sudden and large or a much slower trickle infection. Thus lambs infected with *Nematodirus battus* rapidly develop resistance, while immunity to *Teladorsagia circumcincta* is slow to develop. It takes up to a year for lambs to fully develop their resistance to nematodes. Adult sheep generally harbor only a few adult nematodes but periodic re-exposure is required to maintain their immunity. Resistance to abomasal worms develops more slowly than resistance to intestinal worms. A reduction in resistance to nematodes is seen in ewes from 2 weeks prior to 6 weeks after lambing as a result of periparturient immunosuppression. One consistent feature of intestinal nematode infestations is the very wide variation in parasite load within a population. Most animals harbor a few worms, but a few animals harbor a lot of worms.

Innate Immunity

Innate factors of host origin that influence helminth burdens include the age, sex, and most important, the genetic background of the host.

The influence of sex on helminth burdens appears to be largely hormonal. In animals whose sexual cycle is seasonal, parasites tend to synchronize their reproductive cycle with that of their hosts. For instance, ewes show a “spring rise” in fecal nematode ova, which coincides with lambing and the onset of lactation. Similarly, the development of helminth larvae in cattle in early winter tends to be inhibited until spring in a phenomenon called hypobiosis. The larvae of *Toxocara canis* may migrate from an infected bitch to the liver of the fetal puppy, resulting in a congenital infection. Once

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born, the infected pups can re-infect their mother by the more conventional fecal-oral route.

Genetically mediated resistance to helminths is seen in the superior resistance of sheep with hemoglobin A (HbA) to *Haemonchus contortus* and *Teladorsagia circumcincta* compared with sheep with hemoglobin B. The reasons for this are unclear, but sheep with HbA mount a more effective self-cure reaction and a better immune response to many other antigens as well. Another example is the enhanced resistance to *Cooperia oncophora* seen in Zebu cattle compared to European cattle. In many cases, resistance to parasites is linked to MHC polymorphisms. Thus cattle possessing BoLA-Aw7 and A36 tend to have low fecal egg counts, whereas animals with Aw3 tend to have high fecal egg counts. Some BoLA haplotypes may also be associated with high antibody levels against *Ostertagia.* The effects of the SLA complex on parasite immunity in pigs have also been assessed. Thus there was a 50% lower muscle larval burden in *Trichinella spiralis*–infected *cc* minipigs compared with pigs with the *dd* or *aa* haplotype. Resistance was characterized by a predominance of lymphocytes and macrophages in the cellular reaction around each larva. Polymorphisms in the IFN-γ gene affect the inflammatory response in sheep and their responses to helminths.

Innate factors that influence helminth infestations include not only host effects but also the influence of other parasites within the same host. For example, calves infected with *Cysticercus bovis* show increased resistance to further infestation by this parasite. Similarly, lambs can acquire resistance to *Echinococcus granulosus* so that multiple dosing with large numbers of ova does not result in the development of massive worm burdens. The original dose of ova may stimulate rejection of subsequent doses. Interspecies competition among helminths for mutual habitats and nutrients in the intestinal tract will also influence the numbers, location, and composition of an animal's helminth population.

Adaptive Immunity

Helminths present the immune system with a challenge. Most parasitic worms migrate through the tissues as larval forms and

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eventually reach the intestine or lungs where they develop into adults. Clearly, the mechanisms that destroy these migrating larvae in the tissues must be very different from those that expel large adult worms from the intestine or airways.

In general, larvae and adult worms within tissues trigger type 2 immune responses and as a result are attacked by eosinophils and basophils. Adult worms attached to mucosal surfaces are expelled by IgE and cytokine-mediated mechanisms. Type1, cell-mediated responses are relatively less important.

Because nematodes trigger type 2 immune responses, IgE levels and eosinophil numbers are usually elevated in parasitized animals. Additionally, many helminth infestations are associated with the characteristic signs of type I hypersensitivity, including eosinophilia, edema, asthma, and urticarial dermatitis. For example, pigs infested with *Ascaris suum* show cutaneous allergic reactions to injected parasite antigens, as well as degranulation of intestinal mucosal mast cells. Mammals eventually develop limited immunity to tissue helminths after several months. One parasite, *Ostertagia ostertagi*, is an exception. Cattle remain susceptible to reinfection by *Ostertagia* for many months, and immunity that can inhibit the production of viable larvae is not seen until an animal is more than 2 years old. It is not surprising that this is the most economically important bovine helminth parasite.

Immunity to Tissue Helminths

Larvae and some adult worms migrate through tissues where they are attacked by inflammatory cells and molecules. Unlike bacteria or protozoa, however, larval worms have a thick cuticle that protects their vulnerable hypodermal plasma membrane. As larvae develop and undergo periodic molts, damaged cuticles may be shed to be replaced with new ones. These cuticles cannot be penetrated by the terminal complement complex nor by T cell perforins. Thus eosinophils and macrophages are the key to destroying migrating larvae. Both possess FcεR (CD23) and can therefore bind and kill IgE-coated parasites. Chitinases can degrade helminth cuticles. Chitinases are produced by mast cells, macrophages, and neutrophils. Some mammalian chitinases may lack enzyme activity but can bind to helminth cuticles and serve as

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opsonins or chemoattractants.

If migrating worms cannot be killed, at least they can be walled off. M2 cells produce arginase. Arginase generates proline and polyamines. Proline and the polyamines are required for collagen synthesis and can induce fibroblast proliferation. The granulomas that develop around tissue helminths such as schistosomes or liver fluke are driven by arginase from M2 cells.

Eosinophils and Parasite Destruction

Invading worms attract eosinophils. This is provoked by mast cell or parasite-derived products, as well as by IL-5 and other cytokines from Th2 cells. IL-5 from Th2 cells and GM-CSF from Th17 cells mobilize bone marrow eosinophils, releasing large numbers into the circulation. Chemokines such as the eotaxins also attract eosinophils (Fig. 28.6). Eosinophils exposed to helminth antigens can increase their expression of MHC class II and become antigen-presenting cells. These antigen-presenting eosinophils are highly effective in promoting Th2 responses against worm antigens.

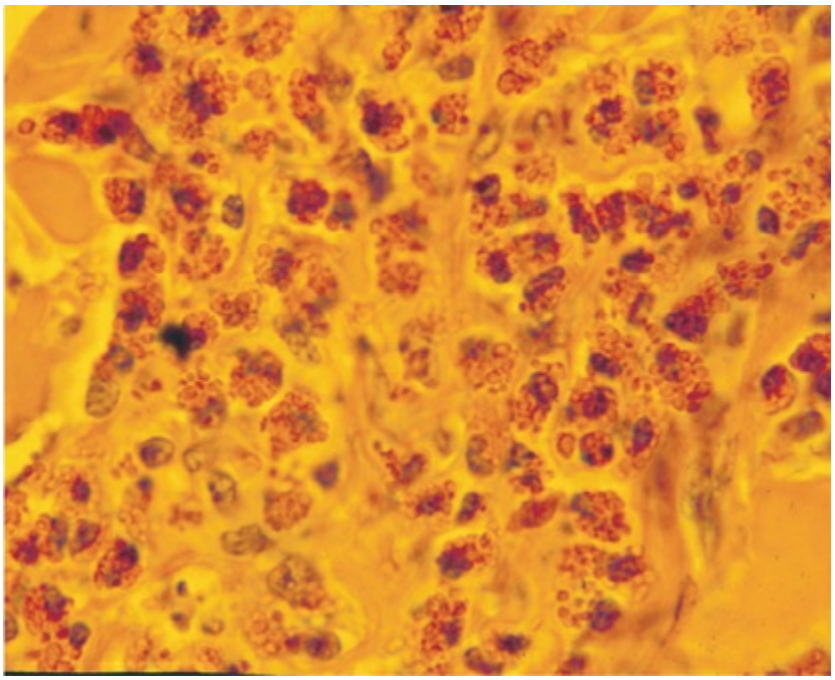


FIG. 28.6 Photomicrograph of a lesion in horse skin caused by allergy to migrating parasitic helminth larvae. The granular cells are eosinophils, and their presence indicates the occurrence of a type I hypersensitivity reaction.

Eosinophils express many different PRRs including TLR1-5, TLR7, TLR9, NOD1, NOD2, and dectin-1. When these are stimulated, they trigger an oxidative burst, increase adhesiveness, and release IL-1, IL-6, TNF-α, and GM-CSF, as well as their cytotoxic granule contents (see Fig. 29.18).

Eosinophils employ Fc receptors to bind antibody-coated worms, they then degranulate and release their granule contents directly onto the worm cuticle (Fig. 28.7). These contents include oxidants, nitric oxide, lysophospholipase, and phospholipase D. Major basic protein, the crystalline core of the eosinophil specific granules, can damage the cuticles of schistosomula, *Fasciola,* and *Trichinella.* Eosinophil cationic protein and eosinophil neurotoxin are ribonucleases that are lethal for helminths. It is important to point out, however, that some larval worms may evade destruction by eosinophils. For example, larvae of *Toxocara canis* simply shed their outer coat together with the attached cells.

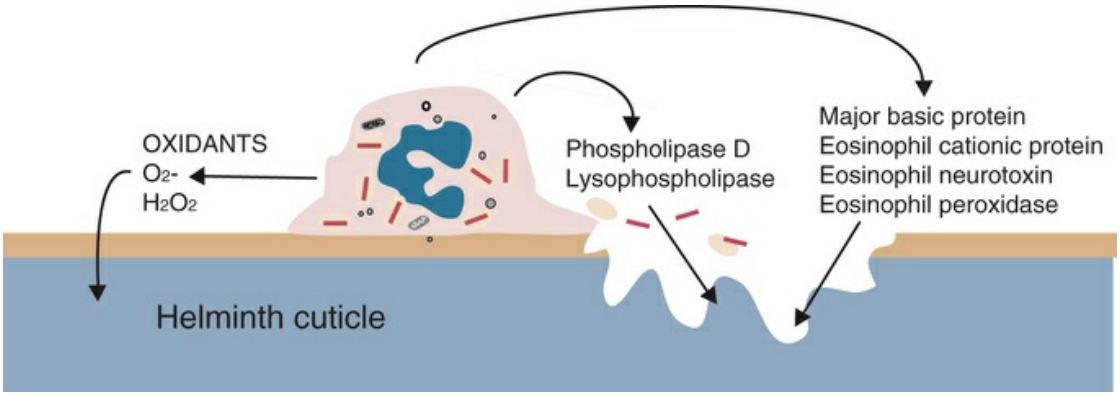


FIG. 28.7 Some of the molecules released from eosinophils that cause damage to the cuticle of parasitic helminths.

Eosinophil granules released from cells remain functional. These free granules express membrane receptors for IFN-γ and eotaxins, that when triggered, stimulate the granules to secrete their contents. Thus eosinophil granules function autonomously to contribute to the defenses against helminths. Eosinophils also generate extracellular NETs using released mitochondrial DNA.

Many nematodes are damaged or killed by eosinophil toxic products, supporting the idea that eosinophils serve a protective function. Nevertheless, this may not apply to all worms. For

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example, both *Teladorsagia circumcincta* and *Haemonchus contortus* produce chemoattractants for eosinophils, whereas the free-living nematode *Caenorhabditis elegans* does not. This suggests that some nematodes actively encourage eosinophil recruitment. Likewise, when *Trichinella spiralis* survival was studied in mice lacking eosinophils, it was found that the larvae in the muscles of these mice died in much larger numbers than in wild-type mice.

Although the IgE-dependent eosinophil-mediated response is the most significant mechanism of resistance to larval helminths, other antibodies also play a protective role. The mechanisms involved include antibody-mediated neutralization of larval proteases, blocking of the anal and oral pores of larvae by immune-complexes, and prevention of molting and inhibition of larval development by antibodies directed against exsheathing antigens. Antibodies to the enzyme glutathione-S-transferase protect sheep against *Fasciola hepatica.* Antibodies against adult worms may stop egg production or interfere with worm development (Fig. 28.8). Thus female *Ostertagia ostertagi* fail to develop vulvar flaps when grown in immune calves. Similarly, spicule morphology may be altered in *Cooperia* males from immune hosts.

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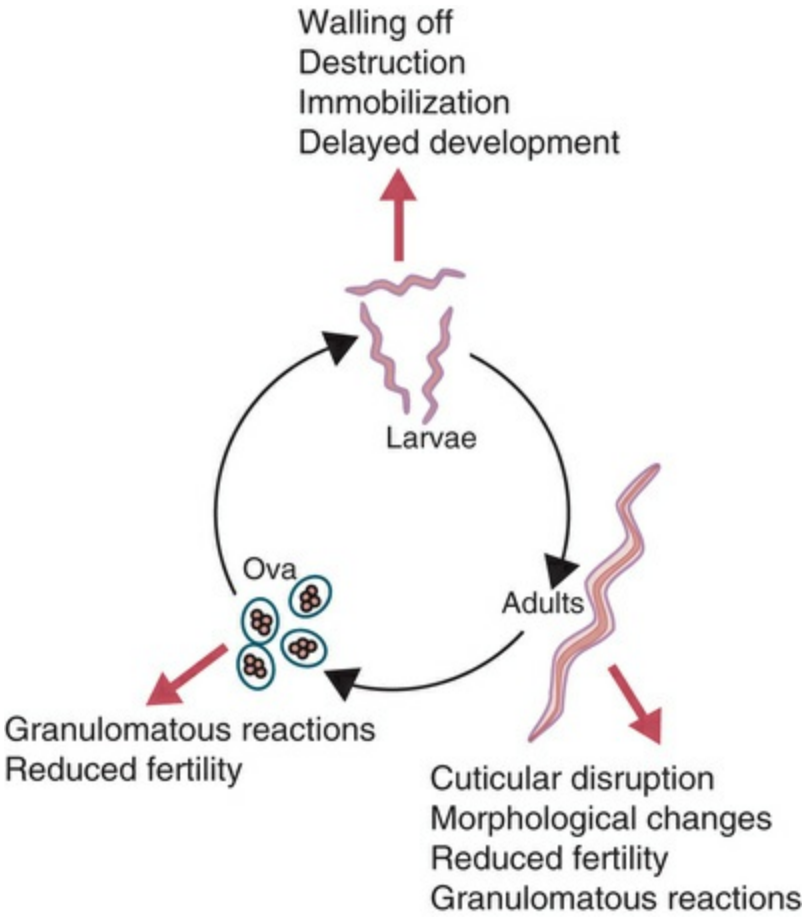


FIG. 28.8 Some effects of the immune responses on the stages of helminth development.

Immunity to Adult Helminths

The expulsion of adult worms from the intestine requires a coordinated response by multiple cell types under the control of T cell–derived cytokines. These cells collectively trigger a strong type 2 response that results in mast cell degranulation, acute inflammation, and ideally, expulsion of the parasites.

The presence of adult worms and their excretory and secretory products in the intestinal wall will trigger PRRs (Box 28.1). Not a lot is known about the role of PRRs in resistance to nematodes, but some helminth products do trigger TLRs and the mannose receptor is known to bind the excretory/secretory proteins of *Trichuris muris.* If the worms cause tissue damage, this will also trigger the release of alarmins including the three Th2-stimulating cytokines, TSLP, IL-25, and IL-33 from enterocytes. These cytokines target dendritic cells and ILC2s. For example, TSLP activates cDC2 dendritic cells.

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IL-25 stimulates the production of type 2 cytokines by ILC2 cells and facilitates differentiation of Th2 cells. IL-33 acts on Th2 cells, ILC2 cells, basophils, and mast cells to drive production of IL-4, IL-5, and IL-13. ILC2s are an important early source of IL-4, IL-13, and IL-5 (Box 28.2).

Box 28.1

Parasites, Microbiota and Host Immunity

Intestinal helminth parasites are members of the microbiota. Their presence alters the composition of the microbiota because the worms release metabolites and excretory/secretory products, and they stimulate their host's immune system. The composition of the bacterial microbiota also influences helminth colonization. Both helminths and the bacterial microbiota influence the Th17-Treg cell balance as well as Th2 responses. Alterations in the Th17-Treg cell balance, especially those that reduce intestinal inflammation, promote helminth survival. For example, premature initiation of Treg responses in *Trichuris muris* infections can inhibit protective immunity. Conversely, Treg depletion reduces the worm burden and enhances intestinal Th1 inflammatory responses. Tregs appear to limit Th2 cell expansion, especially early in infections.

Box 28.2

Tuft Cells

Among the less common cells that line the small intestine are “tuft cells”, so-called because they have a small tuft of microvilli that extends into the intestinal lumen. Until recently, their function was unknown. Now it has been shown that these cells play a key role in initiating type 2 immune responses to helminths. Tuft cells apparently can sense the “taste” of intestinal helminths. In response they produce large amounts of IL-25, a cytokine that recruits eosinophils, activates both Th2 cells and ILC2 cells, and causes them to secrete more IL-13 and IL-4 (Fig. 28.10). IL-13 and IL-4 from ILC2 cells stimulates further tuft cell proliferation and IL-

25 production. In the presence of parasitic helminths, the IL-25 promotes hyperplasia of tuft cells as well as mucus-producing goblet cells. The IL-13 acts together with IL-4 to increase gut motility and mucus production. The combination of increased motility with increased mucus production can expel many helminth parasites.

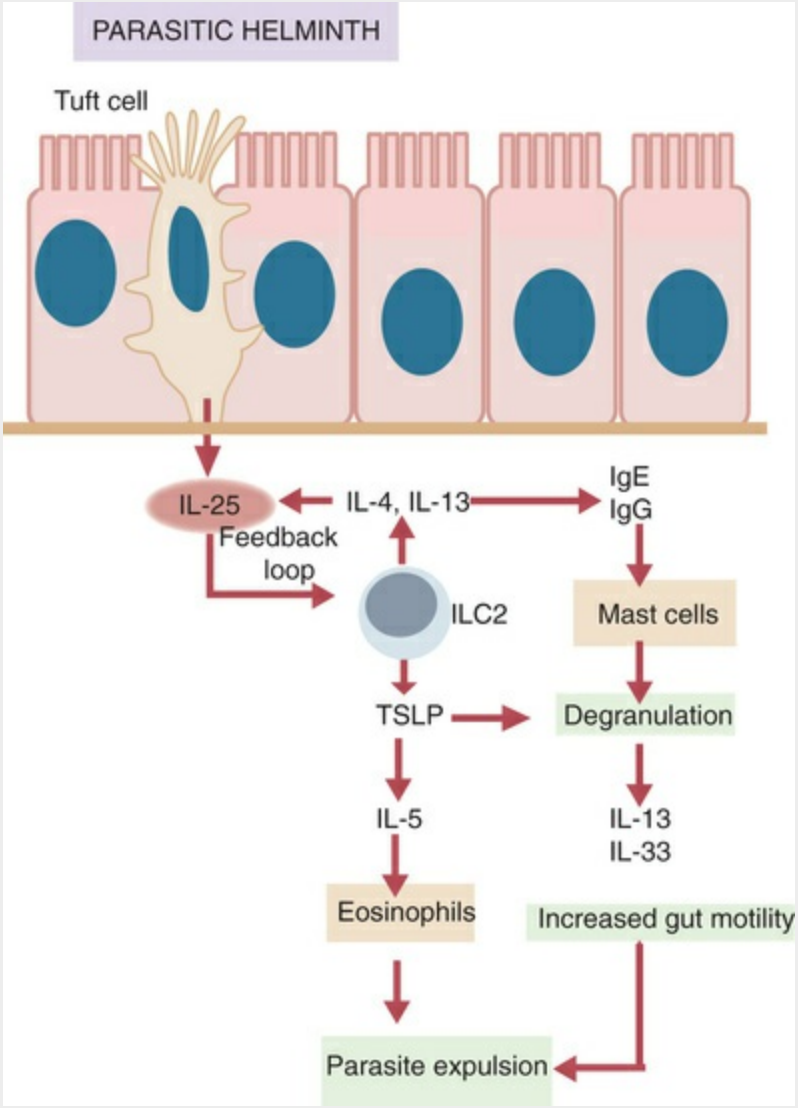


FIG. 28.10 The mechanisms by which tuft cells participate in parasite expulsion.

In response to these stimuli, T cells release their own effector cytokines. For example, γ/δ T cells in the intestinal epithelium produce IL-4 and IL-25. Likewise, Th2 cells produce more IL-4 in a

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positive feedback loop generating the other Th2 cytokines and IL-25. The IL-4 activates STAT6, which upregulates GATA3 that causes differentiation into Th2 cells and suppresses Th1 responses. IL-6 acts with TGF-β to induce Th17 responses. IL-13 repairs epithelia, enhances mucus production, and with IL-9 recruits and activates mucosal mast cells. Both IL-4 and IL-13 activate enterocytes, smooth muscle cells, and macrophages. Increased enterocyte proliferation and turnover may cause worm disengagement. These cytokines also increase intestinal permeability and fluid secretion. The Th2 cytokines stimulate Paneth cells and the consequent increase in defensins may damage helminths. These cytokines also stimulate goblet cells to produce a resistin-like molecule that interferes with worm feeding. Mucins and other goblet cell products may also promote worm expulsion.

While embedded in the intestinal and abomasal mucosa, worms secrete multiple antigenic proteins (Fig. 28.9) and these will stimulate production of both IgA and IgE. IgA is associated with resistance to *Teladorsagia circumcincta,* affecting both worm size and fecundity. IgE responses are probably more important than IgA since they play a key role in controlling adult worms on mucosal surfaces. This is most evident in the "self-cure" reaction in sheep infected with gastrointestinal nematodes, particularly *Haemonchus contortus*.

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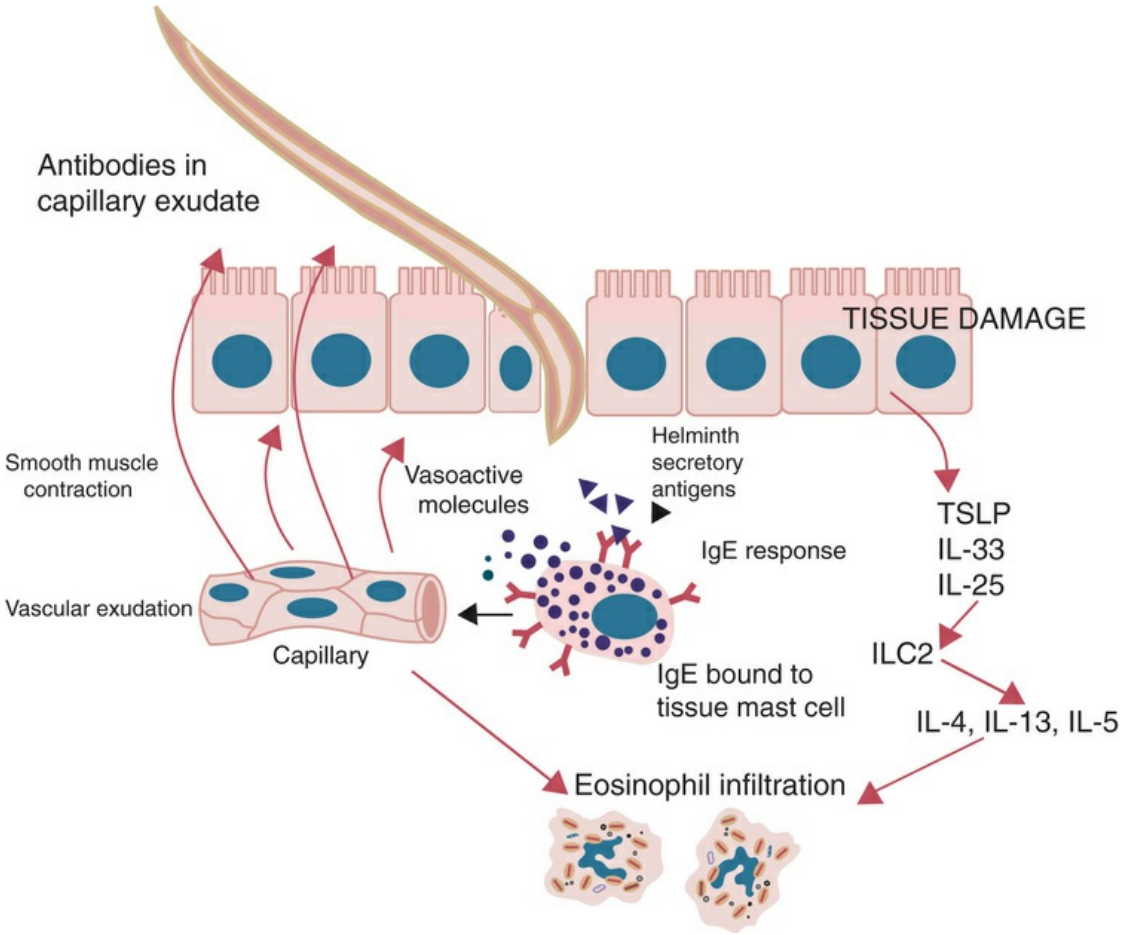


FIG. 28.9 The mechanisms involved in the self-cure reaction against intestinal helminths. In essence, the animal mounts an allergic response to the salivary antigens of attached nematodes. This acute inflammatory response causes the worms to detach from the intestinal wall and pass out in the feces.

Mast cells are also required for helminth expulsion. Mast cell numbers increase in the tissues during helminth infection. They can also be activated directly by PAMPs through PRRs, although it is unclear which parasite PAMPs do this. In response, these mast cells produce IL-4, -13, and -5, plus chemotactic factors. Mast cell proteases degrade tight junctions and allow fluid efflux into the intestine. Mast cell products may be directly toxic to helminths.

Basophils are also attracted to attached worms and are a major source of IL-4. Basophils can act as antigen-presenting cells in helminth *(Trichuris muris)* infections.

The combination of helminth antigens with mast cell–bound IgE triggers mast cell degranulation and the release of vasoactive amines such as histamine, cytokines such as IL-13 and IL-33, chitinases, and proteases. These molecules stimulate vigorous

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smooth muscle contraction and increase vascular permeability. The IL-13 promotes parasite expulsion by stimulating epithelial cell proliferation. Presumably the rapid epithelial cell turnover acts as an "epithelial elevator" to assist in expelling worms. IL-33 also induces the expulsion of adult worms. Tissue edema can inhibit helminth invasion. Increased permeability, epithelial cell proliferation, smooth muscle contraction, and mucus production all contribute to worm expulsion. This expulsion is accompanied by mucosal mast cell infiltration, intestinal eosinophilia, elevated serum IgE, and elevated parasite-specific IgG1 levels.

The violent contractions of the intestinal muscles caused by histamine and the increase in the permeability of intestinal capillaries cause an efflux of fluid into the intestinal lumen, resulting in dislodgment and expulsion of many worms. (This has been characterized as "weep and sweep.") In sheep that have just undergone "self-cure," IgE antibody levels are high, and experimental administration of helminth antigens will cause acute anaphylaxis (Chapter 30). A similar reaction is seen in fascioliasis in calves, where peak PCA antibody titers coincide with expulsion of the parasite.

Variations among worms.

Tissue helminths may be thought of as xenografts. That is, grafts between individuals of two different species. The intensity of the graft rejection process can vary between different hosts and between different worms. Inbred mouse strains differ in their ability to expel intestinal nematodes such as *T. muris.* Since inbred mice are genetically homogeneous, these variations in resistance to *T. muris* must be due to differences among the worms. We know that strains of worms differ in their ability to trigger Th1 and Th2 responses. This may be due to manipulation of the immune response by each worm. For example, *T. muris* can produce a protein related to IFN-γ that suppresses Th2 responses and so enhances worm survival. Alternatively, these differences may be due to parasite numbers. Thus low-level trickle infestations of *T. muris* provoke a Th1 response, and the parasites persist. If higher doses of parasites are administered, mice mount Th2 responses and expel the parasites. A threshold of infection is likely critical for the

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development of resistance.

Cell-Mediated Immunity

Live cysts of the tapeworm *Taenia solium* trigger a Th2 response and thus IgE production. However, after the cysts die, they stimulate a Th1 response and granuloma formation. Biopsies show IL-12, IL-2, and IFN-γ associated with the granulomas surrounding dying tapeworm cysts. It may be that the Th1 response occurs only when the parasite can no longer influence the host's immune response.

Sensitized T cells attack helminths by two mechanisms. First, mononuclear cells are attracted to the site of larval invasion and render the local environment unsuitable for growth or migration. Second, cytotoxic lymphocytes may kill the larvae. Thus treatment of experimental animals with the T cell stimulant, bacille Calmette-Guérin (BCG) vaccine (Chapter 41), inhibits the metastases of hydatid cysts *(Echinococcus granulosus).* In tapeworm infestations in which the parasite cyst (metacestode) grows within the host, the parasite must obtain protein for nourishment. However, the cysticerci of *Taenia ovis* actually grow larger in the presence of immune serum than nonimmune serum. The parasites possess Fc receptors, and it is possible that host immunoglobulins may feed the parasite. Since cyst fluid contains lymphocyte mitogens, it has also been suggested that these might stimulate productions of immunoglobulins that may then be used by the parasite.

The complexity of resistance to helminths is well demonstrated in sheep bred for resistance to *Haemonchus contortus.* Compared with susceptible sheep, there are differences in B cell function; resistant sheep have significantly more IgA- and IgG1-containing cells. There is also evidence for differences in T cell function because resistant sheep respond better to a T-dependent antigens, and treatment of resistant lambs with a monoclonal antibody to CD4 completely blocks their resistance to *H. contortus.* Mucosal mast cell numbers and tissue eosinophilia are also reduced in these treated sheep. In contrast, depletion of $CD8^+$ cells has no effect on resistance.

Evasion of the Immune Response

Although there are multiple mechanisms whereby animals resist

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helminth infection, it is obvious, even to a casual observer, that these defenses are not very effective. Successful parasitic helminths survive and function in the presence of a fully functional host immune system. In general, helminths are most vulnerable to immunological attack while migrating through tissues. Thus most evasive strategies work at the larval stage.

Evasion of Innate Responses

Brugia malayi secretes serpins that inhibit neutrophil serine proteases. *Echinococcus granulosus* secretes an elastase inhibitor that blocks neutrophil attraction by C5a or platelet-activating factor (PAF). Many helminths express surface antioxidants such as superoxide dismutase, glutathione peroxidase, and glutathione S-transferase, that neutralize the host's respiratory burst and protect their surfaces from oxidation (Fig. 28.11). A secreted antioxidant from *Fasciola hepatica* called peroxiredoxin causes alternative activation of bovine macrophages resulting in high arginase activity, low nitric oxide, low IFN-γ, and high IL-10 production. This, plus elevated IL-4, IL-5, and IL-13 production, promotes Th2 responses.

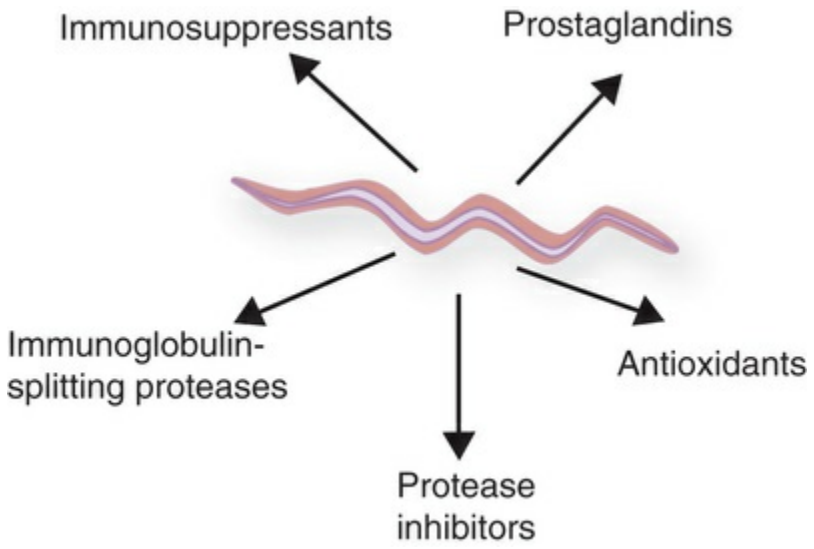


FIG. 28.11 Some methods by which migrating helminth larvae evade host defenses.

Many parasites interfere with the complement system. For

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example, schistosomes can neutralize the alternative complement pathway by inserting decay accelerating factor (CD55) from their host into their outer lipid bilayer. Tapeworms can secrete sulfated proteoglycans, which activate complement in the tissue fluid. Parasites such as *Necator americanus* and *H. contortus* secrete calreticulin homologs that bind C1q and block its activities.

Evasion of Adaptive Responses

Helminths become progressively less antigenic as they evolve in the presence of a functioning immune system. Presumably, natural selection favors the survival of parasites with reduced antigenicity. *H. contortus* is much less antigenic in sheep, its natural host, than in rabbits, which it does not normally infect. Sheep therefore respond to fewer *H. contortus* antigens than do rabbits.

Helminths living within tissues may reduce their antigenicity by adsorbing host antigens onto their surface and masking parasite antigens. This occurs in *Taenia solium* infestations in swine, where the parasites are coated with IgG. Cysticerci can also adsorb MHC molecules to their surface.

Another mechanism of immune evasion is the use of sequential antigenic variation. Although helminths have not evolved a system as complex as that in trypanosomiasis, gradual antigenic variation is recognized. The cuticular antigens of *Trichinella spiralis* larvae change after each molt. Even during their growth phase, these larvae change the expression of surface antigens. Some parasites such as *Fasciola hepatica* shed their glycocalyx and hence their surface antigens when exposed to antibodies.

Some worms interfere with antigen processing. Macrophages from schistosome-infested animals are incompetent antigen-presenting cells. Filarial worms secrete inhibitors that block macrophage proteases. *Taenia taeniaeformis* secretes taeniastatin, a protease inhibitor that inhibits neutrophil chemotaxis, complement activation, T cell proliferation, and IL-2 production.

Immunosuppression is a consistent feature of parasitized animals. This may be due to the production of immunosuppressive molecules or to redirection of immune responses toward Treg cell production and tolerance. *F. hepatica* secretes proteases that destroy

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immunoglobulins. These proteases generate Fab fragments that bind and mask parasite antigens. In addition, *F. hepatica* tegumental protein suppresses production of IFN-γ and IL-12 by acting directly on dendritic cells and possibly suppressing signaling by NF-κB.

Many helminths suppress host immunity by promoting the production of Treg cells and IL-10. Because *F. hepatica* infestation is such a strong inducer of Th2 responses, it can adversely affect an animal's ability to mount Th1 responses and interfere with diagnostic tests such as the whole blood IFN-γ assay used to diagnose bovine tuberculosis (Chapter 33). Sheep infected with *H. contortus* may become specifically suppressed so that they are unreactive to *H. contortus,* even though they remain responsive to unrelated parasites. *Ostertagia ostertagi, Oesophagostomum radiatum,* and *Trichostrongylus axei* infestations depress calf lymphocyte responses to mitogens. In other helminth infections, such as trichinosis, infected animals are nonspecifically immunosuppressed. This immunosuppression is reflected in a lowered resistance to other infections, a poor response to vaccines, and prolongation of skin graft survival.

Parasitic helminths produce a family of immunomodulatory peptides called helminth defense molecules that resemble mammalian cathelicidins. One of these molecules produced by *F. hepatica* can be endocytosed by host macrophages in which it prevents endosomal maturation, impedes antigen presentation, and inhibits antigen carriage to the cell surface in conjunction with MHC class II molecules

Vaccination

It is not surprising, considering the nature of the host response to parasitic worms and the availability of cheap and effective anthelmintics, that vaccines against helminth parasites are not widely available. Nevertheless, the emergence of anthelmintic resistance and environmental concerns raised by excessive chemical use have resulted in an increased interest in anti-parasite vaccines. Vaccine use is predicated on the assumption that a host's immune response can control or prevent an infestation. This is not always obvious in helminth infestations, and traditional vaccines may be of

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little use. Despite this, a recombinant *T. ovis* vaccine has been produced that can induce protective immunity in sheep. This vaccine contains a cloned oncosphere antigen (To45W) with a saponin-based adjuvant. It stimulates a response that prevents parasite penetration of the intestinal wall. Similar single-antigen recombinant vaccines are highly effective against *E. granulosus* in sheep and cattle.

Protection against some helminths has been obtained by the use of live irradiated organisms. The most important of these is the vaccine used to protect calves against pneumonia caused by the lungworm *Dictyocaulus viviparus*. In this vaccine, second-stage larvae hatched from ova in culture are exposed to 40,000 R X-irradiation, and two doses of these larvae are then fed to calves. The larvae can penetrate the calf's intestine, but since they are unable to develop to the third stage, they never reach the lung and are thus nonpathogenic. During their exsheathing process, the larvae stimulate the production of antibodies that can block reinfection. The efficiency of this vaccine depends very much on timing and on the size of the challenge dose since even vaccinated calves may show mild pneumonic signs if placed on grossly infected pastures.

The major helminth antigens are of two types, soluble excretory-secretory products and antigens bound to the parasite surface (somatic antigens). Important secretory products include periredoxin, annexins, galectins, heat-shock proteins, HMGB homologs, and S100 homologs. The immunodominant antigens of nematodes are polyprotein allergens and antigens that act as lipid-binding proteins. Another important somatic antigen is the enzyme γ-glutamyl transpeptidase. Some somatic antigens, such as those in the parasite gut, are hidden since they are not normally exposed to the host's immune response and may therefore be potential candidates for vaccines. For example, vaccination of lambs and kids against the intestinal aminopeptidase of *H. contortus* (called H11) has resulted in the development of antibodies against *Hemonchus* intestinal proteases. These presumably interfere with worm digestion and reduce both parasite numbers and fecundity. Because these molecules do not enter the tissues they are not normally recognized by the sheep immune system. As a result, up to five doses of this vaccine are required for optimal protection. This

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product is now commercially available.

Cattle mount protective responses against *Fasciola* infections. These are especially effective against single high doses but are less efficient against low-level trickle infections of the type likely to be encountered in the field. Animals may be protected against fascioliasis by the use of defined parasite antigens, such as fatty acid-binding protein, glutathione S-transferase, recombinant cathepsin L, and liver fluke hemoglobin.

In general, helminth vaccines are not yet widely employed. There has been reluctance on the part of farmers to change established control procedures, especially when the major financial burden of these infestations is often borne by others.

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Immunity to Arthropods

When arthropods such as ticks, fleas, or mosquitos bite an animal, they inject saliva. This saliva contains digestive enzymes that assist the parasite in obtaining its blood meal. The saliva also contains components designed to minimize host responses (Fig. 28.12).

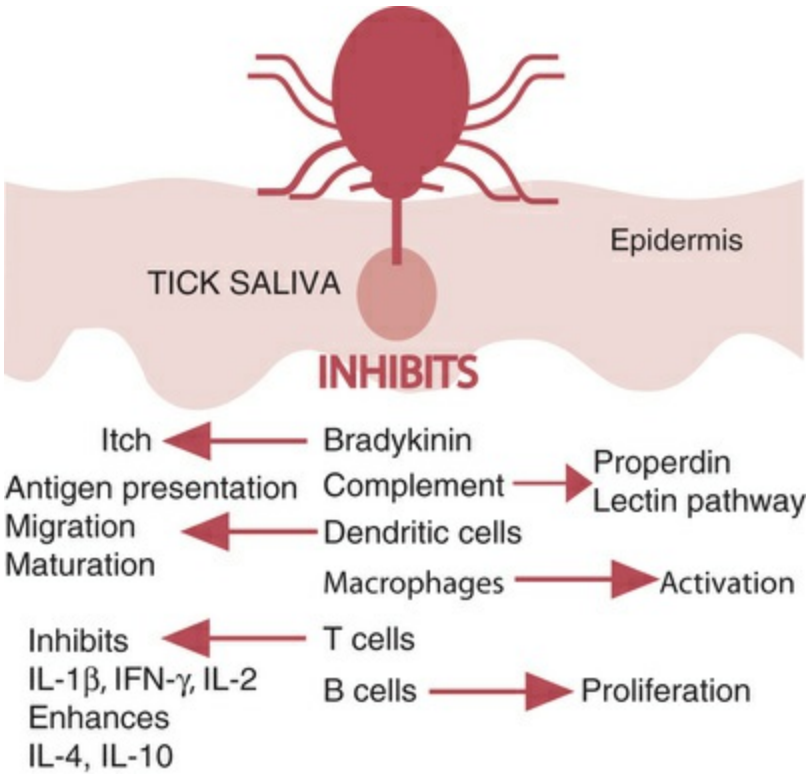


FIG. 28.12 Immunosuppressive and antiinflammatory factors found in tick saliva. Collectively they act to permit prolonged tick attachment and feeding.

Host immune responses to injected arthropod saliva are of three types. Some salivary components are of low molecular weight and cannot function as normal antigens. They may, however, bind to skin proteins such as collagen and then act as haptens, stimulating Th1 responses. On subsequent exposure, these responses may induce a delayed hypersensitivity reaction. Other salivary antigens may bind to epidermal Langerhans cells and induce cutaneous basophil hypersensitivity, a Th1 response associated with the production of IgG antibodies and a basophil infiltration. If these

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basophils are destroyed by antibasophil serum, resistance to biting arthropods is reduced. The third type of response to arthropod saliva is a Th2 response, leading to eosinophil infiltration, IgE production, and type I hypersensitivity. This response may induce severe local inflammation, leading to urticaria, pain, or pruritus. Each of these three types of response may modify the skin in such a way that the feeding of the offending arthropod is impaired and the animal becomes a less attractive source of food. Unfortunately, natural selection and evolution ensure that the biting arthropod is well able to withstand such responses. (These hypersensitivities are discussed further in Chapter 30.)

Demodectic Mange

The mange mites *Demodex folliculorum, D. canis,* and *D. injai* are common in mammalian hair follicles and sebaceous glands. Normally present in low numbers, generalized demodicosis results from mite overgrowth. Under normal circumstances, an innate immune response to mite chitin and an adaptive response to mite antigens maintain mite populations at a low level. The development of generalized demodicosis results from defects in host immunity and has a strong genetic component. The inflammatory reaction around mites and mite fragments contains mononuclear cells with a few plasma cells. The infiltrating lymphocytes are predominantly $CD8^+$ T cells. Type II granuloma formation may occasionally occur. The presence of cytotoxic T cells suggests that this is a type IV hypersensitivity reaction. Dogs do, however, make antibodies to mite proteins. The absence of eosinophils and edema in the lesion suggests that type I hypersensitivity is relatively unimportant. It is of interest to note that immunosuppressive agents such as antilymphocyte serum, azathioprine, or prolonged steroid therapy predispose animals to the development of demodectic mange. Animals with generalized demodicosis have normal neutrophil function and respond normally to vaccines or other foreign proteins. Nevertheless, their T cell response to mitogens such as phytohemagglutinin and concanavalin A is depressed. This is a progressive suppression, and it tends to increase in severe cases. It has been suggested that it reflects T cell "exhaustion." If, however,

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the T cells of dogs with demodicosis are washed free of serum, they regain their ability to respond to mitogens. Serum from these animals is also able to suppress T cell proliferation in normal animals.

Flea-Bite Dermatitis

Biting fleas secrete saliva into the skin wound. Some of the components of flea saliva are of low molecular weight and act as haptens after binding to dermal collagen As a result, a local type IV hypersensitivity reaction characterized by a mononuclear cell infiltration occurs. In some sensitized animals, this type IV reaction is gradually replaced over a period of months by a type I reaction, and the mononuclear cell infiltration gradually changes to an eosinophil infiltration. The immune response mounted by flea-allergic animals is protective. Thus fleas produce fewer eggs on flea-allergic cats than on flea-naïve cats. Flea-allergic cats also appear to remove more fleas by grooming than do flea-naïve animals. Experimental vaccines containing the major antigens from the cat flea midgut have been able to reduce flea populations on dogs, and the female fleas recovered from these immunized animals produced significantly fewer eggs. This suggests that vaccination may eventually be effective in controlling flea populations.

Tick Infestation

Ticks are unique among arthropods in the way they attach and feed from their hosts for several days. This provides time for immune responses to occur and for ticks to employ countermeasures. For example, their saliva contains kininases that destroy bradykinin and histamine-binding proteins, thus reducing pain and itch. The saliva of the tick *Ixodes scapularis* contains a protein that regulates the alternative complement pathway. It displaces properdin and enhances the degradation of C3bBb convertase. Tick saliva also contains an inhibitor of the complement lectin pathway. As a result, host scratching and grooming responses are minimized.

The saliva of *Ixodes ricinus* impairs the antigen-presenting abilities

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of dendritic cells. The salivary protein sialostatin-L2 inhibits inflammasome activation. Tick saliva also inhibits the migration of dendritic cells from inflamed skin to draining lymph nodes and decreases the ability of these dendritic cells to present antigen to T cells. Furthermore, the treated dendritic cells preferentially stimulate Th2 responses. All these effects serve to facilitate the prolonged attachment and feeding of ticks.

Because tick salivary proteins are antigenic, they would be expected to induce immune responses that impair a tick's ability to feed. Ticks, however, have evolved immunosuppressive and antiinflammatory countermeasures that permit them to feed more effectively. Tick saliva impairs macrophage function and suppresses T cell responses to mitogens, as well as production of IL-1β and the Th1 cytokines IFN-γ and IL-2. It suppresses NK cell activity and macrophage nitric oxide production. Saliva from the ticks *Dermacentor andersoni* and *I. ricinus* increases production of the Th2 cytokines IL-4 and IL-10. A tick saliva immunosuppressor binds specifically to T cell CD4 and blocks antigen-induced signaling and T cell responses. Saliva from *I. ricinus* also inhibits host B cell proliferation. Additionally, a serpin from *I. ricinus* interferes with the IL-6 pathway and consequently inhibits Th17 cell differentiation. A salivary protein from *I. scapularis* inhibits the proliferation of B cells exposed to the Osp proteins from the Lyme disease bacterium *Borrelia burgdorferi* but has no effect on T cells. It is abundantly clear that ticks have evolved many mechanisms to prevent immunological attack while they are feeding.

Thus tick saliva contains components that suppress pain and itch, hemostasis, inflammation, adaptive immunity, and wound healing. Nevertheless, it has been observed that ticks on nonimmune animals are larger than those on immune animals. Although the nature of this resistance is unclear, it has been suggested that local hypersensitivity reactions to tick saliva may restrict the blood flow to the tick, reduce its food supply, and stunt its growth. It is possible to immunize guinea pigs with tick homogenates and show that ticks feeding on these animals have reduced fecundity. Although vaccination against salivary antigens is unlikely to be very effective in conferring effective immunity against blood feeding arthropods, there is an alternative approach. Since many of

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the arthropods of veterinary importance take the blood of their host into their digestive tract, it follows that they will also take up immunoglobulins, complement components, and cells. This suggests that if an animal were immunized with internal antigens from the tick, this could cause local damage. These internal antigens have been called "hidden" or "concealed" antigens since under normal circumstances the host would not usually encounter them. Vaccines made against antigens from the intestine of the tick *Boophilus microplus* can inhibit tick reproduction. Indeed, a recombinant tick vaccine based on such a recombinant antigen, Bm86, is available in Australia and Central America. The antibodies produced bind to the brush border of tick intestinal cells, inhibit endocytosis, and prevent the tick from engorging fully. Thus the digestive processes are impaired, and the tick experiences starvation, loss of fecundity, and weakness, and may disengage from its host. As a result, the number of ticks on vaccinated animals is reduced. Experimental multicomponent tick vaccines show even more encouraging results.

Hypoderma Infestation

Unlike the arthropods described previously, the larvae of the warble flies *(Hypoderma bovis* and *Hypoderma lineatum)* actually migrate through body tissues. These larvae must survive or evade the host's xenograft response. In fact, the first instar larvae of these flies do not trigger significant inflammation and are also immunosuppressive. Hypodermin A, the protease secreted by these larvae, can inhibit responses to mitogens and reduce IL-2 production, probably by destroying cell surface receptors. Vaccination with a cloned *Hypoderma* protein has effectively protected cattle against subsequent infestations.

Immune defenses also play a role in preventing invasion by other skin-penetrating arthropods. Body strike results when the larvae of the fly *Lucilia cuprina* attack the skin of a sheep. Sheep can be bred for low and high resistance to body strike. The resistant sheep have greater numbers of IgE-positive B cells in their skin than do susceptible sheep. Resistant sheep also mount a greater inflammatory response and produce more fluid exudate when

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injected with larval excretory and secretory products. On the other hand, larval proteases inhibit complement activation and degrade immunoglobulins.

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29

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Mast Cell and Eosinophil-Mediated Hypersensitivity

CHAPTER OUTLINE

Induction of Type I Hypersensitivity, 324
Immunoglobulin E, 325
- Immunoglobulin E Production, 325
- Immunoglobulin E Receptors, 325

Mast Cells, 326
- Structure and Location, 327
- Life History, 327

Response of Mast Cells to Antigen, 328
- Mast Cell–Derived Mediators, 329
- Interleukin-33, 329
- Regulation of Mast Cell Degranulation, 330
- Regulation of the Response to Mast Cell Mediators, 331
- Mast Cells in Infections, 331
- Late-Phase Reaction, 331

Basophils, 331
Eosinophils, 332

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Eosinophil Activation, 332
Eosinophil Degranulation and Mediators, 332

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Describe the structure, location and properties of mast cells, eosinophils, and basophils.
- Explain how type I hypersensitivities result from type 2 immune responses mediated by immunoglobulin E (IgE).
- Describe how mast cells respond to the binding of antigen to cell bound IgE.
- Describe the structure and properties of IgE.
- Discuss how allergic disease is caused by the release of inflammatory molecules from mast cells, eosinophils, and basophils.
- List the major inflammatory molecules released by each of these cells.
- Describe the ways in which mast cell responses are regulated.
- Understand how these responses probably evolved as a specialized defensive response against parasitic helminths and arthropods.
- Describe the pathways involved in the mobilization of eosinophils.
- Explain how these types of response are affected by the composition of the intestinal microbiota.

Pathologic lesions triggered by immune responses are called hypersensitivities. They are classified into four major types based on their pathogenic mechanisms. Type I hypersensitivity reactions are inflammatory responses that result from the release of the contents of mast cell, basophil, and eosinophil cytoplasmic granules. This granule release is triggered when antigens bind to immunoglobulin E (IgE) on the surface of mast cells or basophils. These granules contain a mixture of molecules that trigger inflammation and attract eosinophils (Fig. 29.1). This inflammation

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plays an important role in resistance to parasitic helminths and arthropods. It is also, however, a cause of unwanted inflammatory disease and thus of major significance in veterinary medicine. IgE production is characteristic of type 2 responses. These responses are triggered by the production of interleukin-4 (IL-4) and other cytokines from Th2 cells.

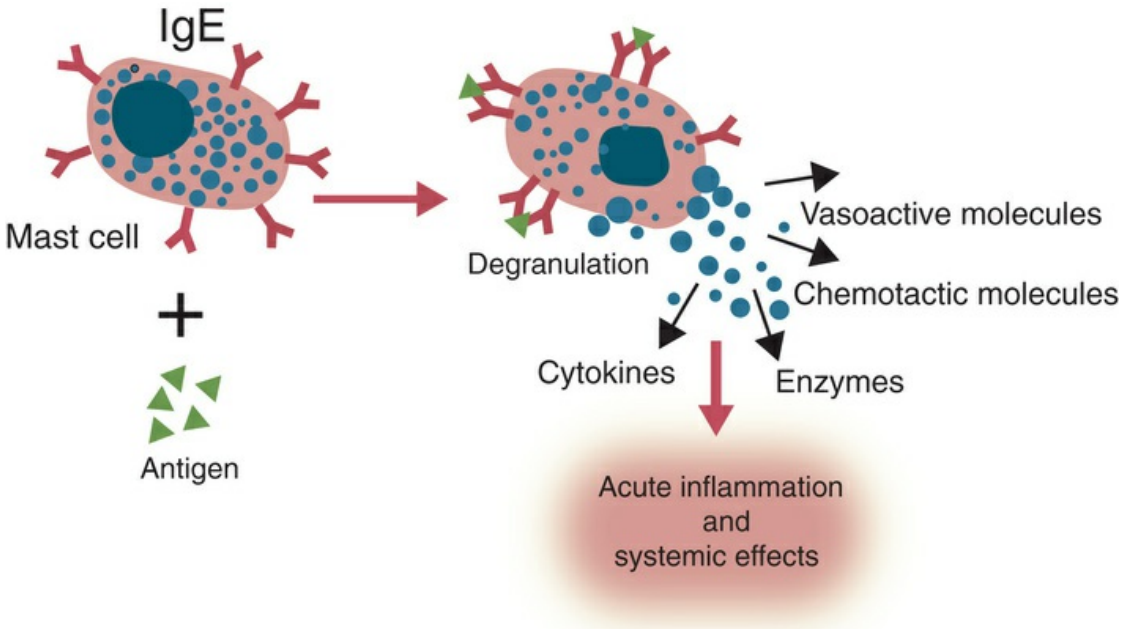


FIG. 29.1 The mechanism of type I hypersensitivity reactions. Numerous biologically active molecules and inflammatory mediators are released by mast cells when antigen cross-links two IgE molecules on the mast cell surface. Some are produced immediately. Others may be synthesized within minutes or hours.

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Induction of Type I Hypersensitivity

Animals are continuously exposed to environmental antigens in food, through skin contact, and in inhaled air. Should they penetrate the body, most individuals will respond to these antigens by producing IgG or IgA antibodies, and there are no obvious clinical consequences. Some animals, however, respond by mounting an exaggerated Th2 response and so produce excessive IgE. It is these animals that develop type I hypersensitivity reactions or allergies. The excessive production of IgE is called atopy, and affected individuals are said to be atopic. The development of atopy and type I hypersensitivity depends on the interaction between genes and environmental factors, especially the intestinal microbiota. The genetics of atopy and allergy are complex. If both parents are atopic, most of their offspring will also be atopic and will suffer from allergies. If only one parent is atopic, the percentage of atopic offspring varies. There is also a breed predisposition to atopy in dogs. For example, atopic dermatitis is most commonly observed in Terriers (Bull, Welsh, Cairn, West Highland White, Scottish), Dalmatians, and Irish Setters, although non-purebred dogs may also be affected. The heritability of atopic dermatitis in Labrador and Golden Retrievers is estimated to be a relatively high 0.47. In horses, high levels of IgE are associated with certain ELA-DRB haplotypes.

Normal animals infested by parasitic worms and insects also produce IgE and it is believed that the IgE response may have evolved specifically to counteract these organisms. Chitin, the biopolymer that confers structural rigidity to fungi, insects, and parasitic helminths, induces the accumulation of eosinophils and basophils in tissues and may be a key trigger of some allergic reactions. Indeed, the “self-cure” reaction seen in parasitized sheep has long been the only recognized benefit of type I hypersensitivity. It is of interest to note that both atopic and parasitized dogs may have reduced IgA levels, an observation supporting the concept that a deficiency of IgA may predispose to a compensatory increase in IgE (Chapter 22).

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Immunoglobulin E

IgE is an immunoglobulin of conventional four-chain structure and about 200 kDa (see Fig. 16.7). It is found in serum in exquisitely small quantities (9 to 700 μg/mL in dogs), and its serum half-life is only 2 days. Most IgE is firmly bound to Fcε receptors on tissue mast cells, where it has a half-life of 11 to 12 days. Some connective tissue mast cells situated close to blood vessels can extend cytoplasmic processes between the endothelial cells and into the vascular lumen to "fish" for IgE that binds to their receptors.

Some IgG subclasses may also bind to mast cell FcR and mediate type I hypersensitivity reactions. For example, IgG4 is associated with some cases of canine atopic dermatitis. However, the affinity of these subclasses for Fc receptors is much lower than that of IgE, and they are of much less clinical significance.

Immunoglobulin E Production

Atopic individuals are predisposed to mount type 2 immune responses. Their Th2 cells produce IL-4, IL-5, and IL-13. These, together with co-stimulation by CD40, trigger B cell IgE synthesis. IL-4 is also produced in significant amounts by degranulating mast cells. This mast cell–derived IL-4 may alter the helper cell balance and enhance yet more Th2 cell production and IL-4 release (Fig. 29.2).

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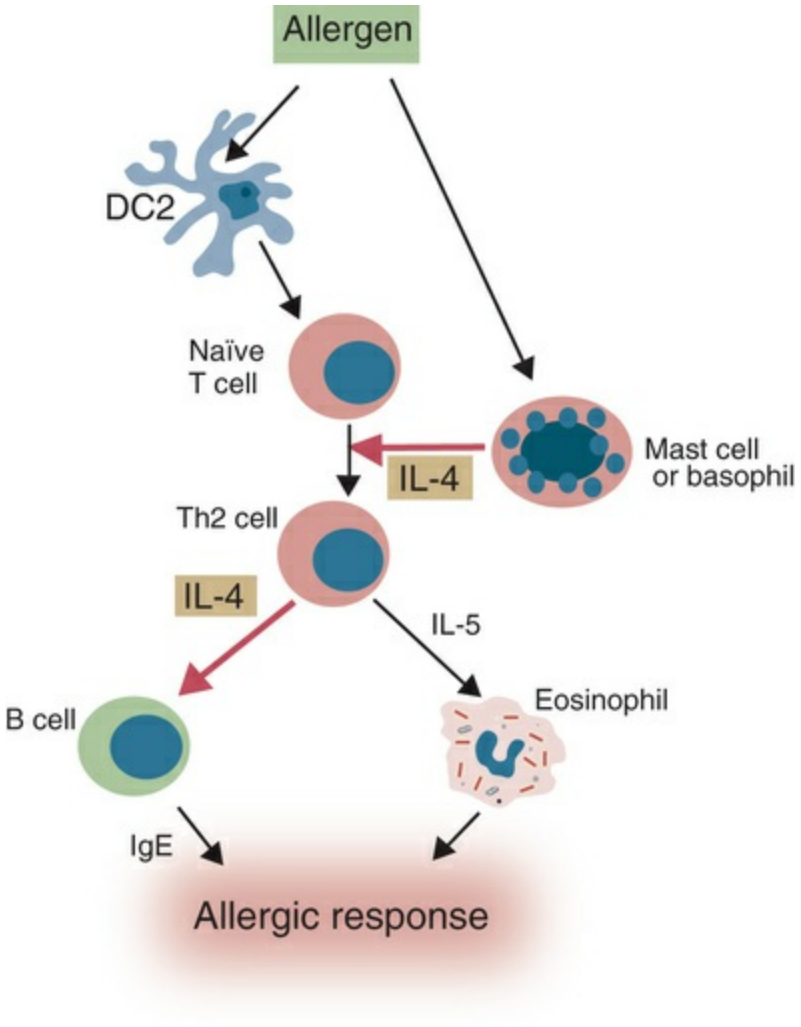


FIG. 29.2 The role of IL-4 in induction of IgE responses. IL-4 is produced by Th2 cells. Once released, it promotes the development of more Th2 cells, which are major sources of this cytokine and promote IgE responses. The degranulation of mast cells also releases IL-4, which further promotes this reaction. NK cells may serve as an initial source of IL-4. The response to IL-4 is inhibited by IFN-γ and IL-12.

Immunoglobulin E Receptors

Animals have two IgE receptors: high-affinity FcεRI and low-affinity FcεRII (CD23). FcεRI occurs in two forms. One form is expressed on mast cells, basophils, neutrophils, and eosinophils. It consists of four peptide chains, one α, one β, and two γ chains ($\alpha\beta\gamma\gamma$) (Fig. 29.3). The α chain binds IgE, the β chain stabilizes the complex, and the γ chains serve as signal transducers. (This same γ chain is also a signal transducer in FcγRI, FcγRIII, and γ/δ TCR.)

The affinity of FcεRI for IgE is very high (10^{-10} M), so they bind almost irreversibly and thus ensure that mast cells are constantly coated with IgE.

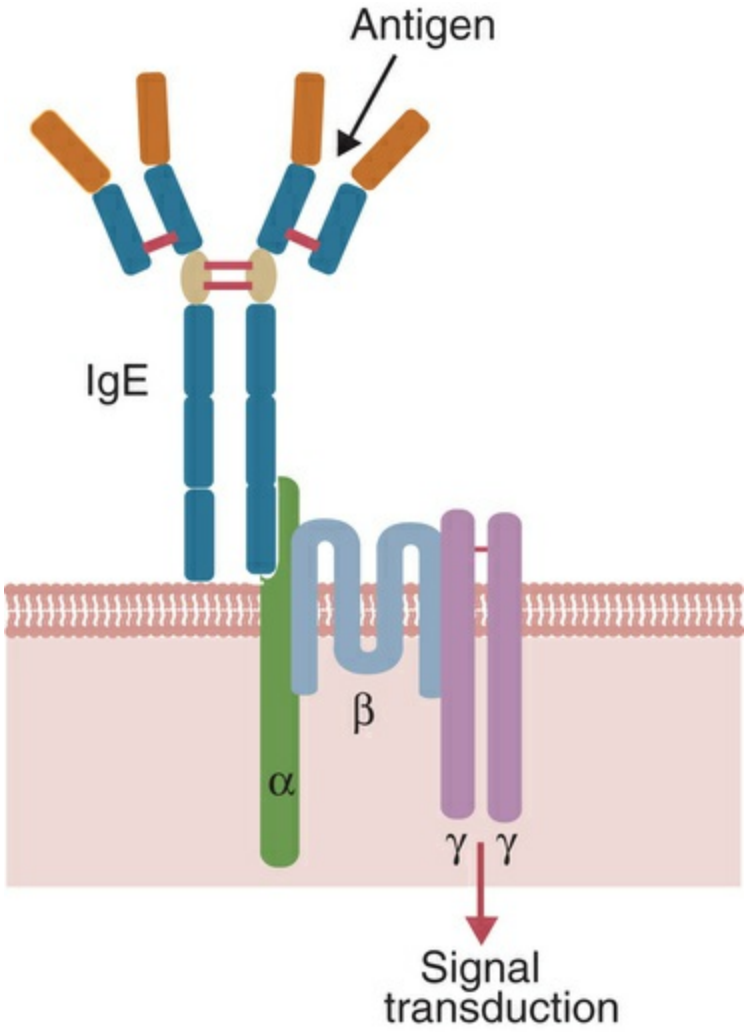


FIG. 29.3 The structure of FcεRI. This tetrameric form of the IgE receptor containing two γ chains is found on mast cells and basophils.

The other form of FcεRI contains only three chains: one α chain and two γ chains ($\alpha\gamma\gamma$). It is expressed on antigen-presenting dendritic cells and monocytes. When an antigen-IgE complex binds to this receptor, it is ingested and treated as exogenous antigen. The expression of FcεRI on antigen-presenting cells is enhanced by IL-4 from Th2 cells. Thus a positive feedback loop (the allergy loop) develops (Fig. 29.4). The antigen processing cells present antigen more effectively to Th2 cells. The Th2 cells then secrete IL-4 and

enhance IgE production.

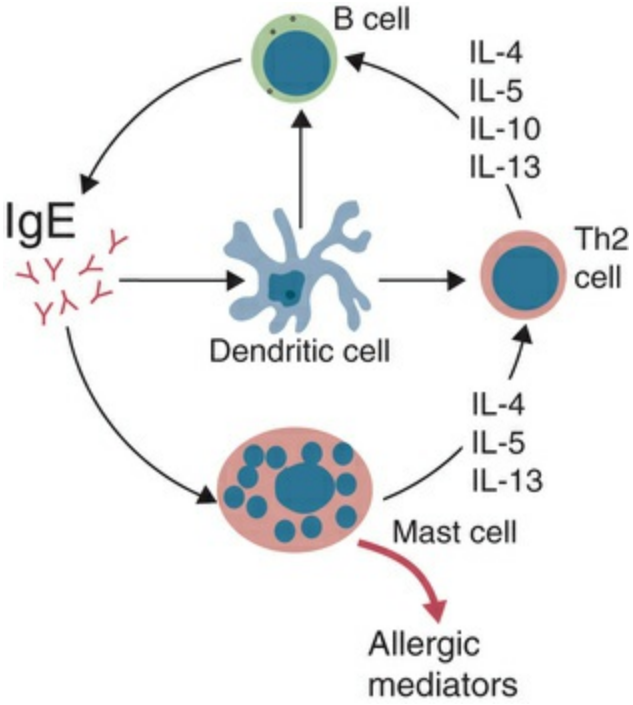


FIG. 29.4 The allergy loop. Dendritic cells express FcεRI and as a result can bind antigen-IgE complexes. This antigen, once processed, stimulates Th2 responses. These Th2 cells in turn secrete cytokines, which further promote the IgE response.

FcεRII (CD23), in contrast, is a selectin found on B cells, NK cells, macrophages, dendritic cells, eosinophils, and platelets. In addition to binding IgE, FcεRII also binds to the complement receptor CR2 (CD21) (Fig. 29.5). Thus B cells expressing FcεRII link to CR2 on other B cells, T cells, and dendritic cells. By linking B cells to dendritic cells in this way, FcεRII enhances B cell survival and regulates IgE production.

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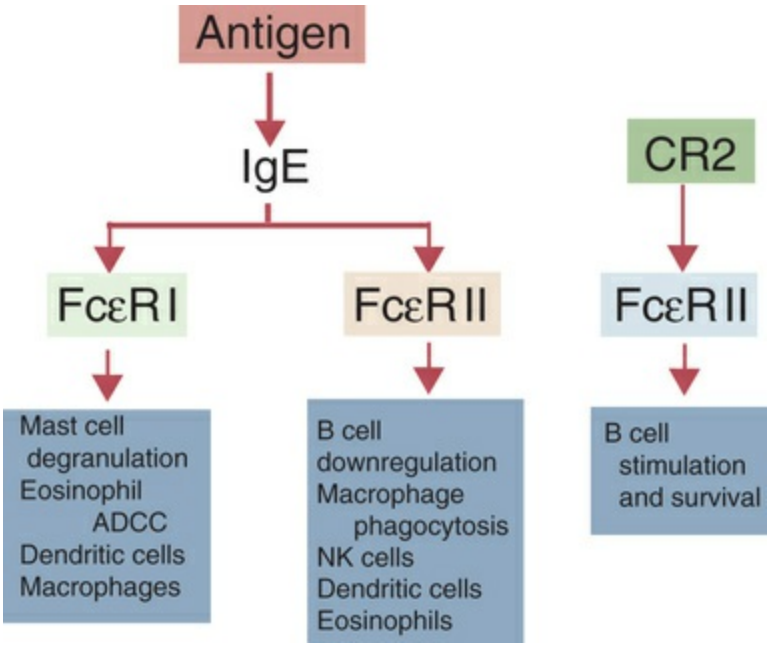


FIG. 29.5 The combination of the Fcε receptors with their ligands stimulates many different responses in mast cells depending on the nature of these stimuli. FcεRII also acts as a complement receptor.

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Mast Cells

Mast cells play a key role in allergic diseases and innate immunity. Because they are located close to body surfaces, they act as sentinel cells and release inflammatory molecules immediately after encountering invaders. They recognize PAMPs and damage-associated molecular patterns (DAMPs) through their PRRs and in response release the contents of their cytoplasmic granules within minutes (Chapter 2). This release normally occurs in a controlled manner and ensures that the severity and type of inflammation are appropriate to the body's immediate needs— but not always!

Structure and Location

Mast cells are large, round cells (15–20 μm in diameter) scattered throughout the body in connective tissue, under mucosal surfaces, and around nerves (Fig. 29.6). They are found in greatest numbers under the skin or in the intestine and airways. Because they are located close to blood vessels, they can regulate blood flow and influence cellular migration. They are easily recognizable because their cytoplasm is densely packed with large granules that stain strongly with dyes such as toluidine blue. These granules often mask the large, bean-shaped nucleus (Fig. 29.7). (Mast cells are so-called because, being full of granules, they were considered to be "well-fed cells" [German *Mastzellen*].)

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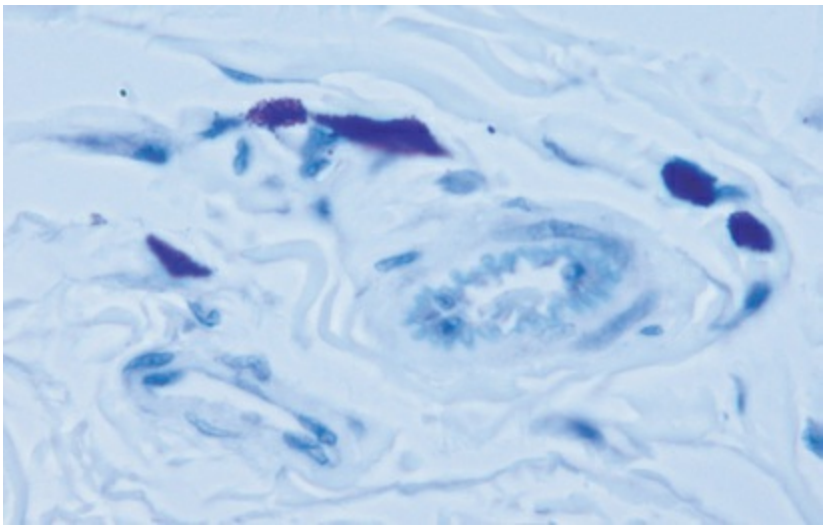


FIG. 29.6 A section of canine skin stained to show mast cells. The mast cells stain intensely because of the heparin in their cytoplasmic granules.

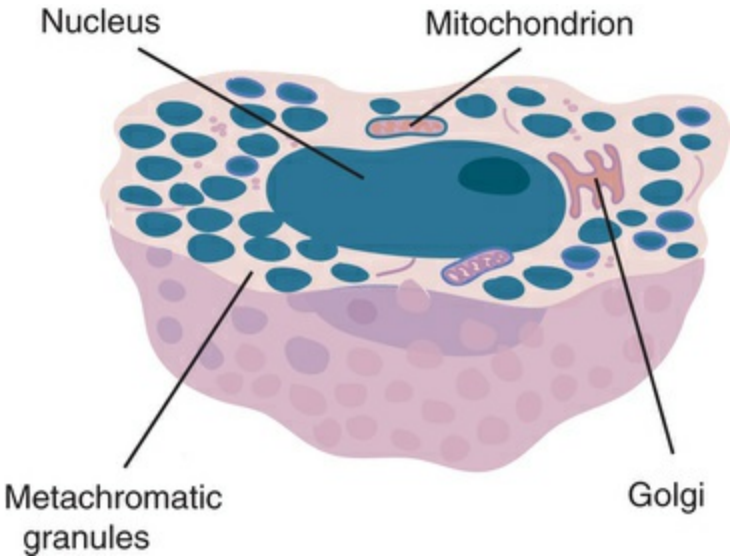


FIG. 29.7 The structural features of a connective tissue mast cell. The term *metachromatic* simply means that the granules stain intensely.

Life History

There are two distinct mast cell populations in rodents. One occurs in the mucosa, the other is found in connective tissues (Table 29.1). Mucosal mast cells arise from bone marrow–derived stem cells and are found between the epithelial cells of the intestinal mucosa and

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lung. They contain chondroitin sulfate but little histamine. Connective tissue mast cells, in contrast, arise from fetal liver stem cells. They are found in the skin, around blood vessels, and in the peritoneal cavity. They are rich in histamine and heparin. Although connective tissue mast cell numbers remain relatively constant, mucosal mast cells proliferate in the presence of intestinal worms. Connective tissue mast cells are constitutive and T cell independent, while mucosal mast cells must be induced and are T cell-dependent. Induced mucosal mast cells disappear within a few weeks after the parasites are eliminated.

TABLE 29.1

Comparison of the Two Major Types of Mast Cell

	Mucosal Mast Cells	Connective Tissue Mast Cells
Structure	Few, variable-sized granules	Many uniform granules
Size	9 to 10 μm diameter	19 to 20 μm diameter
Proteoglycan	Chondroitin sulfate	Heparin
Histamine	1.3 pg/cell	15 pg/cell
Life span	<40 days	>6 months
Location	Intestinal wall, lung	Peritoneal cavity, skin

Many different stimuli trigger mast cell degranulation. The best recognized of these are specific allergens linked to IgE. Allergies, however, are but a special type of inflammation. Numerous other signals can degranulate mast cells, including cytokines, chemokines, chemical agents, physical stimuli, insect and animal venoms, and viruses. Many DAMPs, including the defensins, anaphylatoxins, IL-33, neuropeptides, adenosine, and endothelins (small peptides from endothelial cells), also trigger mast cell degranulation.

Mast cells express multiple pattern-recognition receptors, complement receptors, and the mannose receptor (CD48). Triggering of their toll-like receptors (TLRs) causes mast cells to release different mixtures of mediators. Thus bacterial peptidoglycans acting through TLR2 stimulate histamine release, whereas lipopolysaccharides acting through TLR4 do not. Bacterial binding to mast cell TLRs triggers tumor necrosis factor-α (TNF-α) and IL-6 production. Mast cells can thus use these receptors to distinguish between different pathogens and release a select combination of cytokines, chemokines, and other inflammatory

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mediators, depending on the nature of the stimulus. The inflammatory mediators released by mast cells activate nearby immune cells. They facilitate local neutrophil and eosinophil recruitment, activation, and bactericidal activity.

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Response of Mast Cells to Antigen

Although there are numerous ways by which mast cells can degranulate, the best studied of these is mediated by IgE bound to FcεRI on the cell surface (Fig. 29.8). Mast cells coated in this way are primed to bind antigen. The mast cell can reside in tissues, with its attached IgE acting like a mine in a minefield. If an antigen binds to this IgE, the mast cell will release its granules into the surrounding tissues.

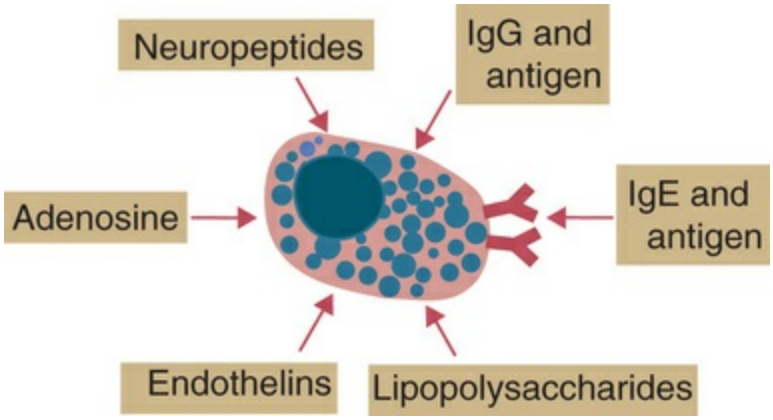


FIG. 29.8 Some of the stimuli that make mast cells degranulate. Antigen bound through IgE causes rapid complete degranulation. The other stimuli shown cause a more gradual, piecemeal degranulation. Thus in normal inflammatory responses, the degree of mast cell degranulation is tailored to local defensive needs.

Degranulation is initiated when an antigen molecule cross-links IgE on two FcεRI and activates their tyrosine kinases. These, in turn, activate phospholipase C, leading to the production of diacylglycerol and inositol triphosphate. These mediators then increase intracellular calcium and activate more protein kinases. The protein kinases phosphorylate myosin in the cytoskeleton so that the granules move to the cell surface. Granule membranes then fuse with the plasma membrane, and their contents are released into the extracellular fluid (Fig. 29.9).

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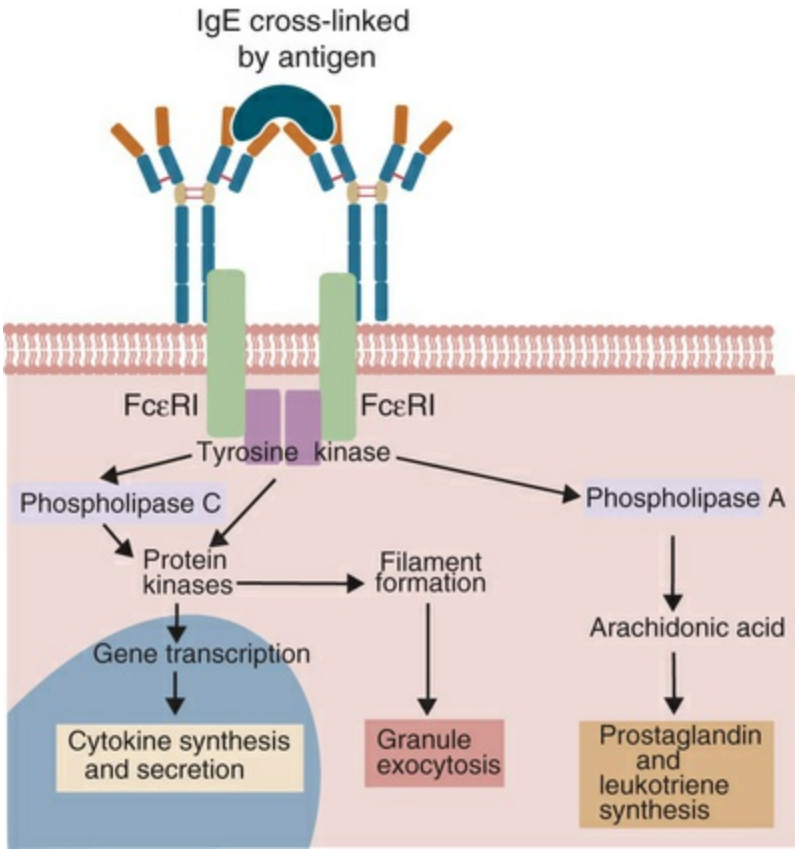


FIG. 29.9 A simplified view of mast cell signal transduction. The process is triggered by cross-linking two bound IgE molecules with antigen. The combined signal eventually leads to degranulation (granule exocytosis), leukotriene and prostaglandin synthesis, and cytokine production.

Cross-linking of two FcεRI by an antigen also activates phospholipase A. This acts on membrane phospholipids to produce arachidonic acid. Other enzymes then convert the arachidonic acid to leukotrienes and prostaglandins (see Fig. 3.7). Finally, the protein kinases promote transcription of genes coding for many different cytokines as well as for cyclooxygenases and lipoxygenase.

The degranulation of mast cells is the central event in the development of allergic (type I hypersensitivity) reactions.

Mast cell granules contain a complex mixture of potent proinflammatory molecules (Chapter 3). Some granules in mouse mast cells contain serotonin or cathepsin D, whereas others contain histamine and TNF-α. Different stimuli determine which granule subsets are exocytosed and thus which mediators are released. It is possible that different clinical forms of allergy may be determined by the granule subset released. Likewise, appropriate treatment of allergies may differ according to the mixture of inflammatory

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mediators involved. The speed of these responses also differs among mast cells. For example, degranulation occurs within seconds after antigen binds to IgE (Fig. 29.10). However, the inflammatory responses induced on mucosal surfaces are much slower than allergic responses in the skin or airways. Mast cell proteins need time to be synthesized, although granule amines are pre-stored and thus available immediately.

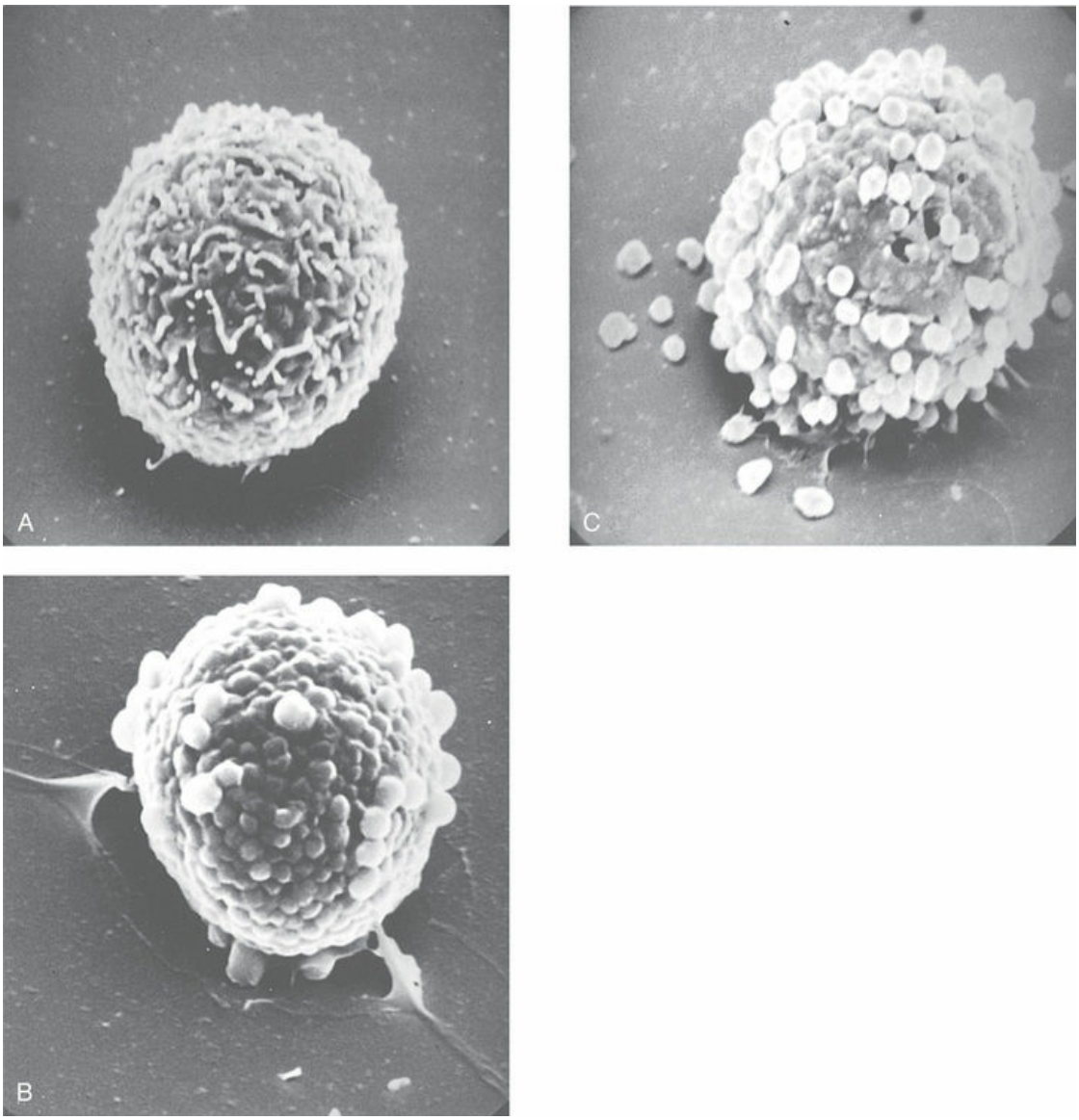


FIG. 29.10 Scanning electron micrographs. **A,** A normal rat mast cell. **B,** A sensitized mast cell fixed 5 seconds after exposure to antigen. **C,** A sensitized mast cell fixed 60 seconds after exposure to antigen. Original magnification ×3000. (From Tizard IR, Holmes WL: Degranulation of sensitised rat peritoneal mast cells in response to antigen, compound 48-80 and polymyxin B. A scanning electron microscope study, *Int Arch Allergy Appl Immunol* 46:867-879, 1974.)

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Mast Cell–Derived Mediators

Mast cell granules are loaded with amines such as histamine, serotonin, and dopamine; lipids such as prostaglandins and leukotrienes; lysosomal enzymes including tryptases, chymases, and cathepsins; cytokines such as TNF-α, IL-4, -5, -6, -15; and some chemokines (Fig. 29.11). Mast cells can capture and store IL-17 by endocytosis. It is then released in allergic reactions. Mast cells also produce chitinases. Chitin is characteristically found in insects, fungi, and helminths, and the production of chitinases supports the suggestion that allergic reactions may have evolved to combat these invaders. Chitin itself is a key allergen in some helminth infections. Mast cells also release small heparin-containing granules that are especially rich in TNF-α. These particles are carried in afferent lymph to draining lymph nodes, where they can trigger changes in cell behavior. The presence of heparin also stabilizes TNF-α so that it persists after delivery.

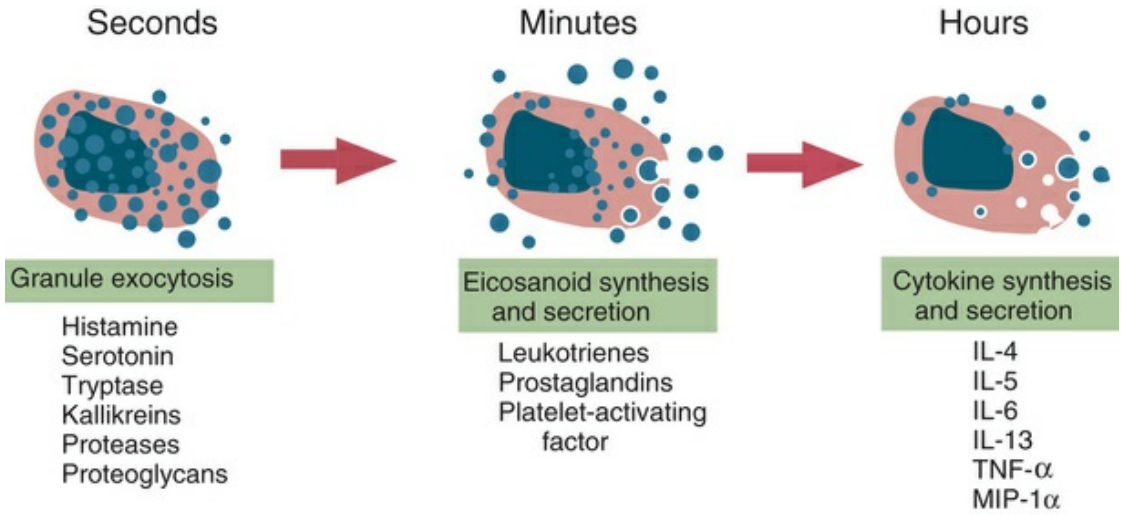


FIG. 29.11 The soluble mediators released from degranulating mast cells. These fall into three categories: molecules released from exocytosed granules, lipids (eicosanoids) synthesized within minutes, and proteins synthesized over several hours.

Interleukin-33

IL-33 is a member of the IL-1 family that is crucial for the induction of type 2 immune responses (Fig. 29.12). It is found in many tissues where it normally regulates DNA transcription. It is not secreted like a conventional cytokine but functions as an alarmin when it escapes from the nuclei of damaged, infected, or dying cells. Its production is increased during inflammation. IL-33 acts on many different targets including Th2 cells, ILC2 cells, NK cells, dendritic cells, mast cells, eosinophils, basophils, B cells, and neurons. It is a key inducer of the Th2 cytokines, IL-4, -5, and -13. (IL-33 together with IL-25 and TSLP are all promoters of Th2 responses. IL-33 is the most potent.) When released by damaged cells, IL-33 is cleaved by inflammatory proteases to shorter peptides that activate group 2 ILCs and triggers them to produce IL-5 and IL-13, and so stimulates eosinophil production. It initiates the Th2 polarizing functions of DC2 dendritic cells.

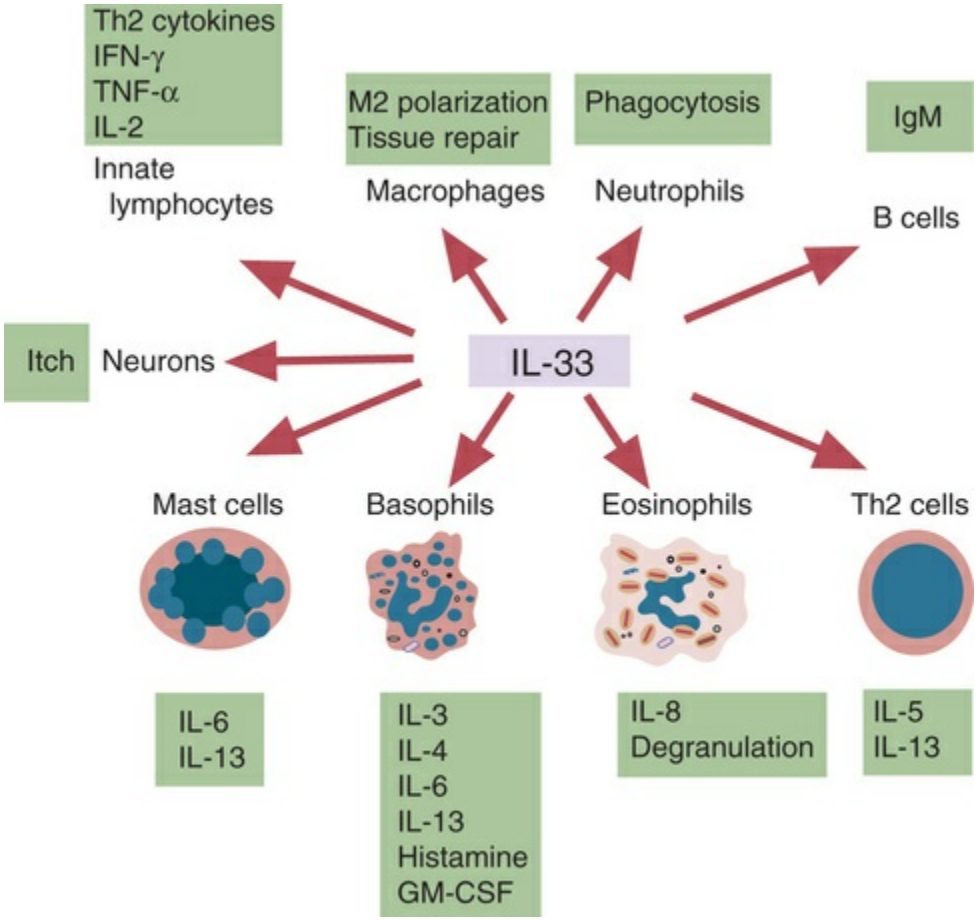


FIG. 29.12 The biological properties of interleukin-33. IL-33 is released by dying cells as well as epithelial cells in response to PAMPs. It stimulates the production of inflammatory cytokines and

chemokines from many different cell types. It stimulates the production of eosinophils. It acts on sensory neurons to induce itch. It is probably therefore a major contributor to the development of type I hypersensitivity.

IL-33 promotes mast cell adherence to blood vessel walls, increases their IgE-mediated degranulation, and enhances their cytokine production. Additionally, it induces the release of leukotrienes and cytokines, and as a result, can trigger acute allergic attacks in the absence of antigen. Similarly, it stimulates basophils to produce their Th2 cytokines. As a result of this enhanced cytokine release, IL-33 recruits and activates eosinophils resulting in eosinophil infiltration (see also Chapter 28). IL-33 binds to sensory neurons triggering intense pruritus and enhancing scratching behavior. Levels of IL-33 are elevated in the blood of humans suffering anaphylactic shock, in helminth infections, in atopic human tissues, and in the lungs of those with severe asthma.

Regulation of Mast Cell Degranulation

Mast cells express two catecholamine receptors called the α- and β-adrenoceptors. These receptors have opposing effects. Molecules that stimulate the α-adrenoceptors (such as norepinephrine and phenylephrine) or block the β-adrenoceptors (such as propranolol) enhance mast cell degranulation (Table 29.2). Conversely, molecules that stimulate the β receptors or block the α receptors inhibit mast cell degranulation. β-stimulators include isoproterenol, epinephrine, and salbutamol and are widely used in the treatment of allergies. β-receptor blockers enhance mast cell degranulation and promote allergies. Some respiratory pathogens such as *Bordetella pertussis* and *Haemophilus influenzae* can cause β-blockade. As a result, the airways of infected animals are more likely to become inflamed because of mast cell degranulation. These infections may also predispose animals to the development of respiratory allergies.

TABLE 29.2

Effects of Stimulating α- and β-Adrenoceptors

System	α Receptor Stimulation or β Blockade	β Receptor Stimulation or α Blockade

Mast cells	Enhances degranulation	Suppresses degranulation
Smooth muscle	Contracts	Relaxes
Blood vessels	Constricts	Dilates

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Regulation of the Response to Mast Cell Mediators

The α- and β-adrenoceptors are found not only on mast cells but also on secretory and smooth muscle cells throughout the body. α-stimulators cause vasoconstriction and may be of use treating severe allergic reactions, in reducing edema, and raising blood pressure. β-stimulators mediate smooth muscle relaxation and may therefore reduce the severity of smooth muscle contraction. Pure α- and β-stimulators are of only limited use in the treatment of allergic diseases because each alone is insufficient to counteract all the effects of mast cell–derived factors. Epinephrine (or adrenalin), on the other hand, has both α- and β-adrenergic activity. In addition to causing vasoconstriction in skin and viscera, its β effects cause smooth muscle to relax. This combination of effects is well suited to combat the vasodilation and smooth muscle contraction produced in type I hypersensitivity reactions. Ideally, epinephrine should be available whenever potential allergens are administered to animals.

Mast Cells in Infections

Mast cells play important roles in both antimicrobial and antiparasite immunity. They can respond within seconds or minutes to microbial invasion. They possess a large array of PRRs as well as being able to recognize antigen indirectly through their Fc receptors. Lipopolysaccharide stimulation of mast cell TLR4 can induce cytokine production in the absence of degranulation. Increased vascular permeability due to mast cell–derived mediators promotes inflammation. They also release preformed antimicrobial peptides such as the cathelicidins. Mast cell tryptase and chymases have antibacterial and anti-parasite activity while neutrophils are attracted by mast cell–derived leukotrienes.

Late-Phase Reaction

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When antigen is injected into the skin of an allergic animal, two waves of inflammation occur. There is an immediate acute inflammatory response that occurs within 10 to 20 minutes as a result of the release of the preformed mast cell mediators. This is followed several hours later by a second wave called the late-phase reaction, which peaks at 6 to 12 hours and then gradually diminishes. This late-phase reaction is characterized by redness, edema, and pruritus. It is believed that this late reaction results from the release of inflammatory mediators by T cells, endothelial cells, neutrophils, and macrophages attracted by mast cell chemotactic factors. Th17 cells also play a role in this late process (Chapter 14).

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Basophils

Basophils, the least common type of blood leukocyte, are functionally similar to mast cells, although their origins and gene expression profiles are very different. Basophils are polymorphonuclear granulocytes (10–14 μm in diameter) whose cytoplasmic granules stain intensely with basic dyes, such as hematoxylin (Fig. 29.13). These granules are fewer and smaller than those in mast cells and they contain histamine as well as a restricted protease profile. Basophils constitute about 0.5% of blood leukocytes. Unlike mast cells they have a very short lifespan of about 60 hours in mice and are actively replenished by stem cells. They are not normally found outside the bloodstream and only enter tissues under the influence of some T cell–derived chemokines.

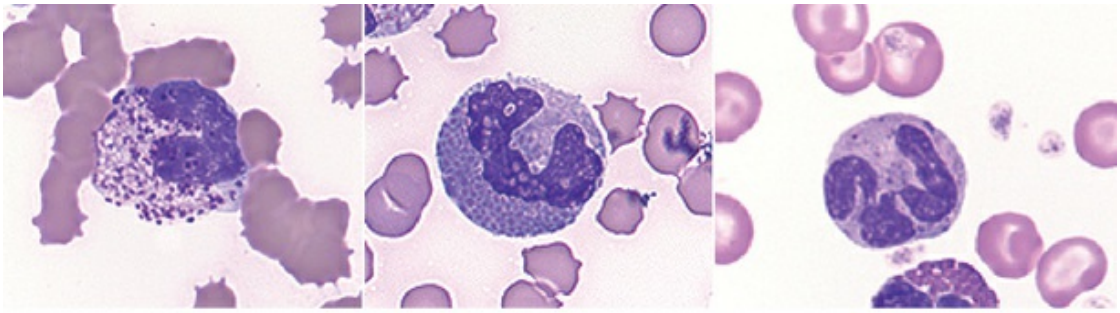


FIG. 29.13 Photomicrographs of peripheral blood basophils from a horse, a cat, and a dog. These cells are about 10 μm in diameter; all were photographed at the same magnification. Giemsa stain. (Courtesy Dr. M.C. Johnson.)

Basophils bind IgE through FcεRI. Antigens binding to this IgE will cause basophil degranulation and the release of their granule contents. Basophil granules contain a mixture of molecules similar to those in mast cells including proteases, amines, and cytokines such as IL-4 and lipid mediators. When activated, they produce IL-4, IL-6, IL-13, and TSLP. Basophils release platelet-activating factor (PAF), a lipid, in response to IgG1-mediated stimulation. PAF is about 10,000 times more effective than histamine in increasing

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vascular permeability.

There are two distinct pathways in mouse anaphylaxis: one mediated by basophils and one mediated by mast cells. Although mast cells induce acute inflammation, basophils probably mediate more long-term allergic states such as chronic allergic dermatitis as well as the late-phase reaction.

Basophils can initiate allergen- and helminth-driven type 2 immune responses by functioning as antigen-presenting cells. Basophils can capture antigen through FcR-bound antibodies. They endocytose, process, and present soluble antigens but not particulate antigens. IL-4 and IL-6 drive Th2 cell differentiation and as a consequence promote IgE responses. Basophils play a protective role in helminth, tick, and bacterial infections; they degrade toxins in venoms; and they contribute to tumor rejection.

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Eosinophils

Tissues undergoing type I hypersensitivity reactions characteristically contain large numbers of eosinophils. These eosinophils are attracted to sites of mast cell degranulation, where they release their own contents. Eosinophils are polymorphonuclear cells, slightly larger than neutrophils or basophils (12–17 μm in diameter), with cytoplasmic granules that stain intensely with the red dye eosin (Figs. 29.14, 29.15, and 29.16). They originate in the bone marrow and spend about 30 minutes circulating in the bloodstream before migrating into the tissues, where they have a half-life of about 12 days. The proportion of eosinophils among the blood leukocytes varies greatly since it is affected by the presence of parasites. Normal values range from about 2% in dogs to about 10% in cattle.

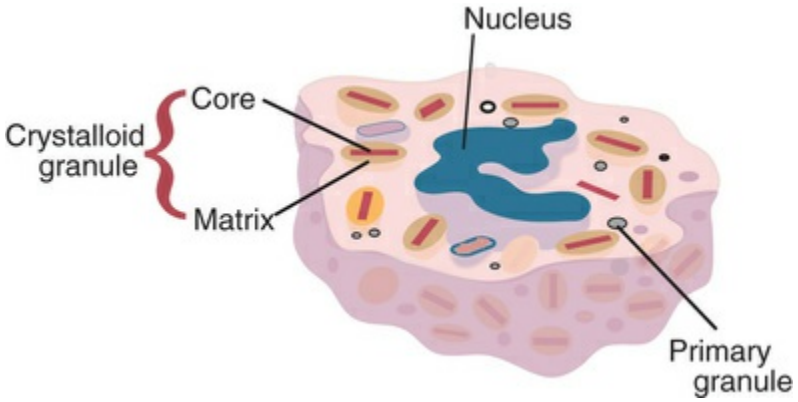


FIG. 29.14 The major structural features of an eosinophil.

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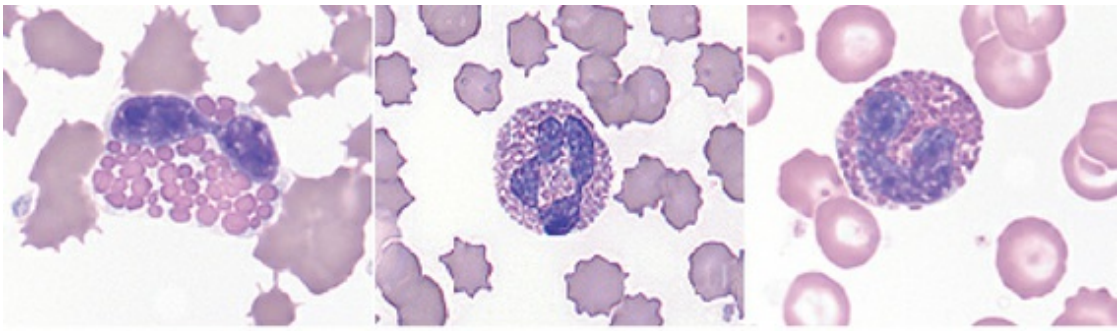


FIG. 29.15 Photomicrographs of peripheral blood eosinophils from a horse, a cat, and a dog. Each cell is about 12 μm in diameter. Giemsa stain. (Courtesy Dr. M.C. Johnson.)

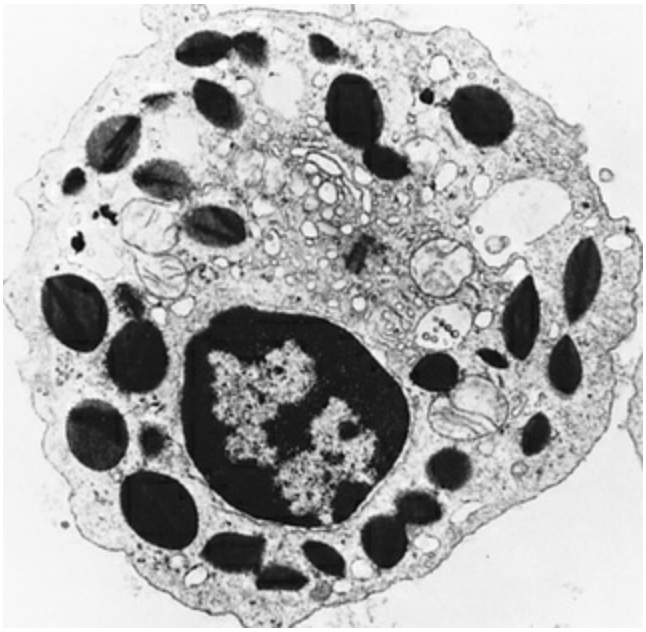


FIG. 29.16 A transmission electron micrograph of a rabbit eosinophil. (Courtesy Dr. S. Linthicum.)

Eosinophil Activation

Eosinophils arise from bone marrow stem cells under the influence of IL-3, IL-5, and GM-CSF (Fig. 29.17). The great majority of the body's eosinophils are located in the intestine. Both Th2 cells and mast cells produce IL-5 and the chemokines known as eotaxins that stimulate the release of eosinophils from the bone marrow. IL-25, IL-33, and TSLP all induce IL-5 production. Thus Th2 cells mobilize

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eosinophils while promoting IgE responses. These eosinophils are attracted to sites of mast cell degranulation by eotaxins, histamine, and its breakdown product imidiazole acetic acid, leukotriene B_4, 5-hydroxytryptamine (5-HT), and by PAF. Eosinophils are especially attracted by CXCL8 (IL-8) complexed to IgA. Some allergens directly activate eosinophils, stimulating their chemotaxis and upregulating CR3 expression. The mobilization and activation of eosinophils enhances their ability to kill parasites and supports the belief that the major function of the IgE-mediated responses is the generation of type 2 responses resulting in the control of helminth parasites (Chapter 28). Activated eosinophils express MHC class II molecules and can serve as antigen-presenting cells.

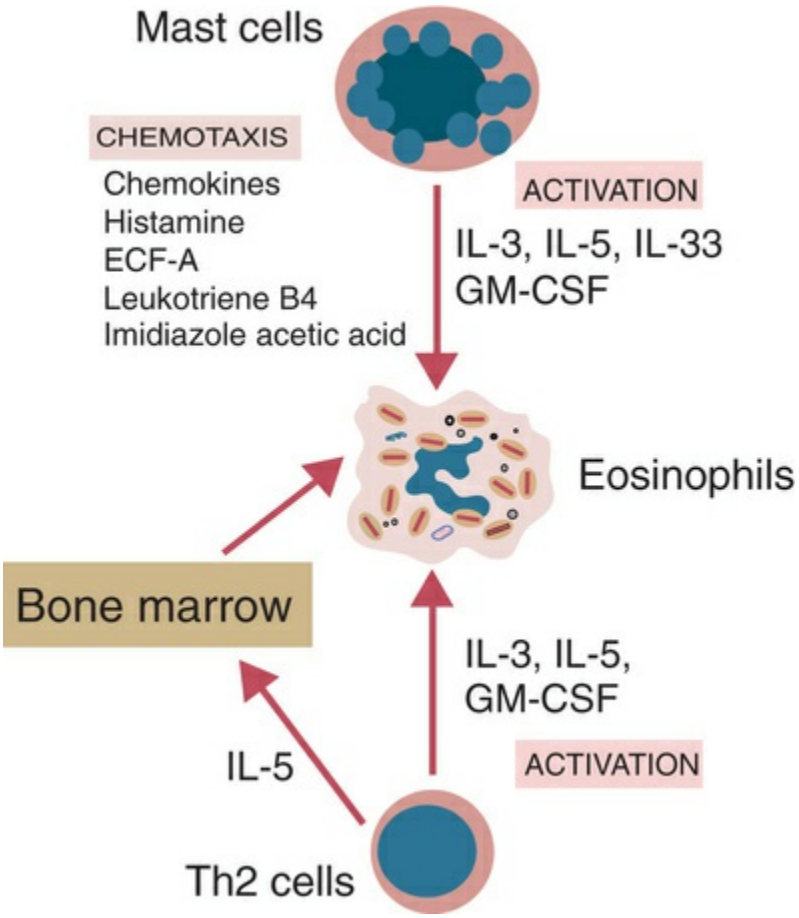


FIG. 29.17 The regulation of eosinophil mobilization, chemotaxis, and activation.

Eosinophil Degranulation and Mediators

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Eosinophils have many PRRs that recognize PAMPs and DAMPs. When triggered, eosinophils release multiple cytokines, chemokines, and their granule contents. Although eosinophils can phagocytose small particles, they are much more suited to extracellular destruction of large parasites since they can degranulate into the surrounding fluid. Eosinophils may release intact granules by exocytosis, or, more commonly, undergo piecemeal degranulation. In this process, small vesicles bud off the secondary granules and are released into the extracellular tissues. This occurs in response to IgE-coated parasites, many chemokines, PAF, and C5a. Once free in extracellular fluid, eosinophil granules can function as independent structures capable of secreting granule proteins in response to IFN-γ or CCL11. Activated eosinophils can also eject extracellular traps containing mitochondrial DNA and granule cationic proteins. They do this in response to bacterial products such as endotoxins. The release of these nets is not accompanied by cell death.

Eosinophils contain two types of granules (see Figs. 29.15 and 29.16). Their small, primary granules contain arylsulfatase, peroxidase, and acid phosphatase. Their large crystalloid granules have a core of major basic protein (MBP) surrounded by a matrix containing eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN) (Fig. 29.18). Eosinophils also produce lipid mediators such as leukotrienes and PAF. Particles bound to eosinophil receptors trigger a powerful respiratory burst. The EPO uses bromide in preference to chloride, thus producing OBr-. It also generates nitric oxide and nitrotyrosine, both potent oxidizing agents. Eosinophil granule proteins can kill helminths and bacteria and are important mediators of tissue pathology. They all, for example, damage respiratory epithelium. The production by eosinophils of multiple Th2 cytokines (Table 29.3) as well as indoleamine dioxygenase inhibits local Th1 responses and ensures that a "Th2 environment" is maintained where eosinophils accumulate.

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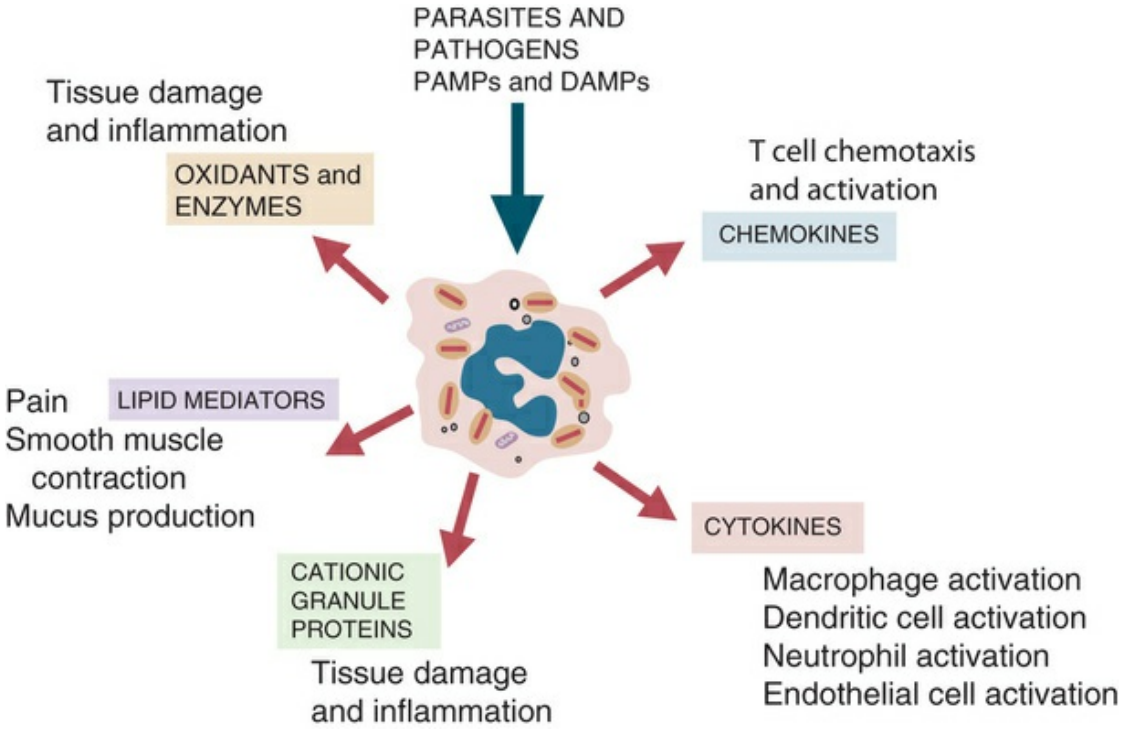


FIG. 29.18 Eosinophils release a complex array of molecules that contribute to the acute inflammatory process. It is clear that, on balance, eosinophils exacerbate the inflammation triggered by mast cells.

TABLE 29.3

The Major Cytokines Produced in Type I Hypersensitivity Reactions

Mast Cells	Basophils	Eosinophils
IL-3	IL-4	IL-1α
IL-4	IL-6	IL-3
IL-5		IL-4
IL-6		IL-5
IL-13		IL-6
IL-16		GM-CSF
IL-22		TNF-α
IL-25		TGF-β
GM-CSF		
TNF-α		

Mast cells and eosinophils interact. Thus eosinophil MBP causes mast cells to release histamine. Mast cells in turn release eosinophil chemotactic agents, activate eosinophils, and enhance the expression of eosinophil receptors. Mast cells can synthesize and secrete IL-3, IL-5, and GM-CSF, all of which promote eosinophil degranulation, growth, and survival.

Eosinophils also play a key role in the regulation of humoral

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immunity. Thus they are the major source of the plasma cell survival factor, APRIL (activation and proliferation-induced ligand) (see Box 15.2). They are required for the homing of plasma cells to the bone marrow and for their long-term survival. An absence of eosinophils prevents efficient B cell class switching to IgA within Peyer's patches. In effect, eosinophils generate and maintain mucosal IgA plasma cells.

In conclusion, type I hypersensitivity reactions are mediated by IgE and recruit three key cells: mast cells, eosinophils, and basophils, to participate in the reaction. Their biological role is likely to mediate antiparasite responses but, under modern conditions, they also mediate allergic diseases.

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30

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Allergic Diseases

CHAPTER OUTLINE

The Hygiene Hypothesis, 335
Specific Allergic Diseases, 336
Anaphylaxis, 336
Milk Allergy, 338
Food Allergies, 338
Allergic Respiratory Disease, 339
Atopic Dermatitis, 340
Clinical Disease, 340
Genetic Background, 340
Environmental Influences, 341
Skin Lesions, Cells, Cytokines, and Chemokines, 341
Pruritus, 342
The Role of IgE, 342
Epidermal Barrier Dysfunction, 343
Infections, 343
Allergies to Vaccines and Drugs, 343
Allergies to Parasites, 344

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Eosinophilic Granuloma Complex, 344
Diagnosis of Type I Hypersensitivity, 345
Treatment of Type I Hypersensitivity, 346
Allergen-Specific Immunotherapy, 346

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Define the hygiene hypothesis.
- Describe how the clinical signs of allergic disease depend in large part on the route by which antigens (allergens) enter the body.
- Explain how the massive systemic release of inflammatory molecules by mast cells and basophils causes anaphylaxis.
- List the significant species differences in anaphylaxis.
- Explain how and why animals commonly suffer from allergies to foods, inhaled antigens, vaccines, or drugs.
- Discuss the evidence suggesting that atopic dermatitis is a very complex disease or set of diseases, not all of which are mediated by IgE.
- Explain the mechanisms and treatment of pruritus.
- Explain the pathogenesis of allergic respiratory disease.
- Discuss the role of epithelial barrier dysfunction in atopic dermatitis.
- Describe the eosinophilic granuloma complex.
- Describe the appropriate treatment for atopic dermatitis.
- Explain the basic principles of treatment of allergic diseases.
- Explain how allergen-specific immunotherapy works.
- Define anaphylaxis, anaphylactoid, atopy, and asthma.

The development of type I hypersensitivity has long been considered to result from the excessive release of inflammatory mediators by mast cells, basophils, and eosinophils. The severity and location of these reactions depends on the number and location of these cells, the degree of sensitization of an animal, the amount

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of antigen involved, and its route of administration. Not all animals develop these hypersensitivity diseases. Nevertheless, their prevalence is increasing and it has been suggested that this increase is due to reductions in the diversity of the body's microbiota—the hygiene hypothesis.

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The Hygiene Hypothesis

The prevalence of allergic disease has increased significantly in Western societies over the past 50 years. While most obvious in humans, this has also affected domestic species, especially dogs and cats. It is likely that this increase is a result of changes in the body's microbiota (Fig. 30.1). The hygiene hypothesis suggests that alterations in Western diets, environmental cleanliness, an urban lifestyle, and overuse of antibiotics together cause long-term changes in the intestinal microbiota that have driven the increases in allergic and inflammatory disease. Since the intestinal microbiota normally influence the Th1/Th2 balance, it is suggested that this dysbiosis causes, Th2 responses to predominate and results in the development of allergies. Additionally, the skin and lung microbiota affect the development of lung and skin allergies.

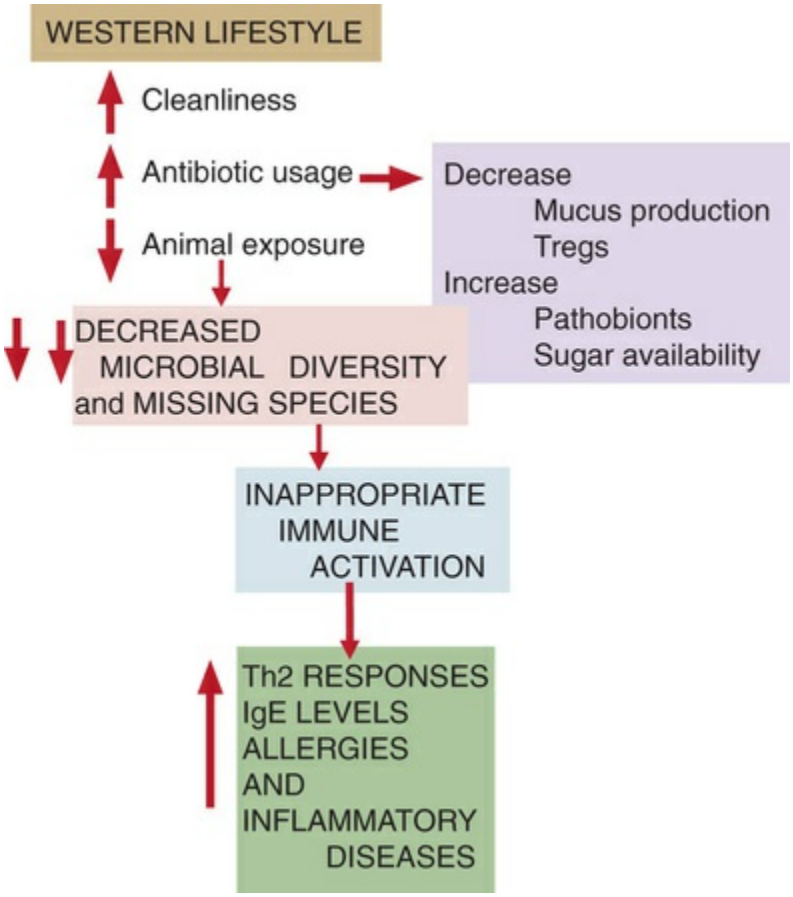


FIG. 30.1 The hygiene hypothesis. A lack of microbial diversity in

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the intestinal microbiota results in a predominant Th2 response and a predisposition to allergic disease.

The hygiene hypothesis has received support from studies on piglets. Major differences can be found in their gut microbiota depending upon the environment in which piglets are raised. These differences also influence the expression of immune system genes. For example, pigs raised in a very clean environment have reduced microbial diversity and express more genes involved in inflammation such as type I interferon genes, MHC class I, antibacterial peptides, and many chemokines. Conversely, outdoor pigs with a diverse microbiota express more genes linked to T cell function such as TCR and CD8.

Similar effects have been observed in rodents. Germ-free mice have high serum IgE levels in early life. These can be greatly reduced by bacterial colonization suggesting that the microbiota regulate IgE production. If low doses of the antibiotic vancomycin are fed to neonatal mice, the diversity of their gut microbiota is reduced, Treg numbers are reduced and they suffer from increased severity of allergic lung disease. Adult mice treated with oral antibiotics have increased IgE levels and blood basophil numbers. They too have increased airway inflammation following allergen challenge.

Conversely, an appropriately balanced microbiota generates antiinflammatory molecules such as short chain fatty acids (SCFAs), polysaccharide A, and peptidoglycans. SCFAs (formate, acetate, butyrate, succinate) and glycans are produced in abundance in high-fiber diets. Human populations that consume large amounts of fiber have a lower prevalence of colitis and inflammatory disease. Among the SCFAs, butyrate has potent antiinflammatory properties and inhibits proinflammatory responses by intestinal macrophages (see Fig. 21.7).

In general, therefore, a balanced and healthy microbiota suppress inflammation in the intestinal wall. They act mainly by inhibition of the NF-κB pathway and block type 2 immune responses by generating Treg cells.

Many allergic reactions resemble those induced by intestinal helminths or biting insects. Both involve Th2 activation and the production of IgE. This has led to the suggestion that the immune

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system in allergic individuals is somehow tricked into reacting to innocuous antigens as if they were parasites. Reactions to parasites decline once the invader has gone. In allergies, however, the reactions persist, suggesting a defect in immunoregulatory pathways.

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Specific Allergic Diseases

Anaphylaxis

In its most extreme form, antigen administered rapidly to an allergic animal will cause generalized mast cell degranulation and massive mediator release. If the rate of release of vasoactive molecules from these mast cells exceeds its ability to adjust to the changes in its vascular system, an animal will undergo anaphylaxis and may die.

Anaphylaxis is a severe, life-threatening systemic hypersensitivity reaction triggered by sudden exposure to an antigen in a highly sensitized animal. Its clinical signs are determined by organ system involvement, which differs among the major domestic animals (Table 30.1). Many of the symptoms result from smooth muscle contraction in the bronchi, gastrointestinal tract, uterus, and bladder, mediated by large amounts of histamine and other amines released by degranulating mast cells and basophils. It is often lethal unless treated promptly.

TABLE 30.1

Anaphylaxis in the Domestic Species and Humans

Species	Shock Organs	Symptoms	Pathology	Major Mediators
Horse	Respiratory tract Intestine	Cough Dyspnea Diarrhea	Emphysema Intestinal hemorrhage	Histamine Serotonin
Ruminants	Respiratory tract	Cough Dyspnea Collapse	Lung edema Emphysema Hemorrhage	Serotonin Leukotrienes Kinins Dopamine
Swine	Respiratory tract Intestine	Cyanosis Pruritus	Systemic hypotension	Histamine
Dog	Hepatic veins	Collapse Dyspnea Diarrhea Vomiting	Hepatic engorgement Visceral hemorrhage	Histamine Leukotrienes Prostaglandins
Cat	Respiratory tract Intestine	Dyspnea Vomiting Diarrhea Pruritus	Lung edema Intestinal edema	Histamine Leukotrienes
Human	Respiratory tract	Dyspnea Urticaria	Lung edema Emphysema	Histamine Leukotrienes
Chicken	Respiratory tract	Dyspnea Convulsions	Lung edema	Histamine Serotonin Leukotrienes

The major shock organs of horses are the lungs and the intestine.

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Bronchial and bronchiolar constriction leads to coughing, dyspnea, and eventually apnea. On necropsy, severe pulmonary emphysema and peribronchiolar edema are commonly seen. In addition to the lung lesions, edematous hemorrhagic enterocolitis may cause severe diarrhea. The major mediators of anaphylaxis in horses are probably histamine and serotonin.

In cattle the major shock organs are the lungs (Fig. 30.2). Anaphylaxis is characterized by profound systemic hypotension and pulmonary hypertension. The pulmonary hypertension results from constriction of the pulmonary vein and leads to pulmonary edema and severe dyspnea. The smooth muscle of the bladder and intestine contract, causing urination, defecation, and bloating. The main mediators of anaphylaxis in cattle are serotonin, kinins, and the leukotrienes. Histamine is of lesser importance. Dopamine enhances histamine and leukotriene release from the lung, thus exerting a form of positive feedback. Because of the anticoagulant properties of heparin from mast cells, blood from affected animals may not coagulate. In cattle, in contrast to the other species, β-stimulants such as isoproterenol potentiate histamine release from leukocytes, whereas α-stimulants such as norepinephrine inhibit histamine release. Epinephrine also potentiates histamine release in cattle. The significance of these anomalous effects is unclear.

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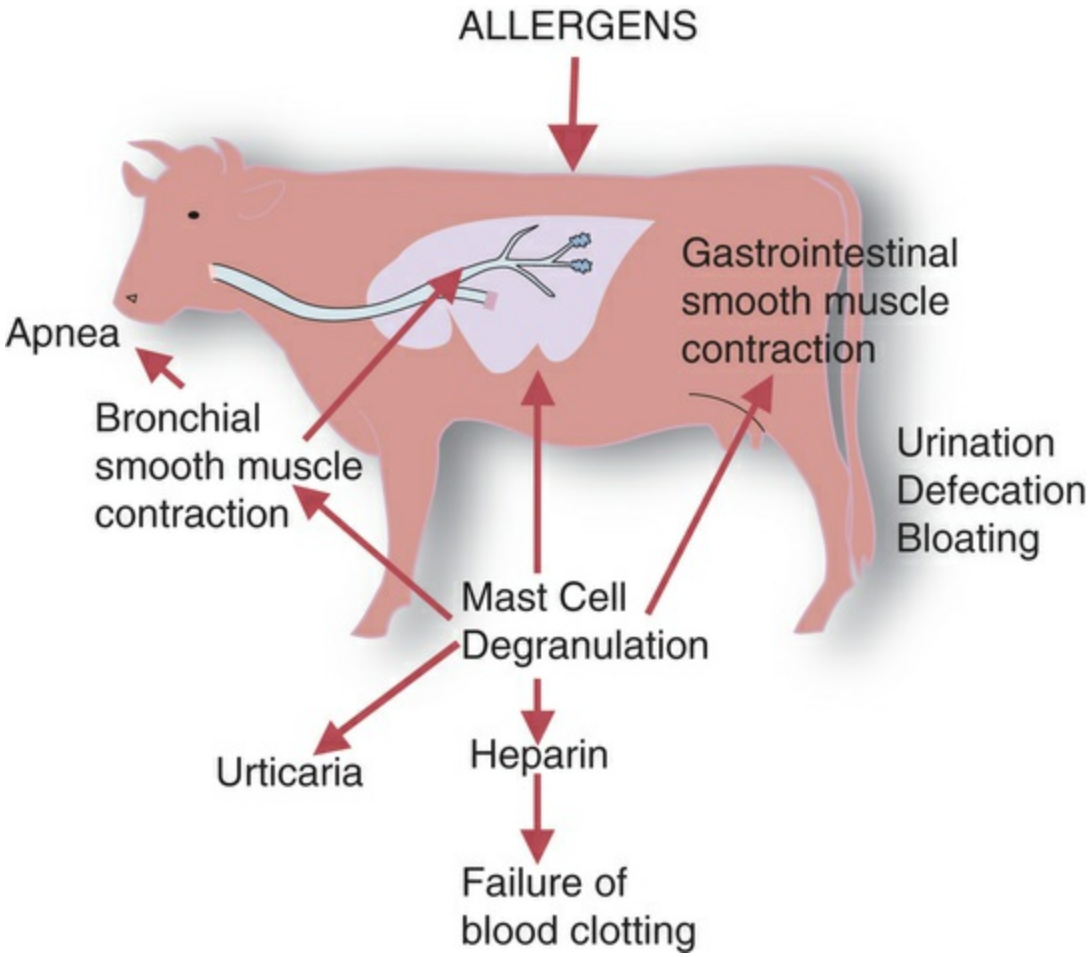


FIG. 30.2 The basic mechanisms of anaphylaxis. The proximal cause of death in most but not all species results from extreme constriction of the bronchial smooth muscle and subsequent asphyxiation.

In sheep, pulmonary signs predominate in anaphylaxis as a result of constriction of the bronchi and pulmonary vessels. Smooth muscle contraction also occurs in the bladder and intestine with predictable results. The major mediators of type I hypersensitivity in sheep are histamine, serotonin, leukotrienes, and kinins.

In pigs, anaphylaxis is largely the result of systemic and pulmonary hypertension, leading to dyspnea and death. In some pigs the intestine is involved, whereas in others no gross intestinal lesions are observed. The most significant mediator identified in this species is histamine.

Dogs differ from the other domestic mammals in that the major shock organ is not the lung but the liver, specifically the hepatic veins. Dogs undergoing anaphylaxis show initial excitement followed by vomiting, defecation, and urination. As the reaction

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progresses, dogs collapse with weakness and depressed respiration, become comatose, convulse, and die. On necropsy, the liver and intestine are massively engorged, perhaps holding up to 60% of the animal's total blood volume. All these signs result from occlusion of the hepatic vein due to a combination of smooth muscle contraction and hepatic swelling. This results in portal hypertension and visceral pooling, as well as a decrease in venous return, cardiac output, and arterial pressure. Identified mediators include histamine, prostaglandins, and leukotrienes.

In cats, the major shock organs are the lungs. Cats undergoing anaphylaxis show vigorous scratching around the face and head as histamine is released into the skin. This is followed by dyspnea, salivation, vomiting, incoordination, collapse, and death. Necropsy reveals bronchoconstriction, emphysema, pulmonary hemorrhage, and edema of the glottis. The major mediators in the cat are histamine and the leukotrienes.

Although anaphylaxis is the most dramatic and severe type I hypersensitivity reaction, it is more usual to observe local allergic reactions, the sites of which are referable to the route of administration of antigens. For example, inhaled antigens (allergens) provoke inflammation in the upper respiratory tract, trachea, and bronchi, resulting in fluid exudation from the nasal mucosa (hay fever) and tracheobronchial constriction (asthma). Aerosolized antigen will also contact the eyes and provoke conjunctivitis and intense lacrimation. Ingested antigens may provoke diarrhea and colic as intestinal smooth muscle contracts violently. If sufficiently severe, the resulting diarrhea may be hemorrhagic. Antigen reaching the skin causes local dermatitis. The reaction is erythematous and edematous and is described as an urticarial type (*Urtica dioica* is the name of the "stinging nettle," a plant that has hollow stinging hairs that inject histamine into the skin when touched; Fig. 30.3). Urticarial lesions are extremely pruritic; consequently, scratching may mask the true nature of the lesion. The inflammatory cell infiltrate in equine urticarial lesions reflects this type 2 response. It contains eosinophils, B cells, macrophages, and mast cells, while expression of interleukin-4 (IL-4), IL-13, and thymic stromal lymphopoietin (TSLP) is increased compared to nonlesional skin.

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FIG. 30.3 Severe urticaria in a Boxer stung by three wasps.
(Courtesy Dr. G. Elissalde.)

Milk Allergy

Jersey cattle may become allergic to the α-casein of their own milk. Normally, this protein is synthesized in the udder, and provided that the animals are milked regularly, nothing untoward occurs. If milking is delayed, however, the increased intra-mammary pressure forces milk proteins into the bloodstream. In allergic cattle, this may result in reactions ranging from mild discomfort with urticarial skin lesions to acute anaphylaxis and death. Prompt milking can treat the condition, although some seriously affected animals may have to go for several lactations without drying off because of the severe reactions that occur on cessation of milking.

Food Allergies

The healthy immune system is tolerant to foods and is unresponsive to potential food allergens. Oral tolerance is a natural process by which ingested antigens are sampled by dendritic cells,

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macrophages, or M cells and presented to intestinal T cells in such a way that they drive the development of IL-10–producing Treg cells. Other tolerance-promoting molecules include retinoic acid, IDO, and TGFβ. The microbiota appear to play a minor role in oral tolerance since it develops in germ-free animals. If this tolerance breaks down, a type 2 response triggered by IL-33 and ILCs may occur. IL-33 increases mucosal permeability and promotes Th2 skewing of the immune response. In such cases, class-switching results in the production of IgE and the development of food allergies. Th2 responses may also occur if Tregs are destroyed or dysfunctional.

Recent experimental evidence suggests that certain intestinal infections may initiate food allergies by breaking oral tolerance. Thus reoviruses can trigger Th1 responses in Peyer's patches. The type I interferons induced by the infection then suppress pTreg activation and so permit food allergens to trigger immune responses. This has been demonstrated in the case of Th1 responses to gluten but could well apply to Th2 responses as well.

About 2% of food protein is absorbed from the intestine as peptide fragments large enough to be recognized as foreign. More may enter if there are defects in the integrity of the enterocyte tight-junction barrier. Antigens may enter the bloodstream and reach skin mast cells within a few minutes. It has been estimated that up to 30% of skin diseases in dogs are due to allergies and that responses to food allergens may account for many cases of cutaneous disease in dogs and cats, although their true prevalence is unknown. The clinical consequences of food allergies are seen both in the digestive tract and on the skin (Fig. 30.4).

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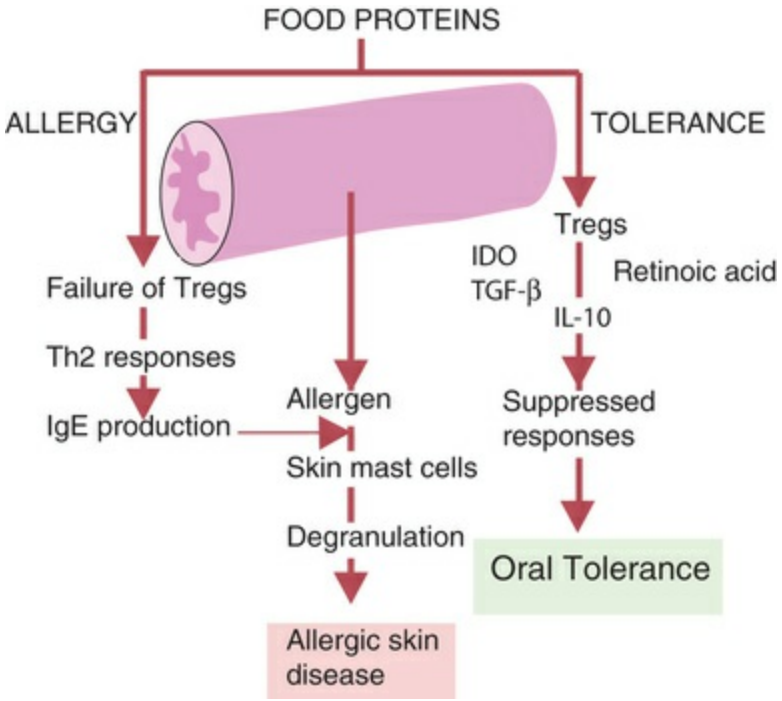


FIG. 30.4 The major mechanisms of food tolerance and food allergies. The failure of Tregs may also be triggered by viral infections.

Only about 10% to 30% of dogs with food allergies have gastrointestinal responses. The intestinal reaction may be mild, perhaps showing only as an irregularity in the consistency of the feces, or it may be severe, with vomiting, cramps, and violent, sometimes hemorrhagic diarrhea occurring soon after feeding. Most dogs show cutaneous symptoms, and these may be indistinguishable from atopic dermatitis. The skin reactions are usually papular and erythematous and may involve the feet, eyes, ears, and axillae or perianal area. The lesion itself is highly pruritic and is commonly masked by self-inflicted trauma and secondary bacterial or yeast infections. This pruritus tends to respond poorly to corticosteroids. In chronic cases the skin may be hyperpigmented, lichenified, and infected, leading to pyoderma. Chronic pruritic otitis externa may also develop. The foods involved vary but are usually protein-rich such as dairy products, wheat meal, fish, chicken, beef, or eggs. In pigs, fishmeal and alfalfa have been incriminated. Analysis of serum from dogs that are allergic to beef and cow's milk shows that the major allergens are bovine IgG heavy chains. A second major antigen in lamb and beef extracts is phosphoglucomutase. Analysis of the skin infiltrate in

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these dogs shows that $CD8^+$ T cells predominate and that expression of IL-4, IL-13, and FoxP3 are increased. Food allergies have been reported in the horse but are uncommon. Wild oats, white clover, and alfalfa have been recognized as allergens in this species.

The most reliable test for suspected food allergies is to remove all potential allergens and then feed a hypoallergenic diet. These elimination diets usually contain meat and carbohydrates from sources to which the animal is unlikely to have been exposed. Examples include mutton, duck, venison, or rabbit with brown rice or potato. An alternative solution is to feed a hydrolyzed diet that contains smaller and less allergenic protein fragments. Several commercial hypoallergenic diets are available to facilitate this diagnosis. Elimination diet trials generally show evidence of remission by 5 to 6 weeks but should last for at least 8 weeks. These diets may be supplemented by adding back other ingredients until the allergen is identified by a recurrence of clinical signs. Treatment involves eliminating the responsible food after correctly identifying it.

Parasitized cats develop significantly higher levels of antibodies to food antigens than unparasitized cats. Most importantly they develop higher levels of IgE antibodies, suggesting that the presence of parasitic worms in the intestine provokes food allergies.

Allergic Respiratory Disease

While common in humans, nasolacrimal urticaria (hay fever) is an uncommon manifestation of respiratory allergy in dogs and cats. Pollens usually provoke rhinitis and conjunctivitis characterized by a profuse watery nasal discharge and excessive lacrimation. If the allergenic particles are sufficiently small, they may reach the bronchi or bronchioles, where the resulting reaction can cause bronchoconstriction, wheezing, and recurrent asthma-like paroxysmal dyspnea (Fig. 30.5). It should be noted that Basenji dogs have unusually sensitive airways and experience a disease similar to some types of human asthma. Cats are also recognized as suffering from asthma manifested by paroxysmal wheezing, dyspnea, and coughing. Although its pathogenesis has not been

clarified, asthmatic cats respond well to corticosteroids and inhaled bronchodilators as well as allergen avoidance. It is of interest to note that there is a concordance between asthma in cats and in their owners, suggesting the involvement of similar allergens.

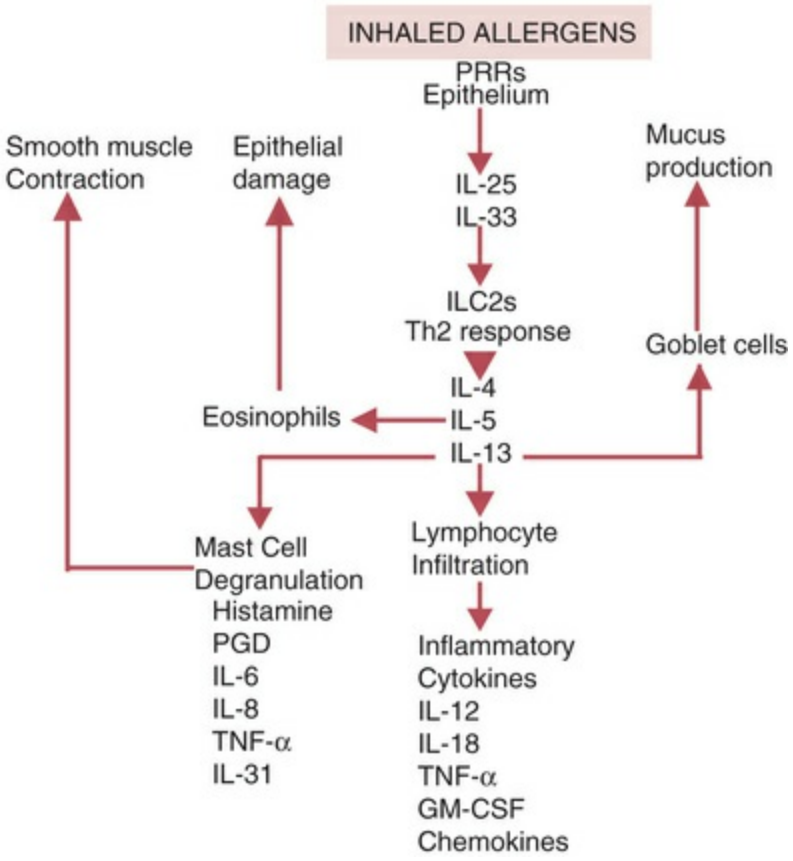


FIG. 30.5 The pathogenesis of allergic asthma.

Asthma in humans is now recognized as a syndrome mediated by several different pathogenic processes. Thus the majority of cases are eosinophilic (>3% eosinophils in sputum) and regulated by ILC2s and basophils. (ILC2s produce the type II cytokines IL-5 and IL-13). These cases are commonly allergic in origin and steroid responsive. About 30% of human asthma cases are, however, neutrophilic (>60% in sputum), associated with high Th17 cell levels, and are often steroid unresponsive. Other asthma subtypes may have both neutrophils and eosinophils or even neither, in sputum. IL-33 is also generated in the lungs of many asthmatics. It acts as an alarmin and activates ILC2 cells, basophils, and mast cells

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to promote inflammation.

A familial allergic rhinitis characterized by extreme nasal pruritus, violent sneezing, dyspnea, mucoid nasal discharge, and excessive lacrimation has been observed in cattle. Depending on the allergen, it may be seasonal. The antigens involved are inhaled and come from a variety of plant and fungal sources. Diagnosis may be confirmed by skin testing. Nasal granulomas may form in chronically affected cattle. These consist of numerous polypoid nodules, 1 to 4 mm in diameter, situated in the anterior nasal mucosa. The nodules contain large numbers of mast cells, eosinophils, and plasma cells.

Atopic Dermatitis

Atopic dermatitis (AD) is a complex, multifactorial syndrome characterized by chronically inflamed and itchy skin. It is very common in humans and dogs (as many as 15% are affected) and has been recognized in cats, horses, and goats. It is not simply an allergic disease driven by allergen exposure because it also involves immune dysregulation, skin barrier defects, and microbial colonization. It is better considered to be a manifestation of multiple diseases, mainly driven by Th2/Th22 responses with some contributions from Th17 and Th1 cells.

Transcriptome profiles of the blood lymphocytes in dogs with AD showed increases in CD8+ and Treg cells Likewise, there is increased expression of IL-13 and tumor necrosis factor-α, as well as decreased expression of IL-10 and TGF-β. The increase in Tregs together with a decline in IL-10 and TGF-β suggests that Treg function is somehow impaired.

Some forms of AD are due to IgE-mediated allergic responses to environmental allergens and so can be classified as extrinsic AD. Conversely, other cases are not due to IgE responses and are classified as intrinsic AD or “atopic-like” dermatitis. Extrinsic AD cases develop high serum IgE levels, and this IgE is directed against environmental and food allergens. Intrinsic IgE cases have normal IgE levels.

Clinical Disease

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Atopic dogs commonly present with pruritus. Initially there may be no obvious skin lesions, but this progresses to diffuse erythema (Table 30.2). Animals may present with the allergic triad: face rubbing, axillary pruritus, and foot licking. Lesions occur most commonly on front feet, the ventral abdomen, and in the inguinal and axillary regions, although they may be found anywhere on the body. These lesions are secondary to the intense pruritus and vary from acute erythema and edema to chronic secondary changes including crusting, scaling, hyperpigmentation, lichenification, and pyoderma. Some animals also develop rhinitis, otitis externa, or conjunctivitis. Dogs may also develop focal "hot spots" or urticaria (Fig. 30.6). Secondary bacterial or yeast infections complicate the disease. Depending on the inducing allergen, the extrinsic disease may or may not be seasonal and relapsing. Once it starts, it tends to get progressively more severe unless treated.

TABLE 30.2

The Diagnostic Criteria for Canine Atopic Dermatitis

The International Task Force on Canine Atopic Dermatitis has listed eight diagnostic criteria for this disease: 1. The disease mainly occurs in indoor dogs. 2. Disease onset occurs in animals under 3 years of age. 3. Animals develop a corticosteroid-responsive pruritus. 4. This pruritus is initially without obvious lesions. 5. The pruritus eventually affects the front feet. 6. The ear pinnae are affected next. 7. The ear margins are affected next. 8. The dorsolumbar area remains unaffected. Any combination of five criteria from this set will diagnose atopic dermatitis with a sensitivity of 85% and a specificity of 79% (Chapter 42).

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FIG. 30.6 A moist "hot spot" on a Golden Retriever. (Courtesy Dr. R. Kennis.)

Genetic Background

The development of canine AD is determined in part by genetic factors. While not highly heritable, disease occurrence differs between breeds. AD is most common in West Highland White Terriers, Labrador and Golden Retrievers, German Shepherds, Cocker Spaniels, Boxers, French Bulldogs, and Shar-Peis. Both susceptibility and protective gene loci have been identified in dogs. Careful clinical evaluation has recognized several disease phenotypes that differ in such features as age of onset, the presence of hot spots, gastrointestinal disorders, flexural dermatitis, and the distribution of the skin lesions. Some breeds may develop lesions in specific areas. French Bulldogs develop lesions in the axillae, eyelids, and flexural surfaces. German Shepherds, in contrast, tend to develop lesions in the elbows, hind limbs, and thorax.

Environmental Influences

Extrinsic AD is commonly associated with reactions to environmental allergens such as molds; and tree, weed, and grass pollens (especially pollens that are small and light and are produced in very large quantities); and as a result may be seasonal. Nonseasonal AD is mainly associated with allergies to house dust

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mites *(Dermatophagoides farinae* and *D. pteronyssinus),* animal danders, and the yeast *Malassezia pachydermatis.* There are no significant clinical differences between food-induced AD and that associated with environmental allergens.

Skin Lesions, Cells, Cytokines, and Chemokines

It was long believed that AD was an allergic reaction to inhaled antigens despite the fact that respiratory signs are not commonly associated with the disease. It is more likely, however, that allergen exposure occurs through the skin. Antigens are trapped by Langerhans cells and then presented to γ/δ T cells. T cells appear to play a critical role in the pathogenesis of AD, with type 2 responses predominating in the first 24 hours and mixed type 1 and 2 responses in the later, chronic disease (48–96 hours) (Fig. 30.7). Lesions from patients with both forms of AD are infiltrated with T cells and dendritic cells. However, Th17- and Th22-derived cytokines are elevated only in those with intrinsic AD. Conversely, lesions from patients with extrinsic AD have higher Th2 cytokines (IL-4, IL-13, and IL-5). The suppressive cytokine TGF-β is underexpressed in both.

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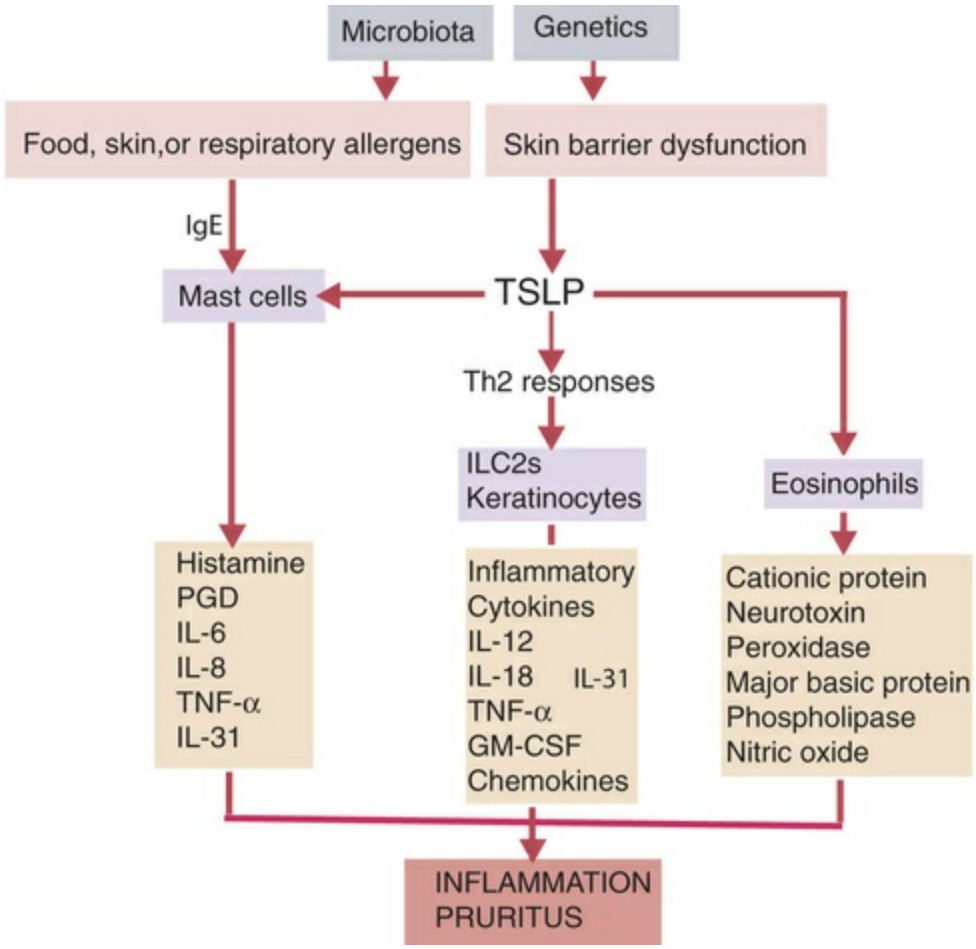


FIG. 30.7 A simplified view of the pathogenesis of atopic dermatitis. Note the central role of Thymic stromal lymphopoietin (TSLP).

Thymic stromal lymphopoietin (TSLP) is upregulated in inflamed skin, especially when associated with epidermal barrier dysfunction. This TSLP is produced by Th2 cells, keratinocytes, and mast cells in response to antimicrobial peptides such as β-defensins and cathelicidins. TSLP is a potent stimulator of Th2 cytokines IL-4, IL-5, and IL-13, and these in turn promote IgE production. Its concentration is very high in lesional skin of humans. Intrinsic AD cases express lower levels of IL-4, IL-5, and IL-13.

Pruritus

Chronic severe itching is one of the most important and distressing features of canine AD. It is caused by mediators released from resident skin cells (Fig. 30.8). These mediators bind to itch-specific neuronal receptors (pruriceptors) that are linked to specific areas of the brain. Itch mediators include histamine, some prostaglandins

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and leukotrienes, some neuropeptides, and IL-31.

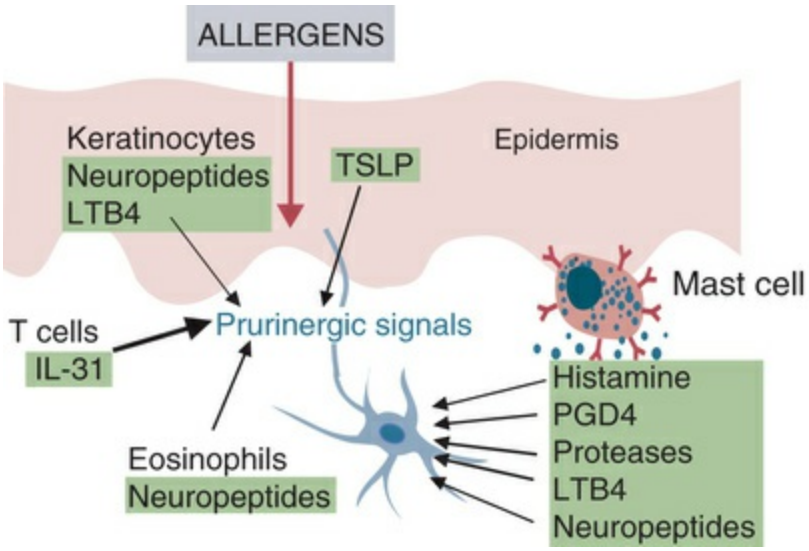


FIG. 30.8 The major mediators associated with pruritus. The most significant of these include histamine, thymic stromal lymphopoietin (TSLP), and interleukin-31 (IL-31). These act on specialized nerve receptors within the skin to induce itch.

In addition, epithelial cells can communicate directly with cutaneous sensory neurons using TSLP. TSLP from keratinocytes enables them to communicate directly with TSLP-sensitive neurons through a pathway that does not require immune stimulation. It is unclear what the relationship is between itch induced by TSLP and itch induced through immune-cell neuronal communication and histamine.

Since canine mast cells contain histamine, it has long been assumed that histamine is the primary mediator of AD. It causes vasodilatation, pain, and itching. However, histamine levels do not correlate well with disease severity, and plasma histamine levels do not differ significantly between normal and atopic dogs. Thus this too suggests that AD is not simply a type I hypersensitivity reaction.

The cytokine IL-31 causes severe pruritus in dogs and is elevated in AD. Peripheral blood mononuclear cells from dogs exposed to allergen (house dust mites) plus Staphylococcal enterotoxin B produce high levels of IL-31. T cell mitogens also stimulate IL-31 production suggesting that T cells are the source of this cytokine.

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The IL-31 receptor is expressed on canine mononuclear cells, keratinocytes, and dorsal root ganglia. In humans with AD or allergic contact dermatitis, the level of IL-31 mRNA is higher in atopic skin lesions than in normal skin. Thus the combination of allergens and the presence of bacteria may stimulate T cells, keratinocytes, and neurons to trigger the inflammation and pruritus of atopic dermatitis using IL-31. The synthetic Janus kinase (JAK) inhibitor oclacitinib maleate may reduce pruritus in many dogs and cats with AD. It blocks signal transduction by JAK1 and JAK3 and as a result inhibits the activities of IL-31 and several other cytokines. It can therefore reduce both the pruritus and the severity of the dermatitis and improve the quality of life for many of these animals. Excessive blocking of IL-31 function may be undesirable since IL-31 regulated genes are also involved in forming an intact skin barrier, and IL-31 stimulates the production of antimicrobial peptides (Box 30.1).

Box 30.1

Monoclonal Antibody Therapy for Atopic Dermatitis

As noted in the text, interleukin-31(IL-31) is the major cause of the severe itching observed in atopic dermatitis (AD) in dogs. The production of IL-31 in affected skin can be inhibited by the JAK inhibitor oclacitinib. It can also be neutralized by administration of a caninized monoclonal antibody—Lokivetmab, directed specifically at canine IL-31. The antibody is injected subcutaneously. It binds to circulating IL-31 and inhibits its binding to the IL-31 receptor. In double-blind, placebo-controlled trials, a single dose has provided relief from itch and a reduction in disease severity in dogs with chronic AD.

Michels GM, Ramsey DS, Walsh KF, et al. A blinded, randomized, placebo-controlled, dose determination trial of lokivetmab (ZTS-00103289), a caninized, anti-canine IL-31 monoclonal antibody in client owned dogs with atopic dermatitis. *Vet Dermatol* 27: 478–e129, 2016.

The Role of IgE

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The division of AD into intrinsic and extrinsic disease results from the observation that there is no consistent correlation between IgE levels and the severity of clinical AD in dogs, and not all AD cases are associated with IgE antibodies to environmental antigens (Box 30.2). Serological assays that measure IgE antibodies to the offending allergens rarely correlate with disease severity or the levels of IgE in the skin. Blood IgE levels may drop to undetectable levels, whereas levels in skin and skin reactivity remain high. Intradermal testing of pruritic dogs shows that many are sensitive to house dust mites, various pollens (trees, weeds, grasses), and epidermal allergens, as well as some seasonal allergens (Fig. 30.9). However, skin testing of clinically normal dogs also elicits positive reactions to these same antigens. For example, 50% to 90% of clinically normal dogs react to house dust allergens. Allergen-specific IgE may also be detected in dogs that have no clinical signs of AD; however, IgE levels cannot discriminate between normal dogs, dogs with intestinal parasites, or dogs with AD. Additionally, about a quarter of canine cases are unreactive to intradermal antigens and lack any allergen-specific IgE.

Box 30.2

Nomenclature

IgE mediates immediate hypersensitivity reactions, so-called because they develop within seconds or minutes after exposure to antigen. This type of hypersensitivity reaction is also commonly called an allergy. Antigens that stimulate allergies may be called allergens. If an immediate hypersensitivity reaction is systemic and life threatening, it is called allergic anaphylaxis or anaphylactic shock. Sometimes an animal may have a reaction that is similar to allergic anaphylaxis but is not immunologically mediated. This type of reaction is described as anaphylactoid.

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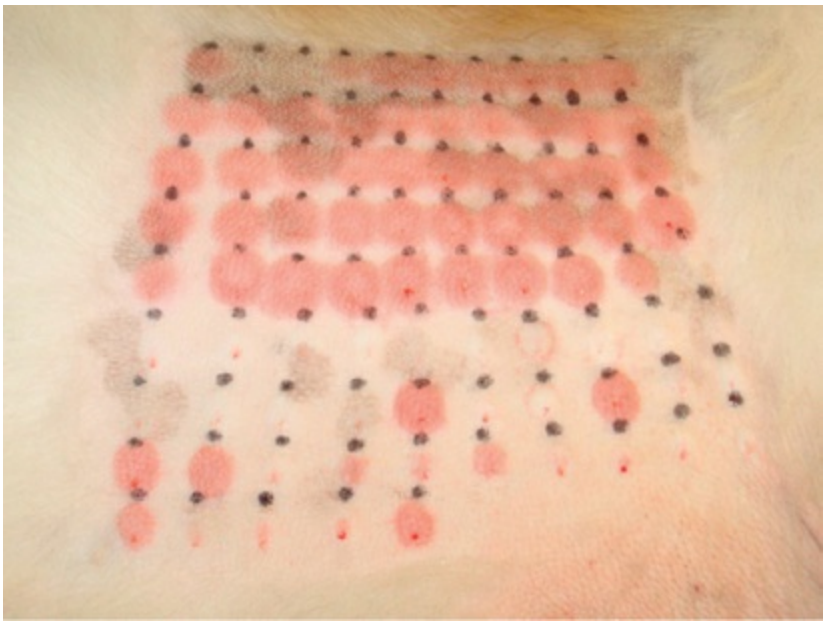


FIG. 30.9 A panel of intradermal skin tests performed on a dog. Each spot denotes a position where a small volume of dilute allergen was injected intradermally. If the dog is allergic to that allergen a local reaction characterized by redness and swelling occur around the injection site. (Courtesy Dr. R. Kennis.)

Epidermal Barrier Dysfunction

In some cases of AD, the initial lesion may be a defect in epithelial cells leading to skin barrier dysfunction. This permits loss of water as well as the penetration of allergens and microbes and increases contacts between the skin immune system and environmental allergens (Fig. 30.10). Defects in either the skin barrier protein filaggrin, or in skin lipids such as ceramides or sphingosine-1-phosphate, may increase skin water loss and its susceptibility to irritation. Atopic dogs may also show defects in the expression of tight junction proteins such as occludin and zonula occludens 1 (ZO-1).

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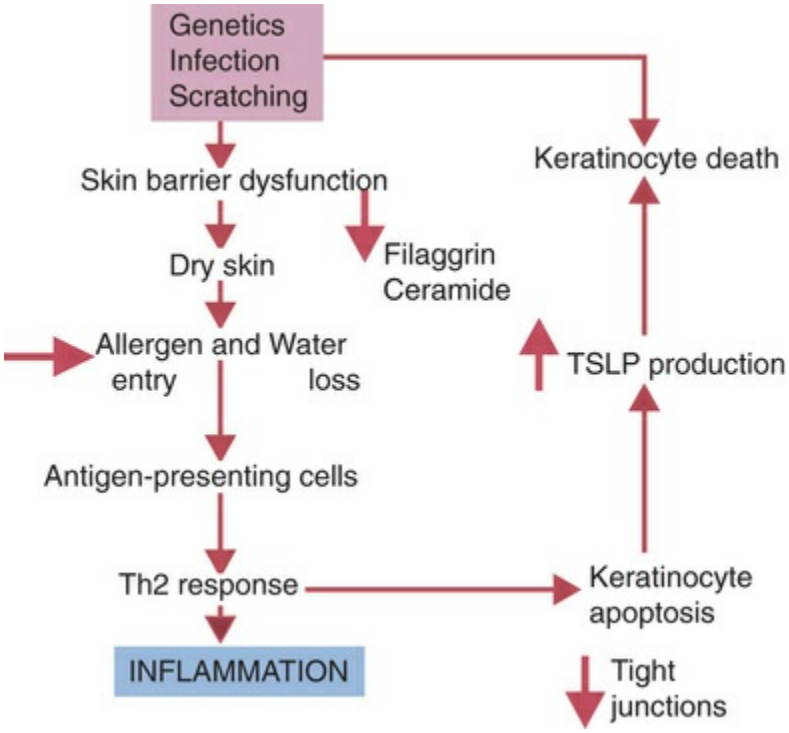


FIG. 30.10 A key contributor to the development of atopic dermatitis is skin barrier dysfunction. This is influenced by genetic, climatic, and allergic factors and exacerbated by scratching.

Filaggrin is a filament-associated protein involved in cross-linking keratin fibers in epidermal cells. It is a major barrier protein that helps the skin retain fluid. Single nucleotide polymorphisms (SNPs) within the canine filaggrin gene have been associated with some forms of canine atopic dermatitis. The importance of filaggrin in canine atopic dermatitis is unclear. It is possible that filaggrin defects only account for a subset of canine AD cases, or alternatively, filaggrin defects are secondary to some other cause of AD. Likewise, there is no clear evidence as yet that improving skin barrier function is of clinical benefit in the treatment of atopic dogs.

Infections

The severity of canine AD is influenced by (and influences) the skin microbiota. The skin microbiota is altered in allergen-induced canine AD where the lesions have evidence of dysbiosis. This is associated with a relative increase in *Staphylococcus pseudintermedius.* Recurrent staphylococcal skin infections are associated with canine AD. These staphylococci may release toxins or superantigens (Chapter 7) that promote skin inflammation. The skin microbiota also influences expression of the C5a receptor that

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regulates some immune defense genes.

Malassezia skin and ear infections are also common in canine AD cases. The yeast *Malassezia pachydermatis* produces a phospholipase that may disrupt skin cell membranes and initiate a secondary dermatitis either by directly triggering inflammation or by acting as an allergen. Dogs with atopic dermatitis have higher mRNA expression of β-defensins in infected, atopic skin, but this may simply reflect the presence of secondary infections. Environmental pollution also influences the development of AD (Box 30.3).

Box 30.3

The Aryl Hydrocarbon Receptor and Atopic Dermatitis

As noted in Box 21.1, the aryl hydrocarbon receptor (AhR) was originally described by toxicologists investigating the toxicity of environmental pollutants such as aromatic hydrocarbons and dioxanes. It has now been shown that there is a direct link between environmental pollution, the AhR, and the development of atopic dermatitis (AD).

Thus air pollutants such as diesel exhaust particles bind to the AhR. The receptor then translocates to the cell nucleus and upregulates multiple genes. These include the genes encoding IL-33 and TSLP, both of which trigger pruritus. Additionally, the AhR upregulates a protein called artemin in keratinocytes. Artemin acts on peripheral sensory nerves to promote sprouting of peripheral nerve fibers into the epidermis and upregulates expression of the capsaicin and wasabi receptors. (Both of these detect chemical irritants.) Collectively, these changes induce extreme skin sensitivity. This can explain how allergic individuals develop "allokinesis"—where mild skin stimulation triggers intense pruritus.

Interestingly, the endogenous ligands of AhR include products from the yeast *Malassezia* as well as from intestinal bacteria such as lactobacilli and some foods. All have been associated with the development of AD in pets. Likewise, it is no coincidence that dogs in households where smoking occurs are significantly more likely to develop AD than those in non-smoking homes.

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Hidaka T, Ogawa E, Kobayashi EH, et al: The aryl hydrocarbon receptor AhR links atopic dermatitis and air pollution via induction of the neurotrophic factor artemin. *Nature Immunology,* 18:64-73, 2017.

Ka D, Marignac G, Desquilbet L, et al: Association between passive smoking and atopic dermatitis in dogs. *Food and Chemical Toxicology,* 66:329-333, 2014.

Allergies to Vaccines and Drugs

An IgE response may result from the administration of any antigen, including vaccines. It is most likely to occur in vaccines that contain trace amounts of fetal calf serum (specifically bovine serum albumin), gelatin, or casein. Severe allergies have been associated with the use of killed foot-and-mouth disease, rabies, and contagious bovine pleuropneumonia vaccines in cattle. IgE responses may also occur following administration of drugs. Most drug molecules are too small to be antigenic, but many can bind to host proteins and then act as haptens. Penicillin allergy, for example, may be triggered either by therapeutic exposure or by ingestion of penicillin-contaminated milk. In sensitized animals, injection of penicillin may cause acute systemic anaphylaxis or milder forms of allergy. Feeding of penicillin-contaminated milk to these animals can lead to severe diarrhea. Allergies to many drugs, especially antibiotics and hormones, have been reported in the domestic animals. Even substances contained in leather preservatives used in harnesses, in catgut sutures, or compounds such as methylcellulose or carboxymethylcellulose used as stabilizers in vaccines may provoke allergies. Given the diversity of drugs used in animals, it is unsurprising that multiple mechanisms may be involved. Even hypersensitivities to an individual drug might be mediated through multiple pathways.

Allergies to Parasites

The beneficial role of the IgE–mast cell–eosinophil system in immunity to parasitic worms was first observed in the "self-cure" phenomenon (Chapter 28). Helminths preferentially stimulate IgE responses, and helminth infestations are commonly associated with

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many of the signs of allergy and anaphylaxis; for example, animals with tapeworms may show respiratory distress or urticaria. Anaphylaxis may be provoked by rupture of a hydatid cyst during surgery or through transfusion of blood from a dog infected with *Dirofilaria immitis* to a sensitized animal.

Allergies are also commonly associated with exposure to arthropod antigens. Chitin, the major component of arthropod exoskeletons and fungal cell walls, is an effective trigger of immediate hypersensitivity. Chitin exposure promotes eosinophil recruitment and Th2 responses. Insect stings account for many human deaths each year as a result of acute anaphylaxis following sensitization to venom. Anaphylaxis can also occur in cattle infested with the warble fly *(Hypoderma bovis)* if their subcutaneous pupae rupture and the animal reacts to the released coelomic fluid.

In horses and cattle, hypersensitivity to insect bites may cause an allergic dermatitis variously called Gulf Coast itch or sweet itch. The insects involved include midges (*Culicoides* species), black flies (*Simulium* species), stable flies *(Stomoxys calcitrans),* mosquitoes, and stick-tight fleas *(Echidnophaga gallinacea).* If animals are allergic to antigens in the saliva of these insects, biting results in the development of urticaria accompanied by intense pruritus. The itching may provoke self-mutilation with subsequent secondary infection that may mask the original allergic nature of the lesion. It is interesting to note that IgE sensitization of skin mast cells is common in clinically healthy horses exposed to *Culicoides* midges, so allergic disease is not an inevitable result of exposure and sensitization. There is a major genetic component to this type of hypersensitivity.

Animals do not inevitably respond to arthropod allergens with a type I hypersensitivity. Thus, responses to *Demodex* mites and to flea saliva may be cell mediated (type IV hypersensitivity, Chapter 33). Flea-bite allergic dermatitis is the single most important allergic skin disease. There is no breed or gender predisposition, but atopic animals as well as those exposed to fleas on an intermittent basis tend to get more severe disease. Continual exposure to fleas at an early age appears to result in hyposensitization. Pruritus is a consistent feature, as is a history of flea infestation. Affected animals, in addition to the characteristic clinical signs, show a

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reaction to intradermally injected flea antigen. Most sensitive animals will respond within a few minutes, but up to 30% may show a delayed reaction at 24 to 48 hours. Hyposensitization therapy has not been shown to be successful in treating flea allergy. Flea allergy can be successfully treated only by total flea control.

Eosinophilic Granuloma Complex

The eosinophilic granuloma complex is a group of diseases associated with various types of skin lesions (i.e., ulcer, plaque, granuloma) in cats. Although their cause is unknown, they have been associated with flea or food allergies or atopic dermatitis. The presence of eosinophils in skin is often associated with the development of pathological lesions (Fig. 30.11). Thus when purified eosinophil cationic protein (ECP) or eosinophil-derived neurotoxin (EDN) are injected into guinea pig or rabbit skin, they disrupt skin integrity and cause inflammation. ECP produces ulcers, whereas EDN produces cellular exudates. Purified eosinophil peroxidase and the chemokine MBP-1 produce induration and erythema. The activities of these proteins may explain the development of lesions in eosinophil-associated skin diseases.

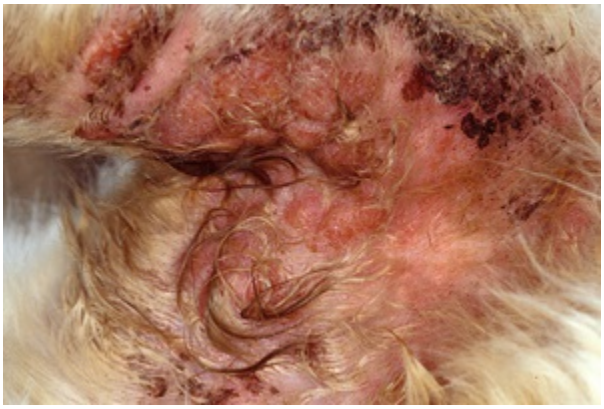


FIG. 30.11 An eosinophilic granuloma on the footpad of a dog.
(Courtesy Dr. R. Kennis.)

A seasonal eosinophilic lesion in cats has been associated with mosquito bites. This may present as scattered individual crusted papules. The eosinophilic plaques in the skin are intensely pruritic.

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As a result, the lesions may be masked by self-inflicted trauma and secondary bacterial infection. Histologically, they are associated with a local mast cell and eosinophil infiltration, as well as an eosinophilia. Eosinophilic granulomas, in contrast, are not pruritic, and present as a line of raised pink plaques on the skin. Some may present as scattered individual crusted papules. Linear eosinophilic ulcers (sometimes called indolent or rodent ulcers) are commonly located in the oral cavity or on the lips. Removal of the offending allergen may result in clinical improvement, and corticosteroid treatment is usually of benefit. The linear form and indolent ulcers may be difficult to treat and may require more aggressive therapy. An idiopathic hypereosinophilic syndrome has been described in humans, cats, and dogs. It is characterized by a prolonged, unexplained eosinophilia, the infiltration of many organs with eosinophils, organ dysfunction (affecting especially the heart, but also the lungs, spleen, liver, skin, bone marrow, gastrointestinal tract, and central nervous system), and death. An eosinophilic enteritis may result from canine hookworm infestation.

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Diagnosis of Type I Hypersensitivity

Diagnosis of allergic disease is based on history, and if possible, the identification of the offending allergens by direct skin testing or serology and the exclusion of other similar conditions.

The term *hypersensitivity* is used to denote inflammation that occurs in response to normally harmless material. For example, animals normally do not react to antigens injected intradermally. If, however, a hypersensitive animal is given an intradermal injection of allergen, it will provoke inflammation (see Fig. 30.9). Vasoactive molecules are released to produce redness (erythema) as a result of capillary dilation, as well as circumscribed edema (a wheal) due to increased vascular permeability. The reaction may also generate an erythematous flare due to arteriolar dilation caused by a local axon reflex. This wheal-and-flare response to an allergen reaches maximal intensity within 30 minutes and then disappears within a few hours. A late-phase reaction sometimes occurs 6 to 12 hours later.

Intradermal skin testing using very dilute aqueous solutions of allergens has been widely used for the diagnosis of allergies, especially canine AD. Following injection, the site is examined for an inflammatory response. The results obtained must be interpreted carefully since both false-positive and false-negative responses may occur. For example, the concentration of antigen in commercial skin testing solutions may be too low. Dogs may be up to 10 times less sensitive than humans to intradermal allergens such as pollens, fungi, or danders. False-positive reactions may be due to the presence of preservatives in allergen solutions. False-negative responses may be due to steroid treatment. The mixture of allergens used for intradermal skin testing commonly includes allergens from trees, grasses, fungi, weeds, danders, feathers, house dust mites, and insects. Intradermal skin testing is less commonly performed in cats because they fail to develop a significant wheal, and the reaction is therefore difficult to evaluate.

An experimental technique used to detect IgE antibodies is called the passive cutaneous anaphylaxis (PCA) test. In this test, dilutions of test serum are injected at different sites into the skin of a normal

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animal. After waiting 24 to 48 hours, the antigen solution is administered intravenously. In a positive reaction, each injection site will show an immediate inflammatory response. The injected antibodies may remain fixed in the skin for a very long period. In the case of the calf, this may be up to 8 weeks. Because it is sometimes difficult to detect very mild inflammatory responses, they can be made more visible by injecting the test animal intravenously with Evans blue dye. The dye binds to serum albumin and does not normally leave the bloodstream. At injection sites where vascular permeability is increased, the dye-labeled albumin enters the tissue fluid and forms a striking blue patch (Fig. 30.12).



FIG. 30.12 PCA reactions in a calf. Several different sera were tested for PCA activity on the flank of a normal calf. (Courtesy Dr. P. Eyre.)

Serological methods of measuring the level of specific IgE in body fluids include the RAST (radioallergosorbent test), Western blotting, and ELISA (Chapter 42). These are not subject to clinical bias, but there has been a poor correlation between the results obtained by serology or skin testing and clinical severity. There is also a poor correlation between ELISA results and intradermal testing. Serological assays are especially prone to a high level of false-positive results (low specificity). A negative ELISA will

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generally rule out extrinsic disease. Best results are obtained by testing for individual allergens rather than groups of allergens. The reasons for this poor correlation between direct IgE measurements and *in vivo* methods such as skin testing probably reflect the occurrence of intrinsic AD. Heavily parasitized dogs may have elevated IgE levels, and this may result in false-positive serological results. It is also possible that immunoglobulins of classes other than IgE may contribute to the development of allergic dermatitis in the dog. For these reasons, many veterinary dermatologists prefer intradermal skin testing to serology.

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Treatment of Type I Hypersensitivity

By far the most satisfactory treatment of extrinsic allergic disease is avoidance of exposure to the allergen. Specific immunotherapy gives good responses in up to 80% of cases, but secondary infections such as bacterial or yeast *(Malassezia)* infections or flea infestations must also be controlled. Allergen-specific immunotherapy may also be used. This has the potential to induce stable, long-term remissions but is not a substitute for avoidance. Topical therapy such as bathing with mild shampoos helps considerably. Diets enriched in the omega-3 fatty acids eicosapentaenoic acid and docosahexaenoic acid may be beneficial when fed to dogs with chronic allergic dermatitis. Omega-3 (fish) oil or omega-6 (evening primrose) oil probably promote the synthesis of antiinflammatory eicosanoids.

The principal indications for drug therapy include short-term temporary relief either while waiting to begin immunotherapy or while waiting for it to take effect. Drugs may also be useful for relief of transient recurrences or in animals in which immunotherapy is not possible. Many different drugs are available to treat type I hypersensitivity, although veterinarians tend to employ only a few of these.

Topical or oral corticosteroids are most commonly used to reduce the irritation and inflammation associated with the acute allergic response. These drugs can suppress all aspects of inflammation by inhibiting NF-κB and blocking the production of inflammatory mediators (Chapter 41). Corticosteroids have a considerable palliative effect on chronic type I hypersensitivities but can have serious side effects. They are immunosuppressive and can increase susceptibility to infection. The effective dose of prednisolone may be significantly reduced by the use of an oral essential fatty acid supplement. Other immunosuppressants such as the calcineurin inhibitors, cyclosporine, and tacrolimus may have similar benefits.

The β-stimulants include epinephrine, isoprenaline, and salbutamol; *α*-antagonists include methoxamine and phenylephrine. All have been used extensively in humans and are available for use in animals. Epinephrine is the most important

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drug used to treat anaphylaxis. It is rapidly absorbed following intramuscular injection and thus can rapidly reverse the clinical signs of shock. Another group of drugs widely employed in the treatment of type I hypersensitivity reactions are the specific pharmacological inhibitors. These drugs, by mimicking the structure of the active mediators, competitively block specific receptors. Thus H1 antihistamines such as diphenhydramine can effectively inhibit the activities of histamine. However, since histamine is but one of a large number of mast cell–derived mediators, and its levels do not correlate well with skin disease severity, antihistamines are of limited effectiveness in controlling allergic diseases in animals.

A multifaceted approach has been recommended for the treatment of AD. Thus any bacterial or yeast infections must be treated. Acute flares may be treated with a combination of skin baths and topical corticosteroids, with oral corticosteroids and antibiotics as needed. Skin hygiene should be improved as much as possible. The severity of the pruritus may be reduced with combinations of antiinflammatory drugs. Treatment of chronic pruritus and skin lesions include oral and topical corticosteroids, oral cyclosporine, and topical tacrolimus, in addition to oclacitinib and possibly oral interferons. Allergen-specific immunotherapy should be offered when feasible.

Allergen-Specific Immunotherapy

Allergies may be controlled by allergen-specific immunotherapy. This involves administering gradually increasing quantities of an allergen to the animal in order to reduce the severity of subsequent allergic disease. Multiple controlled studies have shown that this therapy is effective in humans. It appears to be most effective for the treatment of allergic rhinitis (hay fever), asthma, and allergies to insect stings. Its effectiveness is less clear in the treatment of food allergies and allergic dermatitis. In veterinary medicine, multiple open studies have suggested that this therapy is effective in the treatment of AD, although few randomized controlled trials have been published.

Immunotherapy injections promote IgG rather than IgE

production and reduce the recruitment of inflammatory cells. In humans, this reduces mast cell and eosinophil numbers in the lung, as well as the infiltration of $CD4^+$ T cells and eosinophils in the skin. It induces a shift in the dominant helper cell response from Th2 to Th1 (Fig. 30.13). For example, the IFN-γ/IL-4 ratio is low in atopic dogs, indicating a Th2 cytokine profile. After immunotherapy, the ratio rises, IFN-γ levels increase, and the balance shifts toward a Th1 response. This IFN-γ reduces the effects of Th2 cells on IgE antibody synthesis and shifts allergen-specific immunoglobulin production from IgE to IgG. Immunotherapy may also induce dendritic cell production of IL-12 and IL-18 and promote Th1 responses. It also stimulates Tregs to produce IL-10, inhibiting IgE production, mast cell activation, and histamine and leukotriene release.

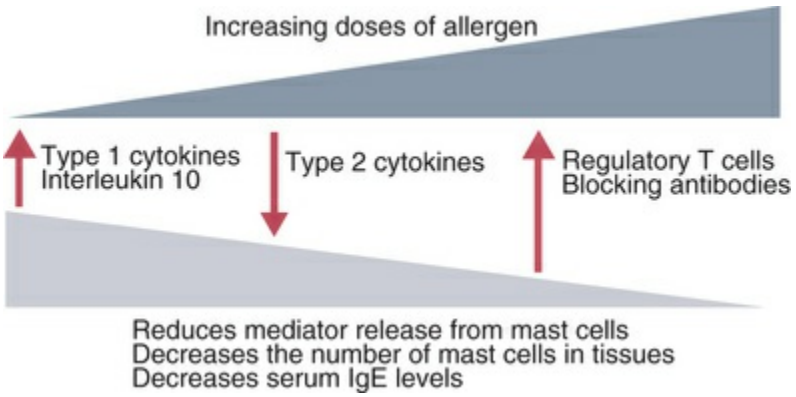


FIG. 30.13 The principles of allergen-specific immunotherapy. Increasing doses of allergen promote a Th1 response, while at the same time reducing the Th2 response and regulating antibody production.

In immunotherapy, small amounts of dilute aqueous solutions of antigen are administered. The first injections contain very little allergen. Over a number of weeks, the dose is gradually increased. If an animal's allergy is of the seasonal type, the course of injections should be timed to reach completion just before the anticipated antigen exposure. It has been estimated that up to 80% of dogs have a good to excellent response to this procedure. This includes clinical improvement and a reduction in the amount of medication required. It may take several months before the benefits of immunotherapy become apparent. Cats may respond even better

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than dogs. On the other hand, horses with hypersensitivity to biting flies have a poor response to immunotherapy. Sublingual immunotherapy may also be an effective treatment modality. It works well in humans but is largely untested in domestic species. It appears to work well against dust mite and food allergy in dogs.

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31

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Red Cell Antigens and Antibody-Mediated Hypersensitivity

CHAPTER OUTLINE

Blood Groups, 348
Blood Transfusion and Incompatible Transfusions, 349
Hemolytic Disease of the Newborn, 349
Blood Groups, Blood Transfusion, and Hemolytic Disease in Domestic Animals, 350
Horses, 350
Cattle, 351
Bovine Neonatal Pancytopenia, 352
Sheep, 353
Pigs, 353
Dogs, 354
Cats, 354
Humans, 355
Parentage Testing, 355
Hemophagocytic Syndrome, 355
Type II Hypersensitivity Reactions to Drugs, 356
Type II Hypersensitivity in Infectious Diseases, 356

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LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Explain how type II hypersensitivity diseases occur when antibodies (and complement) destroy normal cells.
- Describe how the destruction of transfused red blood cells is an example of type II hypersensitivity.
- Explain the clinical signs and treatment of an incompatible transfusion response.
- Explain the pathogenesis of hemolytic disease of the newborn.
- Explain the pathogenesis of neonatal pancytopenia in calves.
- Understand why some drugs may bind to blood cells and make them targets of antibodies in a type II hypersensitivity reaction.
- Describe the treatment of hemolytic disease and neonatal pancytopenia.
- Explain how blood group testing may be used to establish paternity.

Red blood cells, like nucleated cells, express cell surface glycoproteins and glycolipids. Unlike the major histocompatibility complex (MHC) molecules, these molecules are not involved in antigen processing, although they do influence graft rejection (allografts between blood group–incompatible animals are rapidly rejected). Most are functional components of the cell membrane. For example, the ABO glycoproteins in humans are anion and glucose transporter proteins, while the molecules of the M and C systems of sheep red cells are associated with the membrane potassium pump and amino acid transport, respectively (Box 31.1).

Box 31.1

The Intestinal Microbiota and Blood Groups

Intestinal mucus is "decorated" with blood-group antigens. These are synthesized by enterocytes, but only in certain individuals (secretors). These blood group antigens influence the composition of the intestinal microbiota. Thus Bifidobacteria depend on these

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blood group antigens to colonize the gut and so are much less common in nonsecretors. Nonsecretor humans are more susceptible to necrotizing enterocolitis and Gram-negative sepsis in premature infants, presumably because they lack the protective benefits of the Bifidobacteria. On the other hand, noroviruses can also attach through these glycans when they colonize the gut, so nonsecretors are resistant to these agents!

If blood is transfused from one animal to another, genetically different individual, these red cell molecules will act as powerful antigens and stimulate antibody responses in the recipient. These antibodies cause the rapid elimination of the transfused red cells by intravascular hemolysis by complement and by extravascular destruction through opsonization and removal by the mononuclear phagocyte system. Cell destruction by antibodies in this way is classified as a type II hypersensitivity reaction.

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Blood Groups

The molecules expressed on the surface of red blood cells are called blood group antigens or erythrocyte antigens (EAs). There are many different blood group antigens, and they vary in their antigenicity, some being more potent and therefore of greater importance than others. The expression of blood group antigens is controlled by genes and inherited in conventional fashion. For each blood group system, there are a variable number of alleles. (If blood group alleles are inherited together in groups of two or more, they are called phenogroups.) The complexity of erythrocyte blood group systems varies greatly. They range from simple systems like the L system of cattle, which consists of two alleles coding for a single antigen, to the highly complex B system of cattle. The B system contains several hundred alleles or phenogroups that, together with the other cattle blood groups, may yield millions of unique blood group combinations. Although most blood group antigens are integral cell membrane components, some are found free in serum, saliva, and other body fluids, and passively adsorbed onto red cell surfaces. Examples of such soluble antigens include the J antigens of cattle, the R antigens of sheep, the A antigens of pigs, and the DEA 7 antigens of dogs.

Animals may make antibodies against foreign blood group antigens even though they may never have been exposed to foreign red cells. For example, J-negative cattle have anti-J antibodies in their serum, and A-negative pigs have anti-A antibodies. These "natural" antibodies (or isoantibodies) are derived not from previous contact with foreign red cells but from exposure to cross-reacting epitopes that are commonly encountered in nature (see Fig. 9.8). Many blood group antigens are also common structural components of plants, the intestinal microbiota, protozoa, or helminths. The presence of these natural antibodies is not, however, a uniform phenomenon, and not all blood group antigens are accompanied by the production of natural antibodies to their alternative alleles.

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Blood Transfusion and Incompatible Transfusions

Blood is easily transfused from one animal to another. If the donor red cells are identical to those of the recipient, no immune response results. If, however, the recipient possesses preexisting antibodies to donor red cell antigens, the transfused cells will be attacked immediately. The preexisting antibodies are usually of the IgM class. When these antibodies bind red cell antigens, they can cause agglutination or hemolysis, or stimulate opsonization and phagocytosis of the transfused cells. In the absence of preexisting antibodies, the transfused red cells will stimulate an immune response in the recipient. The transfused cells will circulate until antibodies are produced and will then be eliminated. A second transfusion with identical foreign cells results in their immediate destruction.

The rapid destruction of large numbers of foreign red cells can lead to serious illness. The severity of transfusion reactions ranges from a mild febrile response to rapid death, depending on the amount of incompatible blood transfused. Early recognition of a problem may avert the most severe consequences. These reactions occur when large amounts of incompatible blood are transfused to a sensitized recipient. This results in complement activation and lysis of the transfused cells. Large amounts of free hemoglobin escape, resulting in hemoglobinemia and hemoglobinuria. The lysed red cells may trigger blood clotting and disseminated intravascular coagulation. Complement activation also results in anaphylatoxin production, mast cell degranulation, and the release of vasoactive molecules and cytokines. These molecules may provoke shock with hypotension, bradycardia, and apnea. The animal may show sympathetic responses such as sweating, salivation, lacrimation, diarrhea, and vomiting. This may be followed by a second stage in which the animal is hypertensive, with cardiac arrhythmia as well as increased heart and respiratory rates.

If a reaction is suspected, the transfusion must be stopped

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immediately. It is important to maintain urine flow with fluids and a diuretic because hemoglobin may cause renal tubular destruction. Recovery follows elimination of the foreign red cells.

Transfusion reactions can be almost totally prevented by prior testing of the recipient's serum for antibodies against the donor's red cells. The test is called cross-matching. Blood from the donor is centrifuged and the plasma discarded. The red cells are then resuspended in saline and recentrifuged. This washing procedure is repeated (usually three times), and eventually a 2% to 4% suspension of red cells in saline is made. These donor red cells are mixed with recipient serum and incubated at 37° C for 15 to 30 minutes. If the donor red cells are lysed or agglutinated by the recipient's serum, no transfusion should be attempted with those cells. It is occasionally found that the donor's serum may react with the recipient's red cells. This is not of major clinical significance because transfused donor antibodies are rapidly diluted within the recipient. Nevertheless, blood giving such a reaction is best avoided.

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Hemolytic Disease of the Newborn

Female animals may become sensitized to foreign red cells not only by incompatible blood transfusions given for clinical purposes but also by leakage of fetal red cells into their bloodstream through the placenta during pregnancy. Once sensitized, these anti-red cell antibodies may then be concentrated in maternal colostrum. When a newborn animal suckles, these colostral antibodies are absorbed through the intestinal wall and enter its bloodstream. These antibodies, directed against the blood group antigens of the newborn, cause rapid destruction of their red cells. The resulting disease is called hemolytic disease of the newborn (HDN) or neonatal isoerythrolysis.

Four conditions must be met for HDN to occur: The young animal must inherit a red cell antigen from its sire that is not present in its mother. The mother must be sensitized to this red cell antigen. The mother's response to this antigen must be boosted repeatedly by transplacental hemorrhage or repeated pregnancies. Finally, a newborn animal must ingest colostrum containing high-titered antibodies to its red cells.

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Blood Groups, Blood Transfusion, and Hemolytic Disease in Domestic Animals

All mammals possess red cell antigens that can affect blood transfusions and on occasion cause HDN in newborn animals (Table 31.1). Although historically they were named alphabetically in order of their discovery, there is a growing tendency to add the prefix EA (erythrocyte antigen) to reduce confusion with MHC antigens.

TABLE 31.1
Domestic Animal Blood Groups

Species	Blood Group Systems	Serology
Horse	EAA, C, D, K, P, Q, U	Agglutination Hemolytic
Bovine	EAA, B, C, F, J*, L, M, R*, S, Z, T′	Hemolytic
Sheep	EAA, B, C, D, M, R*	Hemolytic Agglutination (D only)
Pig	EAA*, B, C, D, E, F, G, H, I, J, K, L, M, N, O, P	Agglutination Hemolytic Antiglobulin
Dog	DEA 1.1, 1.2, 3, 4, 5, 6, 7*, 8	Agglutination Hemolytic Antiglobulin
Cat	AB	Agglutination Hemolytic

*Soluble blood group substances.

Horses

Horses possess seven internationally recognized blood group systems (EAA, EAC, EAD, EAK, EAP, EAQ, and EAU). Some, such as EAC, EAK, and EAU, are simple, one-factor, two-allele, two-phenotype systems. On the other hand, the EAD system is very complex, with at least 25 alleles identified to date. About 10% of horses have antibodies against other blood groups, especially Aa and Ca. These antibodies can cause severe reactions following incompatible blood transfusions. Their major significance lies in the fact that HDN in foals is relatively common (Fig. 31.1). In mules, in

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which the antigenic differences between dam and sire are great, about 8% to 10% of foals may be affected. In Thoroughbreds and Standardbreds, the prevalence is considerably less, ranging from 0.05% to 2% of foals. This is despite of the fact that in up to 14% of pregnancies, the mare and the stallion have incompatible red cells.

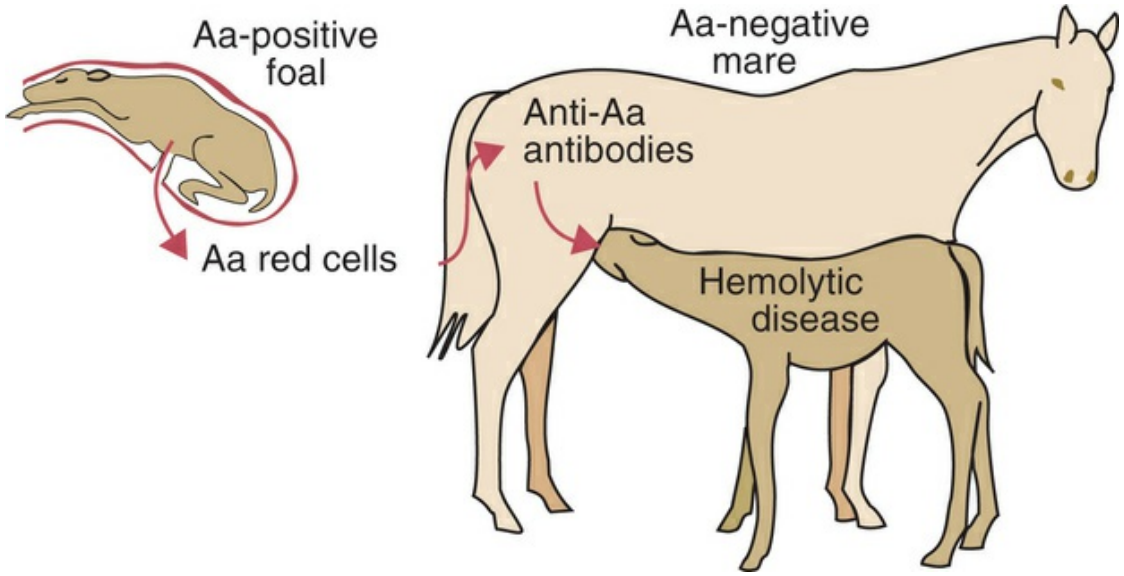


FIG. 31.1 The pathogenesis of hemolytic disease of the newborn in foals. In the first stage, fetal red blood cells leak into the mother's circulation and sensitize her. In the second stage, these antibodies are concentrated in colostrum and are then ingested by the suckling foal. These ingested antibodies enter the foal's circulation and cause red cell destruction.

HDN may occur in foals from mares that have been sensitized by previous blood transfusions or by administration of vaccines containing equine tissues. Most commonly, however, mares are sensitized by exposure to fetal red cells as a result of repeated pregnancies. The mechanism of this sensitization is unclear, but fetal red cells are assumed to gain access to the maternal circulation as a result of transplacental hemorrhage. Mares have been shown to respond to fetal red cells as early as day 56 after conception. The greatest leakage probably occurs during the last month of pregnancy and during foaling as a result of the breakdown of placental blood vessels.

Maternal sensitization is usually minimal following a first pregnancy. However, if repeated pregnancies result in exposure to the same red cell antigens, the maternal response will be boosted.

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Hemolytic disease is therefore usually only a problem in mares that have had several foals. The most severe form of the disease results from the production of antibodies directed against the Aa antigen of the EAA system. Anti-Qa (EAQ system) produces a less severe disease of slower onset. In practice, 90% of clinical cases are attributable to anti-Aa and -Qa. Other minor antigens, such as Pa, Ab, Qc, Ua, Dc, and Db, have been implicated in the remaining cases. Mares that lack Aa and Qa are therefore most likely to produce affected foals. Pregnant mares may also produce antibodies to Ca (EAC system), but these are rarely associated with clinical disease. Indeed, preexisting antibodies to Ca may reduce sensitization by Aa. The presence of this anti-Ca in a mare may eliminate fetal red cells that enter her bloodstream and prevent further sensitization.

Antibodies produced by mares do not cross the placenta but reach the foal through the colostrum. Affected foals are therefore born healthy but sicken several hours after suckling. The severity of the disease is determined by the amount of antibody absorbed and by the sensitizing antigen. The earliest signs are weakness and depression. The mucous membranes of affected foals may be pale and may eventually show a distinct jaundice. Some foals sicken by 6 to 8 hours and die from shock so rapidly that they may not have time to develop jaundice. More commonly the disease presents as lethargy and weakness between 12 and 48 hours of age, although it may be delayed for as long as 5 days. Icterus of the mucous membranes and sclera is consistent in foals that survive for at least 48 hours. Hemoglobinuria, although uncommon, is diagnostic in a newborn foal. As a result of anoxia, some foals in the terminal stages of the disease may convulse or become comatose. The most common causes of death in these foals are liver failure, brain damage, and bacterial sepsis.

Hemolytic disease is readily diagnosed by clinical signs alone. Hematological examination is of little diagnostic use but may be of assistance in indicating appropriate treatment. Definitive diagnosis requires that immunoglobulin be demonstrated on the surface of the red cells of the foal. In the case of anti-Aa or anti-Qa, addition of a source of complement (fresh normal rabbit serum) causes rapid hemolysis. If hemolytic disease is anticipated, the serum of a

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pregnant mare may be tested for antibodies by an indirect antiglobulin test (Chapter 42). By using red cells from horses with a major sensitizing blood group, it is possible to show that the antibody titer increases significantly in the month before parturition.

A test that may be useful for detecting the presence of anti-erythrocyte antibodies in colostrum is the jaundiced foal agglutination test. This involves making serial dilutions of colostrum in saline. A drop of anticoagulated foal blood is added to each tube, and the tubes are centrifuged so that the red cells form pellets at the bottom. In the presence of antibodies, the cells clump tightly, and the pellets remain intact when the tubes are emptied. Non-agglutinated red cells, in contrast, flow down the side of the tube. Concentrated colostrum is viscous and tends to induce rouleaux formation that mimics agglutination. However, if the mare's blood is used as a negative control, this can be accounted for. Mildly affected foals, with a packed cell volume (PCV) of 15% to 25% and a red cell count greater than 4×10^6, will continue to nurse. Those with a PCV of less than 10% will stop nursing and become recumbent. Marked icterus is suggestive of HDN in foals.

The prognosis of uncomplicated hemolytic disease is good provided the condition is diagnosed sufficiently early and the appropriate treatment instituted rapidly. Management of HDN includes prevention of further antibody absorption, adequate nutrition, oxygen therapy, fluid and electrolyte therapy, and maintenance of the acid-base balance. Warmth, adequate hydration, and antimicrobial therapy are also critically important. In acute cases, blood transfusion is necessary. A red cell count of less than $3 \times 10^6/\mu L$ or a PCV of less than 15% warrants a blood transfusion. Transfused equine red cells have a half-life of only 2 to 4 days, so transfusion is only a temporary life-saving measure. Compatible blood may be difficult to find because of the high prevalence of Aa and Qa in the normal equine population. A donor should not only be Aa or Qa negative but should also lack antibodies to these antigens. Exchange transfusion, although efficient, requires a donor capable of providing at least 5 L of blood as well as a double intravenous catheter and an anesthetized foal. A much simpler procedure that avoids many difficulties is transfusion of washed

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cells from the mare. About 3 to 4 L of blood is collected in sodium citrate and centrifuged, after which the plasma is discarded. The red cells are washed once in saline and transfused slowly into the foal. The blood is usually given in divided doses about 6 hours apart. Milder cases of hemolytic disease may require only careful nursing.

If hemolytic disease is anticipated as a result of either a rising antibody titer or the previous birth of a hemolytic foal, stripping off the mare's colostrum and giving the foal colostrum from another mare may prevent its occurrence. The foal should not be allowed to suckle its mare for 24 to 36 hours. Once suckling is permitted, the foal should only be allowed to take small quantities at first and should be observed carefully for adverse side effects.

Neonatal thrombocytopenia has been recorded in the foal. Immunoglobulins can be identified on the foal's platelets, and antibodies to these platelets can be found in the mare's serum.

Serological Testing: Horse blood groups may be identified by tube agglutination, hemolytic, and antiglobulin tests. Gel-based agglutination tests and immunochromatographic techniques under development have produced encouraging results. Each blood group system has a preferred test system. The complement used in the hemolytic test comes from rabbits, but it must be absorbed before use to remove any anti-horse antibodies.

Cattle

Eleven blood group systems—EAA, EAB, EAC, EAF, EAJ, EAL, EAM, EAR′, EAS, EAT′, and EAZ—have been identified in cattle. Two of these (EAB and EAJ) are of the greatest importance. The EAB blood group system is one of the most complex systems known since it is estimated to contain more than 60 different alleles. These alleles are not inherited independently but rather in combinations called phenogroups. Because of the complexity of the EAB system, it is practically impossible to obtain absolutely identical blood from any two unrelated cattle. Indeed, it has been suggested that the complexity of the EAB system is such that there exists sufficient different antigenic combinations to provide a unique identifying character for each bovine in the world.

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Naturally, such a system provides an ideal method for the accurate identification of individual animals, and breed societies may use blood grouping to check the identity of registered animals. The EAC system is also complex, with 10 alleles combining to form about 90 phenogroups.

The J antigen is a lipid found free in body fluids and passively adsorbed onto red cells. It is absent from the red cells of newborn calves but is acquired within the first 6 months of life. J-positive cattle are of two types. Some possess J antigen in high concentration, and this may be detected both on their red cells and in serum. Other animals may have low levels of J in serum, and it is detected only with great difficulty on red cells. (It is probable that a secretor gene controls the expression of J in cattle.) J-negative cattle, lacking the J antigen completely, may possess natural anti-J antibodies, although the level of these antibodies shows seasonal variation, being highest in the summer and fall. Because of the presence of these antibodies, transfusion of J-positive red cells into J-negative recipients may result in a transfusion reaction even in the absence of known previous sensitization.

HDN in calves is rare but has resulted from vaccination against anaplasmosis or babesiosis. These vaccines contain red cells from infected calves. In the case of *Anaplasm*a vaccines, for example, the blood from a large number of infected donors is pooled, freeze-dried, and mixed with adjuvant before being administered to cattle. The vaccine against babesiosis consists of fresh, infected calf blood. Both vaccines cause infection and, consequently, the development of immunity in recipients. They may also stimulate the production of antibodies against blood group antigens of the EAA and EAF systems. Cows sensitized by these vaccines and then mated with bulls carrying the same blood groups can transmit colostral antibodies to their calves, which may then develop hemolytic disease.

The severity of HDN in calves is related to the amount of colostrum ingested. Calves are usually healthy at birth but begin to show symptoms from 12 hours to 5 days later. In acute cases, death may occur within 24 hours after suckling, with the animals developing respiratory distress and hemoglobinuria. On necropsy, these calves have severe pulmonary edema, splenomegaly, and

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dark kidneys. Less severely affected animals develop anemia and jaundice and may die during the first week of life. The red cells of affected calves have antibodies on their surface (detected by an antiglobulin test) and may sometimes be lysed by the addition of complement in the form of fresh normal rabbit serum. Death is due to disseminated intravascular coagulation as a result of activation of the clotting system by red cell ghosts.

Serological Testing: Bovine blood groups are detected by hemolytic tests. Washed red cells are incubated in specific antisera, and rabbit serum is used as a source of complement.

Bovine Neonatal Pancytopenia

Beginning in 2007, multiple outbreaks of an unexplained hemorrhagic disease of newborn beef calves were reported from many countries in Western Europe. It is now called bovine neonatal pancytopenia (BNP). Affected calves show sudden onset bleeding including nasal hemorrhage, petechiation on mucus membranes, internal bleeding, and excessive bleeding from minor wounds such as injection or ear-tag sites. The disease appears 7 to 28 days after birth and affected calves may die within 48 hours of disease onset. Investigation shows an early drop in granulocytes followed by erythrocytes and lymphocytes. The net result is a profound pancytopenia including a thrombocytopenia, anemia, and leucopenia. The bone marrow may be completely aplastic. Mortality may be as high as 90% in clinically affected calves, but there are clearly many subclinical cases as well. Since the disease only occurs in suckled calves and develops within hours of first suckling, it appears to result from the consumption of colostrum. Further investigations showed that the colostrum from cows known to produce affected calves contained antibodies directed against the MHC class I molecules expressed on neonatal leukocytes and bone marrow stem cells. (Precursors and committed cells of the thrombocyte, lymphocyte, and monocyte lineages and precursors of neutrophil, erythrocyte, and eosinophil lineages.) These antibodies were not present in the serum or colostrum of cows with healthy calves. The antibodies also mediate phagocytosis of blood cells since they bind both the α chain of MHC class I antigens and β2-microglobulin.

The disease is triggered by administration of a specific vaccine against bovine viral diarrhea virus (BVDV) (Fig. 31.2). This vaccine contained inactivated BVDV grown in a bovine kidney cell line. A potent, oil-in-water adjuvant containing Quil-A is added (Chapter 24). Immunization with the vaccine induces high levels of antibodies against the class I MHC antigens expressed by the kidney cells.When transferred to calves via colostrum, these antibodies bind to leukocytes and bone marrow stem cells, kill them, and so induce pancytopenia and bone marrow destruction. Only a few of the calves born from mothers that received this specific vaccine develop clinical disease. The reasons for this are unknown but most likely depend upon their MHC haplotype. Antibody levels remain high in cows for many years and may be boosted by each pregnancy. As a result, BNP cases may occur many years after the offending vaccine was removed from the market.

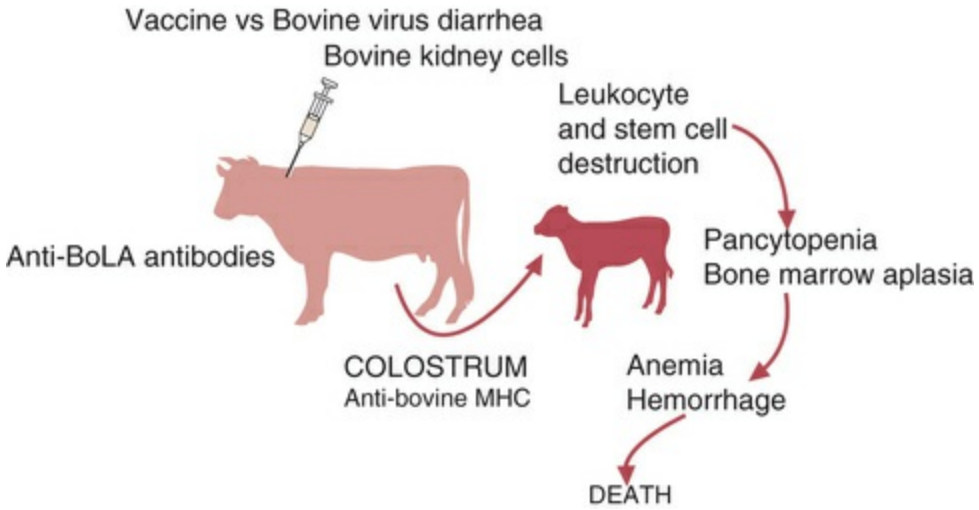


FIG. 31.2 The pathogenesis of bovine neonatal pancytopenia.

Sheep

The blood groups of sheep resemble those of cattle. Six blood group systems (EAA, EAB, EAC, EAD, EAM, and EAR) are currently recognized. The ovine equivalent of bovine EAB is also termed EAB and, like the bovine system, is complex, containing at least 52 different alleles. Sheep also possess an ovine equivalent of the bovine EAJ system, called the EAR system. Two soluble antigens

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are found in this system, R and O, coded for by alleles R and r. The production of R and O substances is controlled by a gene called *I* and its recessive allele i. If a sheep is homozygous for i, it expresses neither R nor O antigens. This interaction between the *I*/*i* genes and the R-O system is called an epistatic effect (Fig. 31.3). R and O antigens are soluble antigens found in the serum of II or Ii sheep and are passively adsorbed onto red cells. Natural anti-R antibodies may be found in R-negative sheep. Sheep also fall into two groups according to whether their red cells have high or low potassium levels. This is regulated by the EAM blood group system. The Mb antigen is an inhibitor of potassium transport.

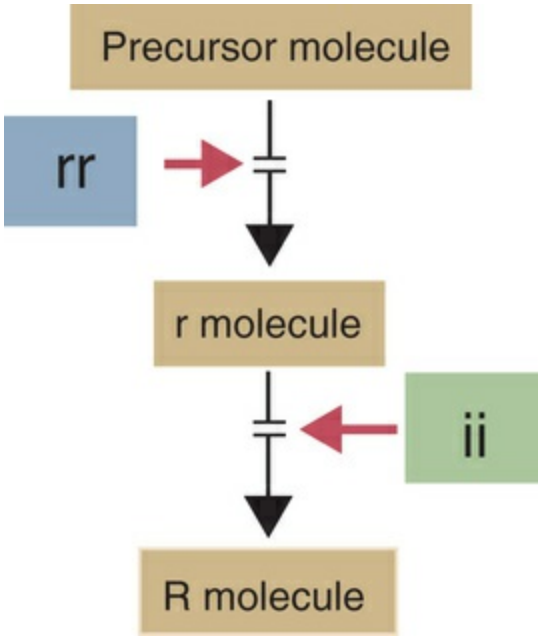


FIG. 31.3 The regulation of expression of EAR blood group antigens in sheep. The *I* gene controls expression of the EAR system.

Serological Testing: Sheep blood groups are detected by hemolytic tests. One exception to this rule is the EAD system, which is detected by agglutination.

Pigs

Sixteen pig blood group systems have been identified (EAA to EAP). Of these, the most important is the EAA system. The EAA

system, like the human ABO system, controls the expression of two carbohydrate antigens, A and O, through the use of glycosyltransferases. Their expression is regulated by a gene called *S* (secretor) with two alleles S and s. In the homozygous recessive state (ss), this gene can prevent the production of the A and O substances (Fig. 31.4). As a result, the amount of these antigens bound to red cells in these animals is reduced to an undetectable level (Box 31.2). A and O substances, like J in cattle and R and O in sheep, are not true red cell antigens but rather are soluble carbohydrates found in serum and passively adsorbed onto red cells after birth. Natural anti-A antibodies may be present in A-negative pigs, and transfusion of A-positive blood into such an animal may cause transient collapse and hemoglobinuria.

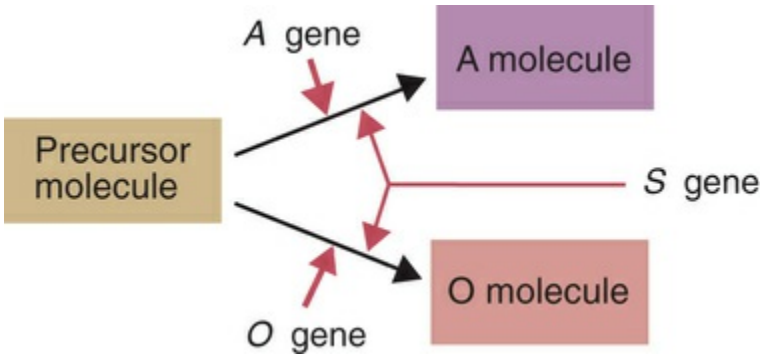


FIG. 31.4 The production of A or O blood group substances by a pig requires the presence of the *S* gene. Pigs that lack this gene (ss animals) produce neither of these blood group substances.

Box 31.2

Inheritance of the EAA Blood Group System in Pigs

In pigs, the expression of the EAA blood group is regulated by two gene loci. One locus, the A locus, contains two alleles, A and O, of which A is dominant. The other, the S locus, also contains two alleles, S and its recessive allele s. The S locus controls the expression of the A system so that A or O blood groups can only be expressed if the animal carries at least one *S* gene. Possible genotypes are therefore AA, AO, and OO, as well as SS, Ss and ss.

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These may be combined thus:

- Animals that are AASS, AASs, AOSS, or AOSs will have A red cells.
- Animals that are OOSS or OOSs will have O red cells.
- Animals that are AAss, Aoss, or Ooss will express neither A nor O and so will have "null" red cells.

HDN in piglets formerly occurred as a result of the use of hog cholera vaccine containing pig blood. This vaccine consisted of pooled blood from viremic pigs inactivated with the dye crystal violet. Sensitization of sows by this vaccine led to the occasional occurrence of hemolytic disease of their offspring. There appeared to be a breed predisposition to this disease, which was most commonly seen in the offspring of Essex and Wessex sows. Affected piglets did not necessarily show clinical disease, although their red cells were sensitized by antibody. Other piglets showed rapidly progressive weakness and pallor of mucous membranes preceding death, and those animals that survived longest showed hemoglobinuria and jaundice. The severity of the reaction did not appear to be directly related to the anti-red cell antibody titer in the piglet serum. Since the withdrawal of all live hog cholera virus vaccines, the problems associated with their use have disappeared.

True HDN has also been recorded in the pig. The antibodies responsible are usually directed against antigens of the EAE system. In addition to the development of hemolytic anemia in newborn piglets, the presence of antibodies to platelet antigens may cause a thrombocytopenia. This is seen clinically as a bleeding problem on tail docking and a tendency to bruise easily (neonatal purpura). On blood smears, the platelets may be clumped, and antiglobulin testing of them will yield a positive result. Deprivation of colostrum in an attempt to prevent piglets from absorbing anti-red cell antibodies may result in the newborn animals being highly susceptible to infection.

Serological Testing: Pig blood groups are detected by agglutination, hemolytic, and antiglobulin tests.

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Dogs

In dogs, seven red cell antigens are internationally recognized (DEA 1.1, 3, 4, 5, 6, 7, 8), but others have been described. (An older nomenclature called them by the traditional alphabetic system, A, Tr, B, C, D, F, J, K, L, M, and N.) Most of these appear to be inherited as simple mendelian dominants. Only the DEA 1 antigens are sufficiently antigenic to be of clinical significance. Based on polyclonal antibody testing, these were initially divided into 1.1 and 1.2, but DEA type 1.2 is now recognized as just a very strong 1.1 (1.1+). About 60% of dogs express a DEA 1 antigen. Naturally occurring antibodies to DEA 1.1 do not occur. Antibodies to DEA 7 may occur in 20% to 50% of DEA 7–negative dogs. Antibodies to DEA 3 and 5 are found in about 10% of negative dogs, but these are usually of low titer and not of clinical significance. Therefore, it is recommended that canine blood donors be negative for DEA 1.1, 1.1+, 3, 5, and 7. More than 98% of the canine population is DEA 4 positive. A universal donor would be an animal negative for all the DEA groups except DEA 4. Unless the blood type of the recipient is known, universal donor blood should only be used and a cross-match performed on all recipients. In practice, the most important canine blood type is DEA 1.1. About 33% to 45% of the dog population is DEA 1.1 positive, and in general these dogs can be considered to be universal recipients. Dogs that are DEA 1.1 negative can also be considered to be universal donors. DEA 1.1–positive blood should never be transfused into a DEA 1.1–negative dog. If so, the recipient will become sensitized to DEA 1.1, and high-titered antibodies will be produced. Subsequent transfusion of positive blood into such an animal could lead to a severe reaction. Similarly, if a negative bitch is sensitized by incompatible transfusions and mated to a positive dog, hemolytic disease may occur in her puppies. Natural HDN in dogs is extremely rare. It occurs when a DEA 1.1–negative breeding bitch is transfused with DEA 1.1–positive blood and subsequently bred to a DEA 1.1–positive male. The puppies develop a hemolytic anemia after 3 to 10 days.

The DEA 7 system (Tr system) is a soluble antigen system related to the human A, cattle J, sheep R, and pig A systems. Two antigens belong to the system—Tr and O. An epistatic secretor gene controls

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their expression. Anti–DEA 7 occurs naturally in some DEA 7–negative dogs. When healthy female dogs with a prior history of pregnancies were examined, the only antibodies detected were directed against DEA 7. However, the prevalence of these antibodies was similar in dogs with a prior history of pregnancy and control dogs. This suggests that pregnancy does not sensitize dogs to these antigens and that females with a prior pregnancy can be employed as blood donors.

A blood group antigen called Dal has been identified on the basis of antibodies produced in Dalmatians following blood transfusion. Presumably some Dalmatians lack this antigen, which is present in other dog breeds. Likewise, two new groups (Kai 1 and Kai 2) unrelated to DEA have been identified. About 94% of North American dogs are Kai 1+/Kai 2-. Five percent are Kai 1-/Kai 2- and 1% are Kai 1-/Kai 2+. Their significance in blood transfusion is unclear.

Serological Testing: Agglutination at 4° C and hemolytic and antiglobulin tests have been used for the detection of canine blood groups. The source of complement can be either fresh dog or rabbit serum. There are several commercial blood typing kits available for dogs. One is a card agglutination test that uses monoclonal DEA 1.1 antibodies to detect positive dogs. The other is an immunochromatographic strip technique (Chapter 42). This test uses a monoclonal antibody against DEA 1.1 to detect the antigen in a blood sample. A third method involves placing a suspension of the red cells on top of a matrix gel column containing ant–DEA 1.1. Matrix gel agglutination relies on performing the agglutination test on top of a viscous gel layer. Non-agglutinated red cells will sink through the gel, whereas agglutinated cells will remain as a layer on top.

Cats

Cats have only one major blood group system, the AB system. Cats may be either type A, type B, or AB. A is completely dominant over B. The AB antigens are glycolipids. The differences between the types are due to differences in the expression of neuraminic acid on the erythrocyte surfaces. Type A cells have predominantly

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glycolylneuraminic acid, while type B cells have acetylneuraminic acid. This difference results from mutations in the cytidine monophospho-*N*-acetylneuraminic acid hydroxylase gene that disrupts the enzyme function. This enzyme converts acetylneuraminic acid to glycolylneuraminic acid. The genetic basis of type AB is unknown. About 75% to 95% of cats are A positive, about 5% to 25% are B positive, and less than 1% are AB. However, this distribution differs among countries and among different purebred cat breeds. In the United States more than 99% of domestic short-hair and long-hair cats are type A, whereas in the United Kingdom only about 40% of short-hair breeds are type A. Severe transfusion reactions have been described in group B cats that received very small quantities of group A blood since 95% of B cats possess IgM anti-A. (Interestingly, only about 35% of A cats possess anti-B, and it is of the IgG and IgM classes and of much lower titer.) If completely matched blood is transfused into cats, its half-life is about 4 to 5 weeks. If, however, group B blood is transfused into cats of blood group A, its half-life is only a few days. If group A blood is transfused into a cat of blood group B, its half-life is just over 1 hour. It is this very rapid destruction that results in severe clinical reactions. Thus a group B cat given as little as 1 mL of group A blood will go into shock, with hypotension, apnea, and atrioventricular block, within a few minutes. Cross-matching is essential in this species.

Occasionally, hemolytic transfusion reactions occur between AB blood group–matched cats. These appear to be due to natural antibodies against a blood group antigen called Mik. Its mode of inheritance is undefined.

HDN has been recorded in Persian and related (Himalayan) breeds but is very rare. It occurs in kittens from queens of blood group B bred to sires of blood group A. The queens subsequently develop high-titered anti-A antibodies. Although healthy at birth, these kittens develop severe anemia as a result of intravascular hemolysis. Affected kittens show depression and possibly hemoglobinuria. Necropsy may reveal splenomegaly and jaundice. Antibodies to the sire's and the kitten's red cells are detectable in the queen's serum.

Serological Testing: Agglutination and immunochromatographic

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tests are used for feline blood typing. Serum from type B cats has strong anti-A activity. Anti-B reagents can use the lectin from *Triticum vulgaris* (wheat germ) or, increasingly, monoclonal antibodies. The agglutination tests can be performed in several different formats, such as in tubes, on cards, in matrix gels, or on glass slides. Results are comparable.

Humans

In humans, HDN is due almost entirely to immunization of the mother against the antigens of the Rhesus (Rh) system (now classified as CD240). The condition should be of historical interest only because a very simple but effective technique is available for its prevention. This depends on preventing an Rh-negative mother from reacting to the Rh-positive fetal red cells that escape from the placenta into her circulation at birth. Strong human anti-Rh globulin is obtained from male volunteers and given to mothers at risk soon after birth. It acts by specifically inhibiting the B cell response to that antigen (Chapter 20). Routine use of this material therefore prevents maternal sensitization, antibody production, and hemolytic disease. The use of a similar system in the domestic mammals is unnecessary because deprivation of colostrum is sufficient to prevent the disease.

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Parentage Testing

Under some circumstances it is necessary to confirm the parentage of an animal. One way of doing this is by examining the blood group antigens of an animal and its alleged parents (Table 31.2). The method is based on the principle that since blood group antigens are inherited, they must be present on the red cells of one or both parents. If a blood group antigen is present in a tested animal but absent from both its putative parents, parentage must be reassigned. Similarly, if one parent is homozygous for a specific blood group antigen, this antigen must inevitably appear in the offspring. However, it must be recognized that blood typing procedures can only exclude, never prove, parentage.

TABLE 31.2

Use of Blood Groups to Assign Paternity

BLOOD GROUP					
	DEA 1.1	DEA 1.2	DEA 6	DEA 7	DEA 8
Sire 1 ?	+	+	–	+	–
Sire 2 ?	+	+	–	–	+
Dam	–	–	+	+	–
Puppy 1	+	+	–	–	–
2	+	+	–	+	–
3	–	–	–	+	+*
4	–	–	+	+	–

*This puppy possesses DEA 8, which could not have come from sire 1 or its dam. Sire 1 could not have sired this litter.

(Courtesy Dr. D. Colling.)

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Hemophagocytic Syndrome

Hemophagocytic syndrome is a disorder of activated macrophages associated with multiple cytopenias in the blood. These cytopenias result from excessive phagocytic activity by macrophages. The syndrome has been described in humans, dogs, and cats. In humans, it may be either inherited or acquired. In dogs, the syndrome has been reported as secondary to infectious, neoplastic, or immune-mediated diseases. Diagnostic criteria include the presence of pancytopenia or bicytopenia and the presence of more than 2% macrophages containing ingested red cells in a bone marrow aspirate. Most of these dogs have an underlying disease. About one-third of canine cases are associated with autoimmune diseases such as lupus or immune-mediated thrombocytopenia (Chapter 37). These animals are commonly anemic, neutropenic, and thrombocytopenic, and it may be argued that autoantibodies opsonized the blood cells leading to their phagocytosis. Other affected dogs suffer from infectious diseases such as pyometra, pleuritis, ehrlichiosis, blastomycosis, or Lyme disease. In some cases, affected dogs recover once their underlying infection is treated. The disease is also associated with some neoplastic diseases such as malignant lymphoma or myelodysplastic syndrome. Canine hemophagocytic syndrome may also occur in the absence of any obvious associated disease. Affected dogs are anemic, neutropenic, thrombocytopenic, febrile, anorexic, and lethargic. In humans this syndrome results from a natural killer (NK) cell deficiency or as a result of excessive macrophage activation resulting from oversecretion of Th1 cytokines.

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Type II Hypersensitivity Reactions to Drugs

Red cells may be destroyed in drug hypersensitivities by three mechanisms. First, the drug and antibody may combine directly and activate complement, and nearby red cells will be destroyed in a bystander effect.

Second, some drugs may bind to cell surface glycoproteins. For example, penicillin, quinine, L-dopa, aminosalicylic acid, and phenacetin may bind to red cells. Since these cells are then modified, they may be recognized as foreign and eliminated by antibodies, resulting in hemolytic anemia. Penicillin-induced hemolytic anemia is not uncommon in horses. These conditions can be suspected based on recent treatment with penicillin and improvement when its use is discontinued. It may also be possible to detect antibodies against penicillin or penicillin-coated red cells in these animals. Sulfonamides, phenylbutazone, aminopyrine, phenothiazine, and possibly chloramphenicol may cause agranulocytosis by binding to granulocytes, and phenylbutazone, quinine, chloramphenicol, and sulfonamides can result in a thrombocytopenia as they bind to platelet surface glycoproteins. If the cells of affected animals are examined using a direct antiglobulin test, antibody may be demonstrated on their surface. If these antibodies are eluted, they can be shown to be directed against the offending drug.

Third, drugs such as the cephalosporins may modify red cell membranes in such a way that the cells passively adsorb antibodies and then are removed by phagocytic cells.

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Type II Hypersensitivity in Infectious Diseases

Just as drugs can be adsorbed onto red cells and render them immunologically foreign, so too can bacterial antigens such as the lipopolysaccharides, viruses such as equine infectious anemia virus and Aleutian disease virus, bacteria such as *Anaplasma,* and protozoa such as the trypanosomes and *Babesia.* These altered red cells are regarded as foreign and are either lysed by antibody and complement or phagocytosed by mononuclear phagocytes. Clinically severe anemia is therefore characteristic of all these infections.

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32

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Immune Complexes and Neutrophil-Mediated Hypersensitivity

CHAPTER OUTLINE

Classification of Type III Hypersensitivity Reactions, 357
Local Type III Hypersensitivity Reactions, 358
Blue-Eye, 360
Hypersensitivity Pneumonitis, 360
Equine Asthma, 360
Staphylococcal Hypersensitivity, 361
Generalized Type III Hypersensitivity Reactions, 361
Serum Sickness, 361
Glomerulonephritis, 362
Type I Membranoproliferative Glomerulonephritis, 362
Type II Membranoproliferative Glomerulonephritis, 363
Type III Membranoproliferative Glomerulonephritis, 363
Clinical Features of Glomerulonephritis, 363

VetBooks.ir

Immunoglobulin A Nephropathy, 365
Swine Glomerulopathy, 365
Dirofilariasis, 365
Finnish-Landrace Glomerulopathy, 365
Canine Glomerulopathy, 366
Other Immune Complex–Mediated Lesions, 366
Purpura Hemorrhagica, 366
Dietary Hypersensitivity, 366
Polyarthritis, 366
Drug Hypersensitivities, 366

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Explain the pathogenesis of immune complex–mediated hypersensitivity reactions.
- Explain how immune complexes cause local inflammation dominated by neutrophils.
- Describe how local deposition of immune complexes in the lungs following inhalation of antigenic dusts results in hypersensitivity pneumonitis.
- Distinguish between recurrent airway obstruction and inflammatory airway disease in horses.
- Explain the pathogenesis of blue-eye, serum sickness, and purpura hemorrhagica.
- Explain how immune complexes formed in the bloodstream are deposited in the glomeruli of the kidney and so cause a membranoproliferative glomerulonephritis leading to renal failure.
- Distinguish between the three forms of membranoproliferative glomerulonephritis.

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• Explain why type III hypersensitivity is a feature of many viral diseases.

Immune complexes formed by the combination of antibodies with antigen activate the classical complement pathway. When these complexes are deposited in tissues, the activated complement generates chemotactic peptides that attract neutrophils. The accumulated neutrophils may then release oxidants and enzymes, causing acute inflammation and tissue destruction. Lesions generated in this way are classified as type III or immune complex–mediated hypersensitivity reactions.

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Classification of Type III Hypersensitivity Reactions

The severity and significance of type III hypersensitivity reactions depend, as might be expected, on the amount and site of deposition of immune complexes. One form of reaction occurs locally when immune complexes are generated within tissues. Another form results when large quantities of immune complexes are produced within the bloodstream. This can occur, for example, when an antigen is administered intravenously to an immune recipient. Immune complexes generated in the bloodstream are deposited in glomeruli in the kidney, and the development of glomerular lesions (glomerulonephritis) is characteristic of this type of hypersensitivity. If the complexes bind to blood cells, anemia, leukopenia, or thrombocytopenia may also result. Immune complexes may also be deposited in blood vessel walls to cause vasculitis or in joints to cause arthritis.

It might reasonably be pointed out that the combination of an antigen with antibody always produces immune complexes. However, the occurrence of clinically significant type III hypersensitivity reactions results from the formation of excessive amounts of these immune complexes in the wrong places. For example, several grams of an antigen are needed to sensitize an animal, such as a rabbit, in order to produce experimental type III reactions. Minor immune complex–mediated lesions probably develop relatively frequently following an antibody response, without causing clinically significant disease or tissue damage.

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Local Type III Hypersensitivity Reactions

If an antigen is injected subcutaneously into an animal that already has a high level of antibodies in its bloodstream, inflammation will develop at the injection site within several hours. This is called an Arthus reaction after the scientist who first described it. It starts as a red, edematous swelling; eventually hemorrhage and thrombosis occur; and, if severe, it culminates in local tissue destruction.

Immediately following antigen injection, neutrophils adhere to vascular endothelium and then emigrate into the tissues. By 6 to 8 hours, when the reaction has reached its greatest intensity, the injection site is densely infiltrated by these cells (Fig. 32.1). As the reaction progresses, damage to blood vessel walls results in hemorrhage and edema, platelet aggregation, and thrombosis. By 8 hours, mononuclear cells appear in the lesion, and by 24 hours or later, depending on the amount of antigen injected, they become the predominant cell type. Eosinophils are not a significant feature of this type of hypersensitivity.

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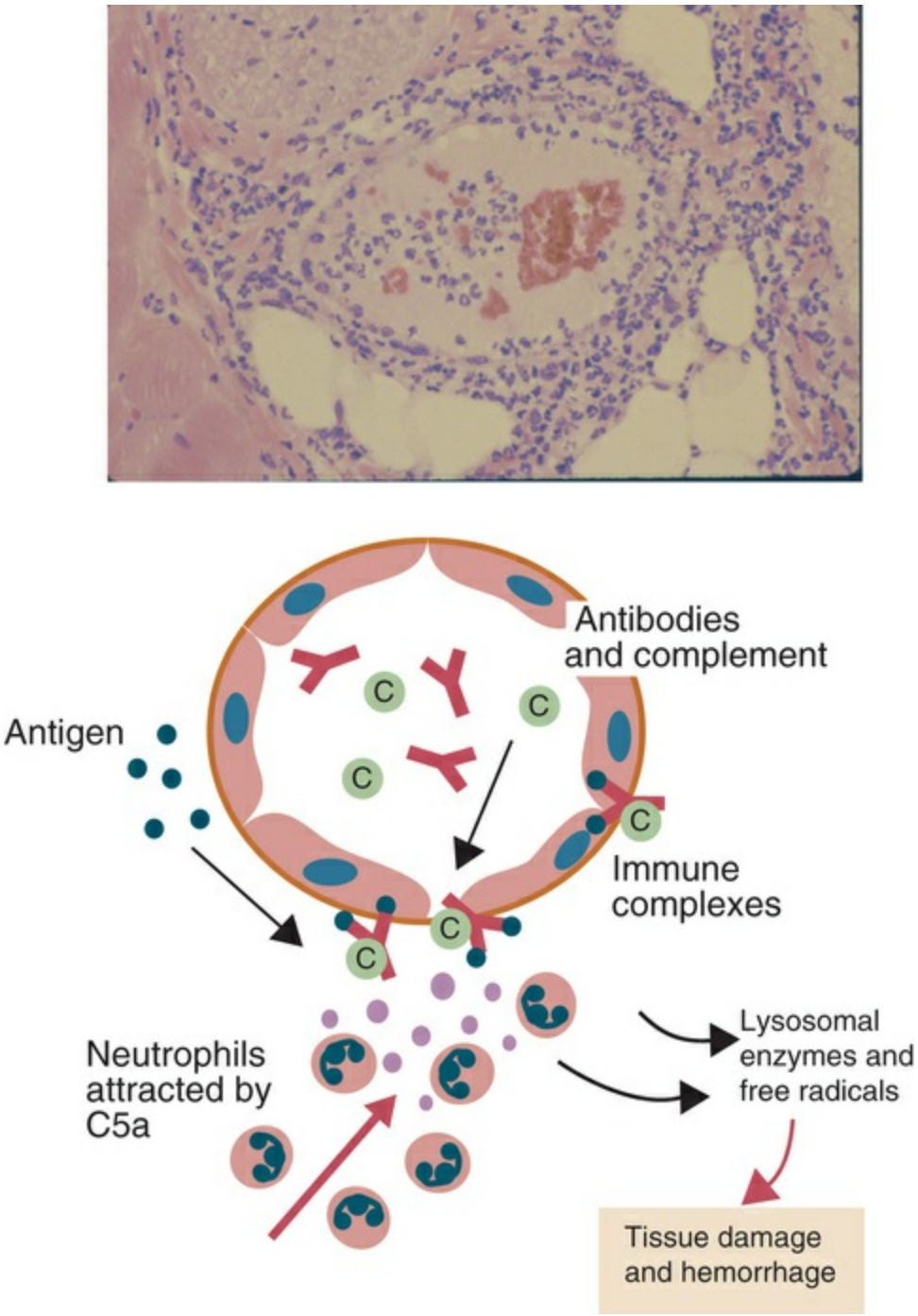


FIG. 32.1 The mechanisms of an Arthus reaction as well as a histological section of an Arthus reaction in the skin of a cat 6 hours after intradermal inoculation of chicken red blood cells. (Courtesy Dr. A. Kier.)

The fate of the injected antigen can be followed if it is labeled with a fluorescent dye. The antigen first diffuses from the injection site through tissue fluid. When small blood vessels are encountered, the antigen diffuses into the vessel walls, where it encounters circulating antibodies. Immune complexes form and are deposited

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between and beneath vascular endothelial cells. Complement components activated by the classical pathway will be deposited here as well.

Immune complexes formed in tissues must be removed. They first bind to Fc and complement receptors on cells. The most widespread of these Fc receptors is FcγRIIa (CD32a) expressed on neutrophils. Immune complexes binding to these receptors activate the neutrophils and stimulate production of oxidants, leukotrienes, prostaglandins, cytokines, and chemokines. Immune complexes also bind to mast cells through FcγRIII and trigger them to release their granule contents. Among the molecules released by mast cells are chemoattractants and proteases that activate complement, cytokines, kinins, and lipid mediators. All these mediators promote inflammation by acting on vascular endothelium and stimulating neutrophil adherence and emigration.

Immune complexes activate complement and generate the chemotactic peptide C5a (Fig. 32.2). Neutrophils, attracted by C5a and mast cell–derived chemokines, emigrate from the blood vessels, adhere to the complexes, and promptly phagocytose them. Eventually the immune complexes are digested and destroyed. During this process, however, more proteases and oxidants are released into the tissues. When neutrophils attempt to ingest immune complexes attached to structures such as basement membranes, they release their granule contents directly into the surrounding tissues. Neutrophil proteases disrupt collagen fibers and destroy ground substances, basement membranes, and elastic tissue. Normally tissues contain antiproteinases that inhibit neutrophil enzymes. However, neutrophils can subvert these inhibitors by secreting OCl^-. The OCl^- destroys the inhibitors and allows tissue destruction to proceed.

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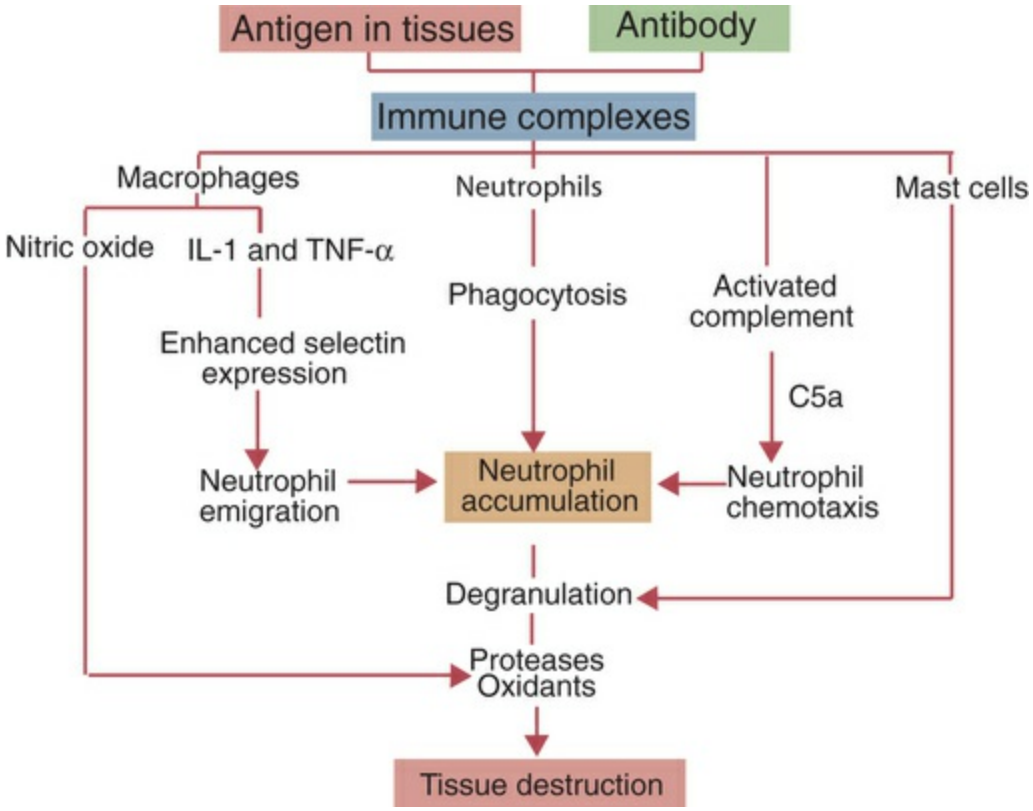


FIG. 32.2 Some of the mechanisms involved in the pathogenesis of the Arthus reaction.

Although it has long been assumed that immunoglobulins do not themselves damage antigens, evidence has shown that they can kill microorganisms and cause tissue damage. When provided with singlet oxygen from phagocytic neutrophils, antibodies can catalyze the production of oxidants such as ozone. This ozone kills not only bacteria but also nearby cells.

Neutrophil proteases also act on C5 to generate C5a, which promotes further neutrophil accumulation and degranulation. Other enzymes released by neutrophils make mast cells degranulate or generate kinins. As a result of all this, inflammation and destruction of blood vessel walls cause edema, vasculitis, and hemorrhage characteristic of an Arthus reaction.

Although the classical direct Arthus reaction is produced by local administration of an antigen to hyperimmunized animals, any technique that generates immune complexes in tissues will stimulate a similar response. A reversed Arthus reaction can therefore be produced if antibodies are administered intradermally to an animal with a high level of circulating antigen. Injected, preformed immune complexes, particularly those containing a

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moderate excess of an antigen, will provoke a similar reaction, although, as might be anticipated, there is less involvement of blood vessel walls, and the reaction is less severe. A passive Arthus reaction can be produced by giving antibody intravenously to a nonsensitized animal, followed by an intradermal injection of an antigen, and real enthusiasts can produce a reversed passive Arthus reaction by giving antibody intradermally, followed by intravenous antigen.

Although it is unusual for pure hypersensitivity reactions of only a single type to occur under natural conditions, there are diseases in domestic animals in which type III reactions play a major role. Experimentally, Arthus reactions are usually produced in the skin since that is the most convenient site at which to inject the antigen. However, local type III reactions can occur in many tissues, with the precise site depending on the location of the antigen.

Blue-Eye

Blue-eye is a condition seen in a small proportion of dogs that have been either infected or vaccinated with live canine adenovirus type I (see Fig. 27.8). These animals develop an anterior uveitis leading to corneal edema and opacity. The cornea is infiltrated by neutrophils, attracted by virus-antibody complexes that are deposited in tissue. Blue-eye develops about 1 to 3 weeks after the onset of infection and usually resolves spontaneously once the virus is eliminated.

Hypersensitivity Pneumonitis

Type III hypersensitivity reactions may occur in the lungs when sensitized animals inhale antigens. For example, cattle housed during the winter are exposed to dust from hay. Normally, these dust particles are relatively large and are deposited in the upper respiratory tract, trapped in mucus, and eliminated. If, however, hay is stored when damp, bacterial growth and metabolism will result in heating. As a result of this warmth, thermophilic actinomycetes will grow. One of the most important of these thermophilic actinomycetes is *Saccharopolyspora rectivirgula*, an

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organism that produces large numbers of very small spores (1 μm diameter). When inhaled, these spores can penetrate as far as the alveoli. If cattle are fed moldy hay for long periods, constant inhalation of *S. rectivirgula* spores will result in the development of high-titered antibodies to *S. rectivirgula* antigens in serum. Eventually, inhaled spore antigens will encounter antibodies within the alveolar walls, and the resulting immune complexes and complement activation will cause a pneumonia (or pneumonitis), the basis of which is a type III hypersensitivity reaction.

Hypersensitivity pneumonitis consists of an acute alveolitis together with vasculitis and exudation of fluid into the alveolar spaces. The alveolar septa may be thickened, and the entire lesion is infiltrated with inflammatory cells. Since many of these cells are eosinophils and lymphocytes, it is obvious that the reaction is not a pure type III reaction. Examination of the lungs of affected cattle by immunofluorescence demonstrates deposits of immune complexes with complement. In animals inhaling small amounts of an antigen over a long period, proliferative bronchiolitis and fibrosis may be observed. Clinically, hypersensitivity pneumonitis presents as a pneumonia occurring between 5 and 10 hours after acute exposure to grossly moldy hay. The animal may have difficulty breathing and develop a severe cough. In chronically affected animals, the dyspnea may be continuous. The most effective method of managing this condition is by removing the source of the antigen. Administration of steroids may be beneficial.

A hypersensitivity pneumonitis also occurs in farmers chronically exposed to *S. rectivirgula* spores from moldy hay and is called farmer's lung. Many other syndromes in humans have an identical pathogenesis and are usually named after the source of the offending antigen. Thus pigeon breeder's lung arises following exposure to the dust from pigeon feces, mushroom grower's disease is due to hypersensitivity to inhaled spores from actinomycetes in the soil used for growing mushrooms, and librarian's lung results from inhalation of dusts from old books! Hay sickness is a hypersensitivity pneumonitis seen in horses in Iceland that is probably an equine equivalent of farmer's lung.

Equine Asthma

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Several forms of chronic obstructive respiratory disease are recognized in horses and collectively called equine asthma. They are most conveniently divided into two syndromes: recurrent airway obstruction (RAO) or "Heaves" and inflammatory airway disease (IAD).

RAO is characterized by chronic airway inflammation, bronchoconstriction, and neutrophil infiltration. RAO is seen in horses over 9 years of age. IAD is a milder condition seen in horses of any age. It is associated with decreased performance and a chronic, intermittent cough. Both are forms of chronic bronchiolitis associated with exposure to molds and other allergens in dusty stable air.

It has been recognized that asthma in humans consists of several distinctly different diseases. Some are associated with high eosinophil counts in bronchoalveolar washes, while others are characterized by high neutrophil counts. Some human cases have both neutrophils and eosinophils while others have neither. A similar spectrum and complexity is seen in equine asthma (Chapter 30).

Recurrent airway obstruction.

RAO resembles severe asthma in humans. Affected animals have respiratory distress episodes triggered by inhalation of airborne allergens even while at rest. During these episodes, horses develop airflow obstruction as a result of bronchospasm, increased mucus production, airway hyperplasia, and airway hyperresponsiveness (Fig. 32.3). Neutrophil recruitment is apparent by 4 to 6 hours after antigen challenge and precedes the development of airway obstruction. Th2 cytokines appear to contribute to the recruitment and activation of these neutrophils and the release of extracellular NETS (Chapter 5). During remissions, horses are clinically normal and their airway function and bronchial cytology appear normal.

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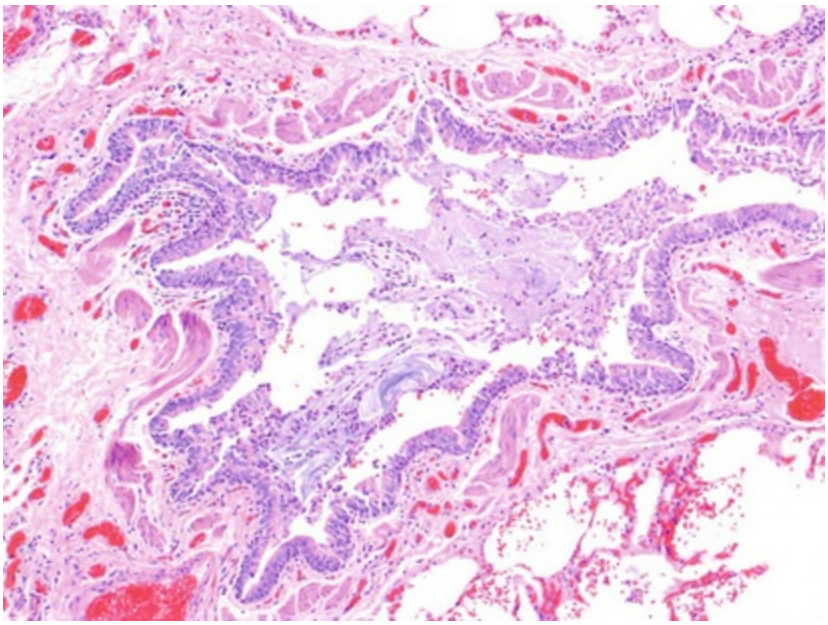


FIG. 32.3 Chronic airway obstructive disease in a horse showing mucus in the lumen, epithelial hyperplasia, smooth muscle hypertrophy, and mildly increased numbers of submucosal inflammatory cells. (Courtesy Dr. B. Porter.)

RAO occurs most obviously in horses that inhale large amounts of organic dusts such as those generated in dusty stables. It includes obstructive pulmonary disease seen in stabled horses and summer pasture–associated obstructive pulmonary disease. It is defined as a severe debilitating disease characterized by coughing and an increased breathing effort due to bronchoconstriction, airway hyperreactivity, neutrophil infiltration and mucus accumulation in the airways. Characteristically, horses with RAO suffer from labored breathing even while at rest.

RAO is probably a hypersensitivity disease associated with an enhanced Th2 response. Thus biopsies show significant increases in type 2 cytokines, IL-1β, IL-8, TNF-α, TGF-β1, as well as TLR4 and NF-κB transcripts. Similar but less marked trends occur in IL-17 and IFN-γ. High concentrations of the chemokine CXCL8 (interleukin-8 [IL-8]) are found in the bronchoalveolar washings of affected animals. Exposure of cultured equine bronchial epithelial cell cultures to hay dust or lipopolysaccharide increases IL-8, CXCL2, and IL-1β expression. The percentage of $CD4^+$, $Foxp3^+$ Treg cells increases in the bronchoalveolar washings of horses with RAO, suggesting that these may influence the course of this disease.

Horses with RAO may show positive skin reactions to

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intradermal inoculation of actinomycete and fungal extracts (such as *Rhizopus nigricans, Candida albicans, S. rectivirgula, Aspergillus fumigatus,* or *Geotrichum deliquescens*). Affected horses may respond to aerosol challenge with extracts of these organisms by developing respiratory distress. Clinical signs may resolve on removal of the moldy hay and reappear on re-exposure. There is no evidence of IgE involvement in RAO and little correlation between skin test results and severity of disease. There is, however, evidence for a genetic predisposition perhaps involving the gene for the IL-4 receptor. The prevalence of RAO is increased three-fold when one parent is affected and five-fold when both are affected.

Affected animals usually have large numbers of neutrophils or eosinophils in their small bronchioles and high titers of antibodies to equine influenza in their bronchial secretions. The significance of the latter is unclear. It has been suggested that continuous prolonged activation of bronchoalveolar epithelial cells by dust particles and air-borne endotoxins leads to excessive production of neutrophil-attracting chemokines. These neutrophils then cause damage by producing proteases, peroxidases, and oxidants. Removal of clinically affected horses to air-conditioned stalls results in improvement of the disease, but this is reversed if the horses are returned to dusty stables. In some cases, RAO may persist even when horses are moved to low dust environments, probably as a result of airway remodeling.

Inflammatory airway disease.

IAD is a milder disease in which no labored breathing is seen at rest. It has minimal clinical signs such as a nasal discharge, cough, and decreased performance. It affects up to 30% of young horses (<5 years old) in training. Although commonly linked to bacterial or viral infections, in many cases no infectious agent can be isolated. Horses with IAD show poor performance, exercise intolerance, and coughing. Excessive airway mucus is apparent. There is evidence of inflammation detected by cytological evaluation of bronchiolar lavage fluid. Cytokine transcripts show elevated IL-1β, -5, -6, -8, -10, -17, and -23, as well as TNF-α and IFN-γ in affected horses compared to controls. However, these differences are influenced by the presence of mast cells, eosinophils, or neutrophils. Thus in

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washes with high neutrophil numbers, IL-17 was also elevated, while IL-4 was depressed compared to those with high mast cell numbers. Its pathogenesis is unknown, but, like RAO, IAD is associated with inhalation of organic dusts and aeroallergens and may be seasonal.

Staphylococcal Hypersensitivity

Staphylococcal hypersensitivity is a pruritic pustular dermatitis of dogs. Skin testing with staphylococcal antigens suggests that types I, III, and IV hypersensitivity may be involved. Histological findings of neutrophilic dermal vasculitis suggest that the type III reaction may predominate.

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Generalized Type III Hypersensitivity Reactions

If an antigen is administered intravenously to animals with circulating antibodies, immune complexes form in the bloodstream. These immune complexes are removed by binding to either erythrocytes or platelets, or if very large, by mononuclear phagocytes (Fig. 32.4). If, however, complexes are produced in excessive amounts, they are deposited in the walls of blood vessels, especially medium-sized arteries, and in vessels where there is a physiological outflow of fluid such as glomeruli, synovia, and the choroid plexus (Fig. 32.5). An example of this type of hypersensitivity is serum sickness.

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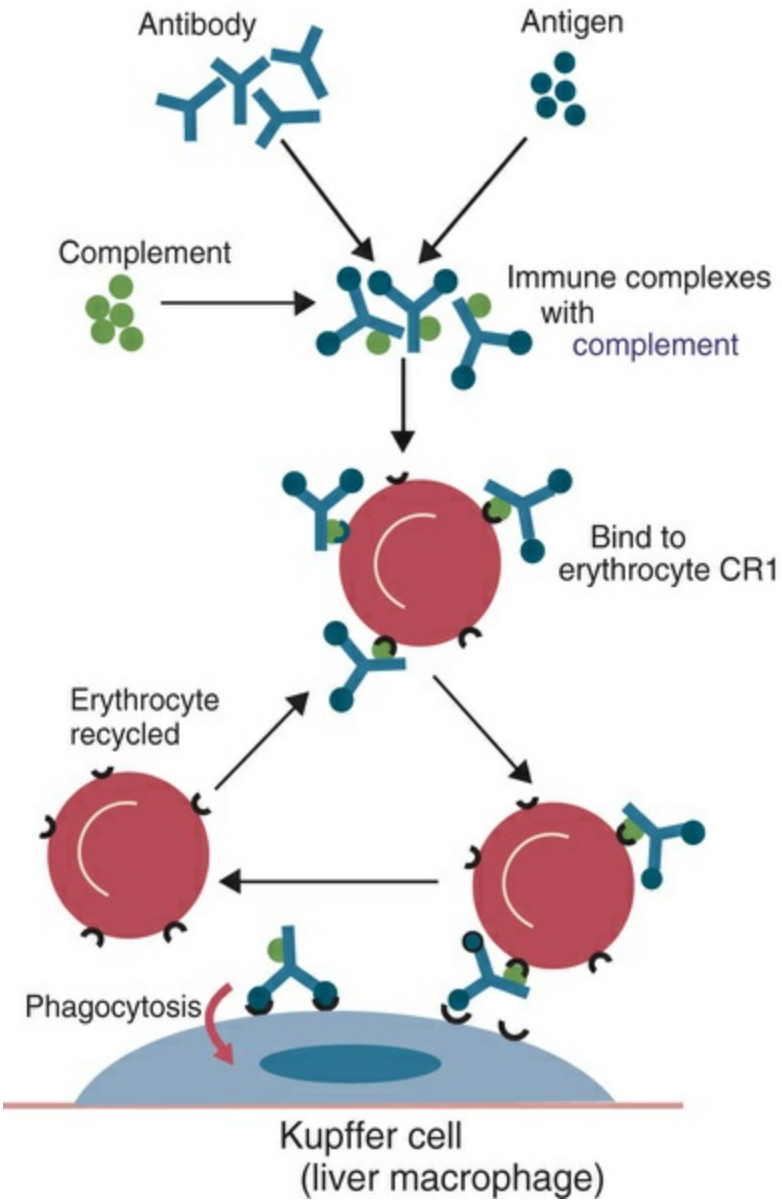


FIG. 32.4 In primates, immune complexes are removed by binding to complement receptors on red blood cells. They are then carried to the liver, where they are transferred to Kupffer cells for phagocytosis. In the absence of complement components, significant accumulation of immune complexes occurs in tissues. In other mammals immune complexes bind to receptors on platelets.

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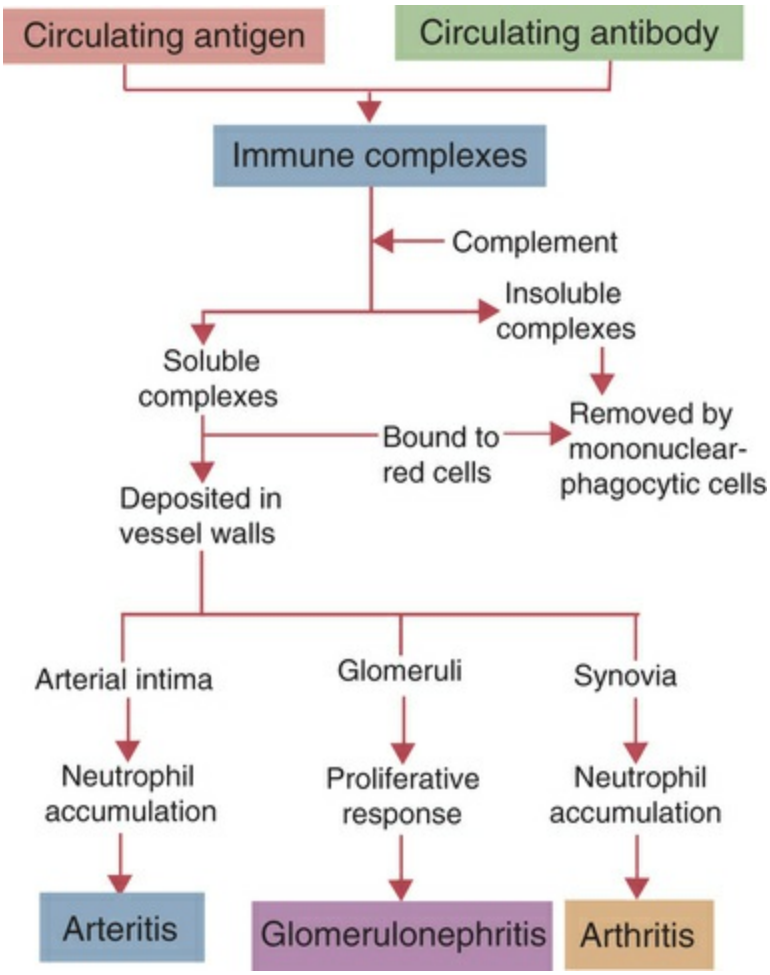


FIG. 32.5 The mechanisms involved in the pathogenesis of acute serum sickness.

Serum Sickness

Many years ago, when the use of antisera for passive immunization was in its infancy, it was observed that wounded soldiers who had received a very large dose of equine anti-tetanus serum developed a characteristic illness about 10 days later. This was called serum sickness, and consisted of a generalized vasculitis with erythema, edema, and urticaria of the skin; neutropenia; lymph node enlargement; joint swelling; and proteinuria. The reaction was usually of short duration and subsided within a few days. A similar reaction can be produced experimentally in rabbits by administration of a large intravenous dose of antigen. The development of sickness coincides with the formation of large amounts of immune complexes in the circulation (Fig. 32.6). The experimental disease may be acute if it is caused by a single, large

injection of an antigen or chronic if caused by multiple small injections. In either case, animals develop glomerulonephritis and arteritis (Fig. 32.7).

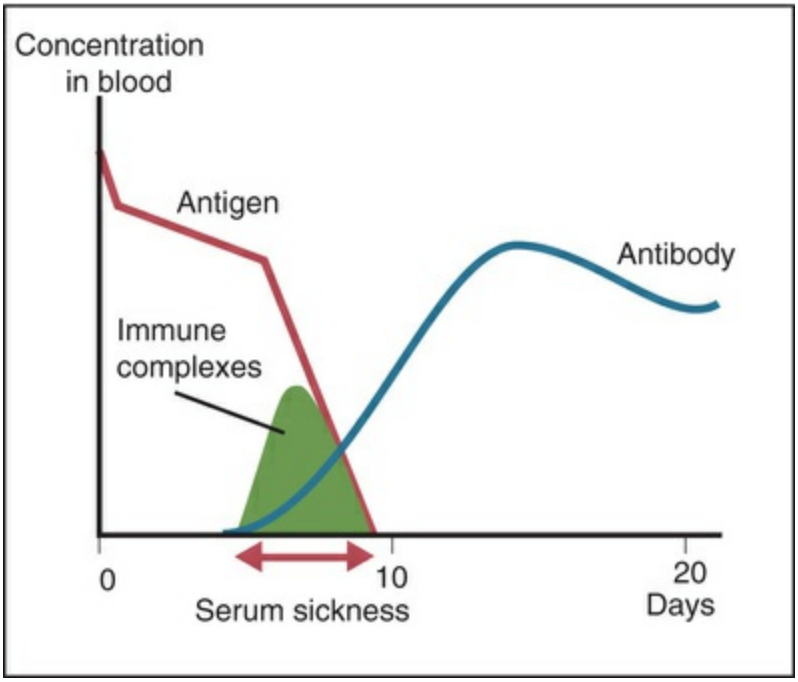


FIG. 32.6 The time course of acute serum sickness. The appearance of the disease coincides with the generation of immune complexes in the bloodstream.

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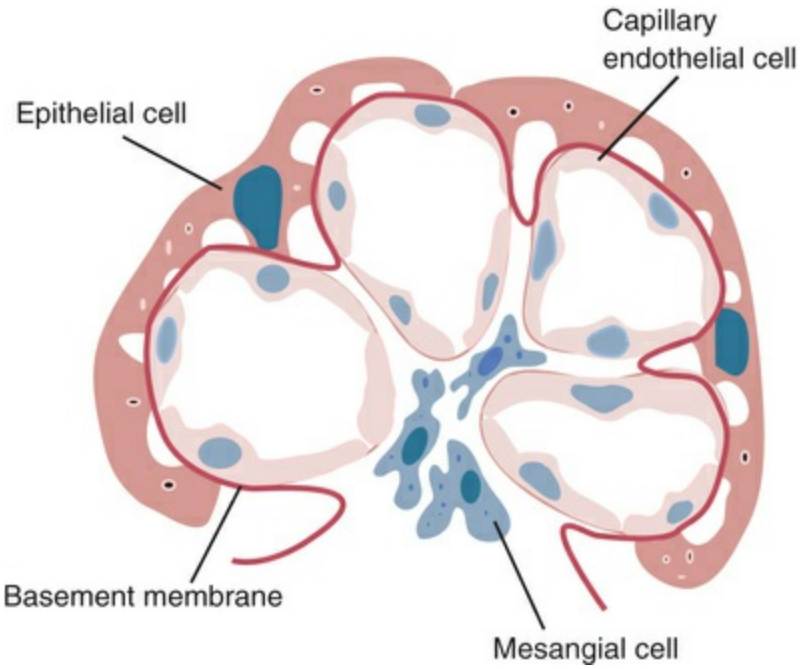


FIG. 32.7 The structure of a typical glomerulus. Immune complexes may be deposited on either side of, or within, the glomerular basement membrane.

Glomerulonephritis

When immune complexes are deposited in the glomeruli, they cause basement membrane thickening and stimulate glomerular cells to proliferate. Any or all of the three glomerular cell populations, epithelial cells, endothelial cells, and mesangial cells, can proliferate. The lesion is therefore called membranoproliferative glomerulonephritis (MPGN). If immune complexes are deposited only in the mesangium, mesangial cell proliferation will result in a mesangioproliferative glomerulonephritis. MPGN lesions are classified into three types based on their histopathology and pathogenesis (Fig. 32.8).

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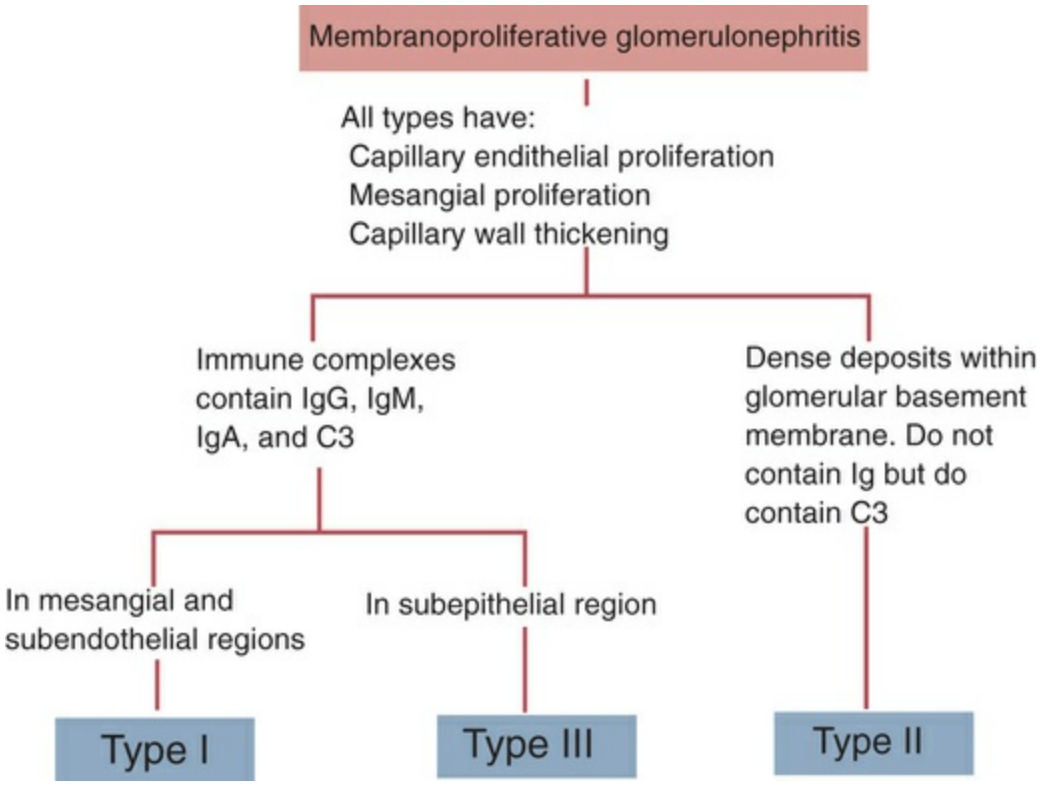


FIG. 32.8 A classification of different forms of membranoproliferative glomerulonephritis.

Type I Membranoproliferative Glomerulonephritis

Type I MPGN is caused by immune complex deposition in glomerular vessels. These complexes usually penetrate the vascular endothelium, but not the basement membrane, and are therefore trapped on the inside, where they stimulate endothelial cell swelling and proliferation (Fig. 32.9). If an animal is given repeated injections of small doses of an antigen over a long period, continued damage to the glomerular cells by immune complexes leads to production of transforming growth factor-β (TGF-β). This cytokine stimulates nearby cells to produce fibronectin, collagen, and proteoglycans. This results in a thickening of the basement membrane to form the so-called "wire loop" lesion (also called a membranous glomerulonephritis). Alternatively, the immune complexes may be deposited in the mesangial region of glomeruli. Mesangial cells are modified smooth muscle cells. As such they can release cytokines and prostaglandins and ingest immune complexes. They respond to these immune complexes by proliferation and production of IL-6 and TGF-β. The IL-6 stimulates

autocrine growth of the mesangial cells. The TGF-β stimulates production of extracellular matrix. This glomerulonephritis eventually interferes with glomerular function. By immunofluorescence, it can be shown that lumpy aggregates of immune complexes are deposited in capillary walls and on the epithelial side of the glomerular basement membrane (Fig. 32.10).

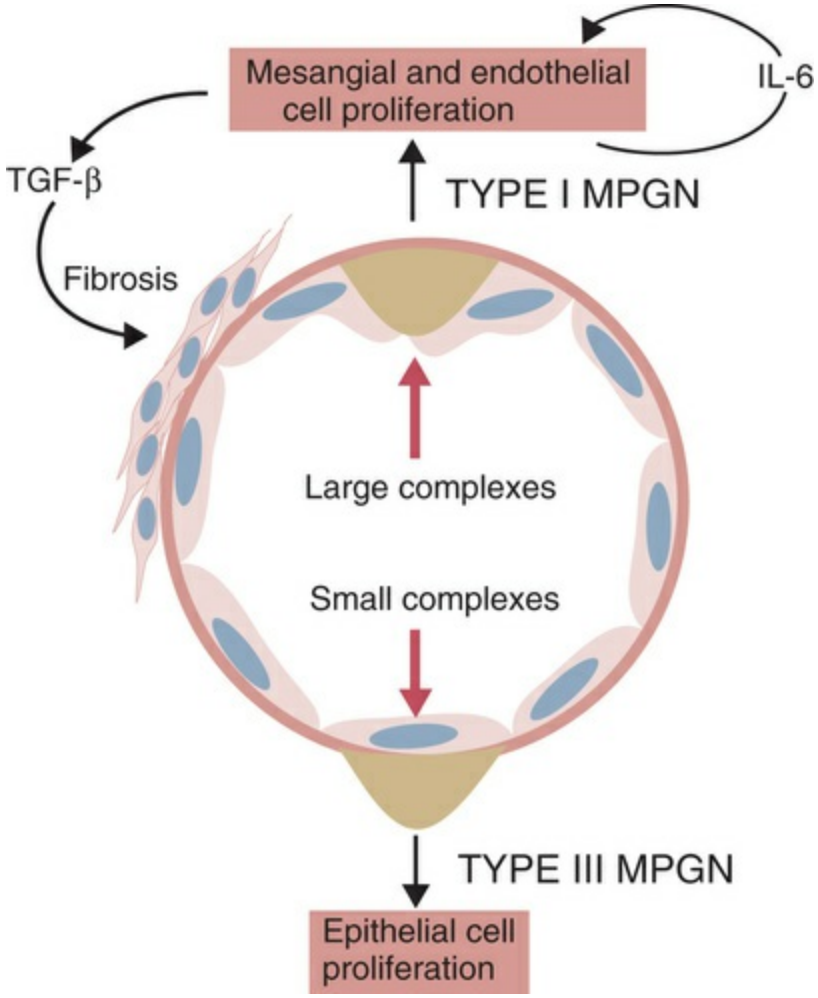


FIG. 32.9 The pathogenesis of different forms of immune complex–mediated glomerulonephritis. Remember, however, that more than one type of lesion may be present in an animal at the same time.

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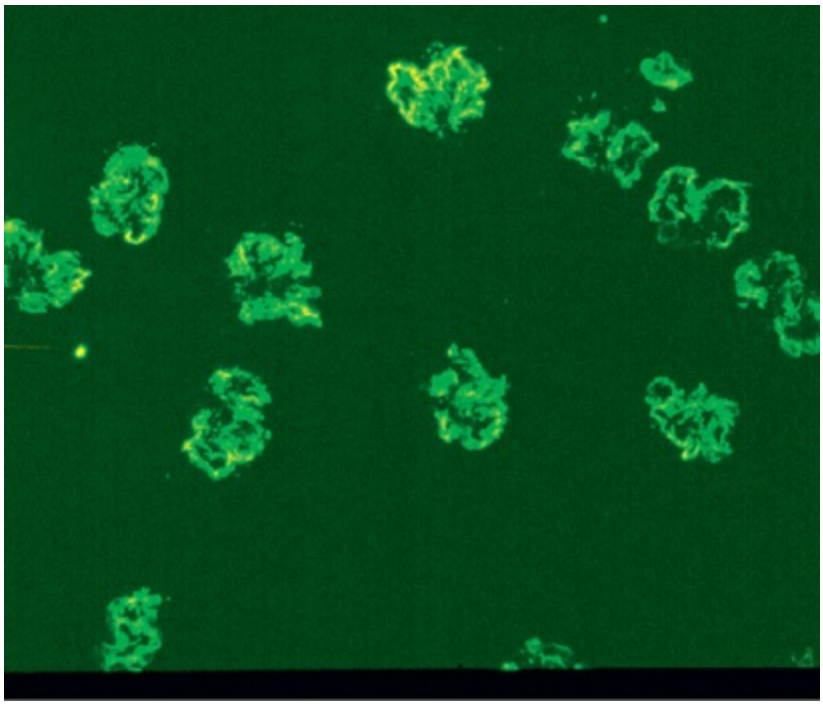


FIG. 32.10 A fluorescent micrograph of a section of glomerulus from a Finnish-Landrace lamb with immune complex–mediated glomerulonephritis. The labeled antisheep globulin reveals the presence of "lumpy-bumpy" deposits characteristic of type I membranoproliferative glomerulonephritis in many glomeruli. (From Angus KW, Gardiner AC, Morgan KT, et al: Mesangiocapillary glomerulonephritis in lambs. II. Pathological findings and electron microscopy of the renal lesions, *J Comp Pathol* 84:319-330, 1974.)

Type II Membranoproliferative Glomerulonephritis

Type II MPGN (or dense deposit disease) is similar to the type I disease in that there is endothelial and mesangial proliferation. However, it is characterized by the presence of homogeneous, dense deposits within the glomerular basement membrane (in the lamina densa) rather than on its surface (see Fig. 4.18). The deposits may contain C3 but not immunoglobulin. Type II MPGN results from uncontrolled complement activation and is seen in factor H deficiency in pigs (Chapter 4).

Type III Membranoproliferative Glomerulonephritis

Type III MPGN is a variant of type I MPGN. It differs from typical type I disease by the presence of immune complexes on both the endothelial and epithelial sides of the basement membrane. It is believed that very small immune complexes penetrate the basement

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membrane and are deposited where they stimulate epithelial cell swelling and proliferation. If excessive, these proliferating cells may fill the glomerular space to form epithelial crescents. A single case of unknown cause has been described in a cat.

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Clinical Features of Glomerulonephritis

Type I MPGN develops when prolonged antigenemia persists in the presence of antibodies. It is therefore characteristic of chronic viral diseases such as equine infectious anemia, infectious canine hepatitis, bovine virus diarrhea, Aleutian disease of mink, and African swine fever; parasitic diseases such as leishmaniasis; and chronic bacterial diseases such as Lyme disease and Ehrlichiosis (Table 32.1). Clinically, it should be suspected in an animal with proteinuria without evidence of infection, although definitive diagnosis requires a renal biopsy and histological evaluation. Type I MPGN has also been reported in dogs with pyometra, chronic pneumonia, distemper encephalitis, acute pancreatic necrosis, and bacterial endocarditis. It is a feature of feline leukemia. In animals with tumors, large amounts of antigen may be shed into the bloodstream and result in a type I MPGN. It has also been associated with lymphosarcomas, osteosarcomas, and mastocytomas. Circulating immune complexes and renal lesions occur in dogs with systemic lupus erythematosus (Chapter 38), discoid lupus, generalized demodicosis, and recurrent staphylococcal pyoderma. Some cases may be due to complement deficiencies. As a result of these deficiencies, removal of immune complexes is impaired, and they accumulate in glomeruli. Many cases of type I MPGN develop in the absence of an obvious predisposing cause.

TABLE 32.1

Infectious Diseases With a Significant Type III Hypersensitivity Component

Organism or Disease	Major Lesion
Erysipelothrix rhusiopathiae	Arthritis
Mycobacterium johnei	Enteritis
Streptococcus equi	Purpura
Staphylococcus aureus	Dermatitis
Borrelia burgdorferi	Glomerulonephritis
Leishmania	Glomerulonephritis
Ehrlichiosis	Glomerulonephritis
Canine adenovirus-1	Uveitis, glomerulonephritis

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Canine adenovirus-2	Glomerulonephritis
Feline leukemia	Glomerulonephritis
Feline infectious peritonitis	Peritonitis, glomerulonephritis
Aleutian disease	Glomerulonephritis, anemia, arteritis
Hog cholera	Glomerulonephritis
African swine fever	Glomerulonephritis
Bovine virus diarrhea	Glomerulonephritis
Equine viral arteritis	Arteritis
Equine infectious anemia	Anemia, glomerulonephritis
Visceral leishmaniasis	Glomerulonephritis
Dirofilaria immitis	Glomerulonephritis

The presence of immune complex lesions within glomeruli stimulates neutrophils, mesangial cells, macrophages, and platelets to release thromboxanes, nitric oxide, and platelet-activating factor. These increase basement membrane permeability so that plasma proteins, especially albumin, are lost in the urine. This loss, if severe, may exceed the ability of the body to replace the protein. As a result, albumin levels drop, the plasma colloid osmotic pressure falls, fluid passes from blood into tissue spaces, and the animal may become edematous and ascitic. The loss of fluid into tissues results in a reduction of blood volume, a compensatory increase in secretion of antidiuretic hormone, increased sodium retention, and accentuation of the edema. The decreased blood volume also results in a drop in renal blood flow, reduction in glomerular filtration, retention of urea and creatinine, azotemia, and hypercholesterolemia. Although all these may occur as a result of immune complex deposition in glomeruli, the development of this nephrotic syndrome is not inevitable. In fact, the clinical course of these conditions is unpredictable, with some animals showing a progressive deterioration in renal function and others showing spontaneous remissions. Many animals may be clinically normal despite the presence of immune complexes in their glomeruli, and immune complexes are commonly observed in old, apparently healthy dogs, horses, and sheep. The most common initial signs are anorexia, weight loss, and vomiting. Polyuria and polydipsia occur when about two-thirds of glomeruli are destroyed. Azotemia occurs when 75% are destroyed. Development of nephrotic syndrome (proteinuria, hypoproteinemia, edema, or ascites) only occurs in about 15% of affected dogs but in up to 75% of affected cats. Some dogs become hypertensive. Thromboembolic disease may also develop. Because of the unpredictable occurrence of spontaneous remissions, it is difficult to judge the effects of treatment. It has been

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usual to treat affected animals with corticosteroids and immunosuppressive drugs, but the rationale and effectiveness of this treatment is unclear except when the glomerulonephritis is associated with concurrent autoimmune disease such as systemic lupus erythematosus. Recently, encouraging responses have been obtained with angiotensin-converting enzyme inhibitors (captopril) and experimental thromboxane synthase inhibitors. Protein restriction may help reduce the clinical signs of renal failure. If the glomerulopathy is secondary to infection as in Lyme-associated glomerular disease or leishmaniasis, the underlying cause should be treated with appropriate antimicrobial therapy. The glomerular lesion is not inflammatory, and although the lesion contains immunoglobulins, there is no evidence to suggest that it is caused by hyperactivity of the immune system. Steroid treatment of rabbits with experimental immune complex disease has been shown to exacerbate the condition. For disease associated with a profound proteinuria, nephrotic syndrome, or progressive azotemia, mycophenolate alone or in combination with prednisolone has been recommended (Chapter 41). For stable or slowly progressive disease, mycophenolate or chlorambucil alone or in combination with azathioprine on alternate days is appropriate. Therapeutic effectiveness should be assessed serially by changes in proteinuria, renal function, or serum albumin concentrations. In the absence of adverse side effects, at least 8 to 12 weeks of therapy should be provided before altering or abandoning a treatment.

Immunoglobulin A Nephropathy

By far the most important cause of renal failure in humans is IgA nephropathy. In this form of type I MPGN, patients have elevated serum IgA, and IgA-containing immune complexes are deposited in the mesangial region. The resulting cellular proliferation and glomerulonephritis can lead to renal failure. The cause of IgA nephropathy is unknown. IgA deposits can be found in the glomeruli of up to 35% of some human populations and up to 47% of dogs. In these dogs, the IgA is deposited in the mesangial and paramesangial areas and is associated with mesangial proliferation. Dogs with enteritis or liver diseases showed the highest prevalence

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of glomerular IgA deposition. A slightly different condition has also been described in dogs aged 4 to 7 years. The animals developed a type III MPGN with mild hematuria, proteinuria, and hypertension. IgA-containing immune complexes were present in both the subepithelial and subendothelial locations.

Swine Glomerulopathy

Spontaneous type I MPGN is observed in pigs. It is especially common in Japan, where it appears to be due to deposition of immune complexes containing IgG (and IgA) antibodies against *Actinobacillus pleuropneumoniae.* In other cases, it may be secondary to chronic virus infections such as hog cholera or African swine fever. Occasionally, however, proliferative glomerulonephritis develops spontaneously. In most cases, epithelial crescent formation suggests that the proliferating cells are epithelial in origin. However, occasional membranoproliferative lesions are observed as well. There is usually strong staining for C3 and weaker staining for IgM using immunofluorescence assays. Pigs rarely have IgG or IgA deposits. Affected pigs are relatively young (<1 year). There is a high prevalence of gastric ulcers in affected animals, but whether this is related is unclear. An inherited complement factor H deficiency in Yorkshire pigs results in the development of a lethal type II MPGN called porcine dense deposit disease (Chapter 4).

Dirofilariasis

Some dogs heavily infected with the heartworm *Dirofilaria immitis* develop glomerular lesions and proteinuria. The lesions include thickening of the glomerular basement membrane with minimal endothelial or mesangial proliferation. Since IgG1-containing deposits may be found on the epithelial side of the basement membrane (type III MPGN), it has been suggested that immune complexes formed by antibodies to heartworm antigens provoke these lesions. Other investigators have disputed the immune complex nature of this condition and claim that the lesions develop in response to the physical presence of microfilariae in glomerular

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blood vessels. The fact that infected dogs may develop amyloidosis (Chapter 7) suggests that they mount a significant immune response to the worms.

Finnish-Landrace Glomerulopathy

Some lambs of the Finnish-Landrace breed die within a few weeks of age as a result of renal failure due to a type I MPGN. The lesions develop *in utero* and are present at birth. The glomerular lesions are similar to those seen in chronic serum sickness, with mesangial cell proliferation and basement membrane thickening (Fig. 32.11). In extreme cases, epithelial cell proliferation may result in epithelial crescent formation. Neutrophils may be present in small numbers within glomeruli, and the rest of the kidney may exhibit diffuse interstitial lymphoid infiltration and necrotizing vasculitis. Deposits containing IgM, IgG, and C3 are found in the glomeruli and choroid plexus, and serum C3 levels are low. The lesions are therefore probably produced as a result of immune complex deposition within these organs, although the nature of the inducing antigen is unknown.

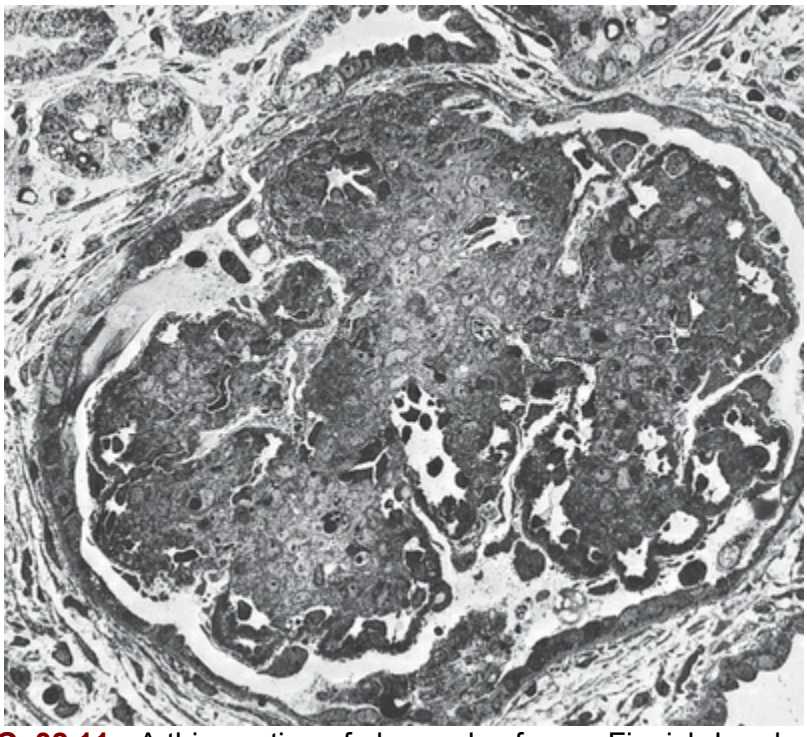


FIG. 32.11 A thin section of glomerulus from a Finnish-Landrace

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lamb with type I membranoproliferative glomerulonephritis. The primary lesion in this case is mesangial proliferation with some basement membrane thickening. (From Angus KW, Gardiner AC, Morgan KT, et al: Mesangiocapillary glomerulonephritis in lambs. II. Pathological findings and electron microscopy of the renal lesions, *J Comp Pathol* 84:319-330, 1974.)

Canine Glomerulopathy

C3 deficiency inherited as an autosomal recessive condition has been described in Brittany Spaniels (Chapter 4). Many of these dogs develop type I MPGN, which may lead to renal failure. The lesions are typical with mesangial proliferation, thickening of the glomerular capillary wall, and deposition of electron-dense deposits in the mesangium and subendothelial space. The deposits contain both IgG and IgM. A familial glomerulopathy has been observed in Bernese Mountain Dogs. It is associated with MPGN and interstitial nephritis.

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Other Immune Complex–Mediated Lesions

Purpura Hemorrhagica

Two to four weeks after an acute *Streptococcus equi* infection (or vaccination against *S. equi*), horses may develop urticaria, followed by severe subcutaneous edema, especially involving the limbs, and the development of hemorrhages in the mucosa and subcutaneous tissues. Affected horses are anorexic and depressed and have a high fever. Immune complexes containing *S. equi* antigens (M-protein or R-protein) are found in the bloodstream of affected animals. These immune complexes cause an acute vasculitis as well as a type I MPGN with resulting proteinuria and azoturia. Other triggers of purpura hemorrhagica in the horse include infections with *Corynebacterium pseudotuberculosis, Rhodococcus equi,* equine influenza virus, and equine herpesvirus type I. In some cases it develops in the absence of any obvious infection. Horses usually recover when aggressively treated with systemic glucocorticosteroids.

Pigs also suffer from sporadic cases of an immune complex–mediated thrombocytopenic purpura syndrome. The animals have thrombocytopenia, anemia, excessive bleeding, and membranoproliferative lesions in their glomeruli. The cause is unknown.

Dietary Hypersensitivity

If an antigenic milk replacer, such as soy protein, is fed to very young calves before the development of ruminal function, the foreign antigen may be absorbed and stimulate antibody formation and a type III hypersensitivity. As a result, the calves become unthrifty and lose weight. However, the precise pathogenesis of this condition is unclear. A small proportion of calves develop an IgE response and a type I hypersensitivity.

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Polyarthritis

Immune complexes can be readily found in the blood and synovial fluid of animals with rheumatoid arthritis and in many with osteoarthritis. In rheumatoid arthritis, they are believed to have a major role in the etiologic progression of disease. Their role in osteoarthritis is unclear, but they may be secondary to local trauma. Important examples of this type of arthritis are the nonerosive polyarthritides seen in foals and puppies and described in Chapter 38.

Drug Hypersensitivities

In the previous chapter, it was pointed out that if a drug attached itself to a cell such as an erythrocyte, the immune response against the drug could lead to elimination of the cell. A similar reaction may occur through type III hypersensitivity reactions if immune complexes bind to host cells. In this case, the cells are recognized as opsonized and are removed by phagocytosis. As might be predicted, if immune complexes bind to erythrocytes, anemia results; if they bind to platelets, thrombocytopenia and purpura result. Binding to granulocytes leads to a granulocytopenia and, consequently, recurrent infection. Severe skin reactions may follow deposition of antibody-drug complexes in the blood vessels of the dermis. However, in many cases, it is difficult to distinguish between the toxic effects of a drug and type III hypersensitivity unless specific antibodies can be eluted from affected cells.

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33

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T Cell–Mediated Hypersensitivity

CHAPTER OUTLINE

The Tuberculin Reaction, 367

- Cutaneous Basophil Hypersensitivity, 369
- Tuberculin Reactions in Cattle, 369
- Tuberculin Reactions in Other Animals, 370
- Johnin Reactions, 370
- Other Skin Tests, 370
- Tuberculosis Serology, 371

Pathological Consequences of Type IV Hypersensitivity, 371

- Tubercle Formation, 371
- Allergic Contact Dermatitis, 371
- Mucocutaneous Diseases, 373

Measurement of Cell-Mediated Immunity, 374

- *In Vivo* Techniques, 374
- *In Vitro* Techniques, 374

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LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Explain how some antigens, when injected into the skin, induce a slowly developing inflammatory response called delayed, or type IV, hypersensitivity.
- Understand how delayed hypersensitivity reactions are mediated by T cells and natural killer (NK) cells.
- Describe the tuberculin response and its importance in detecting tuberculosis.
- Describe the process of tubercle formation.
- Explain the pathogenesis of allergic contact dermatitis.
- Describe the major skin-loss disorders seen in animals.
- Describe why *in vitro* assays for cell-mediated immunity must focus on detecting secreted cytokines or measuring cell division.
- Explain how *in vivo* assays for cell-mediated responses generate a biological response such as the development of a delayed hypersensitivity skin reaction or the rejection of an allograft.
- Describe the basic principles of the IFN release assay, the lymphocyte stimulation assay, the chromium release assay, intradermal mitogen testing, and the ELISpot assay
- Describe how T cell or NK cell cytotoxicity can be measured.

Certain antigens, when injected into the skin of sensitized animals, provoke inflammation at the injection site after a delay of 12 to 24 hours. These delayed hypersensitivity reactions are classified as type IV hypersensitivities and result from interactions between the injected antigen and T cells. An important example of a delayed hypersensitivity reaction is the tuberculin response. This is an inflammatory response that develops in the skin of an animal infected with tuberculosis following intradermal injection of tuberculin. Delayed hypersensitivity reactions can be considered T cell–mediated inflammatory responses directed against organisms that are resistant to elimination by conventional responses.

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The Tuberculin Reaction

Tuberculin is the name given to extracts of mycobacteria used to skin-test animals in order to identify those suffering from tuberculosis. Several types of tuberculin have been employed for this purpose. The most important is purified protein derivative (PPD) tuberculin, prepared by growing organisms in synthetic medium, killing them with steam, and filtering. The PPD tuberculin is precipitated from this filtrate with trichloroacetic acid, washed, and resuspended in buffer ready for use. Thus PPD tuberculin is a crude mixture of proteins, carbohydrates, and lipids. Its major antigenic component is probably the heat-shock protein (HSP) 65. Many of its proteins are shared among different mycobacterial species, so that tests that use PPD tuberculin are relatively nonspecific. It is possible to increase the specificity of the tuberculin test with a defined mycobacterial protein such as early secretory antigenic target-6 (ESAT-6). ESAT-6 is a protein of unknown function that is recognized strongly by T cells. However, the reactions induced by very pure proteins tend to be minimal and require greater amounts of antigen to induce a satisfactory response.

When tuberculin is injected into the skin of a normal animal, there is no obvious response. On the other hand, if it is injected into an animal infected with mycobacteria, a delayed hypersensitivity response occurs. In these animals, a red, indurated (firm) swelling develops at the injection site. The inflammation begins after 12 to 24 hours, reaches its greatest intensity by 24 to 72 hours, and may persist for several weeks before fading gradually. In very severe reactions, tissue necrosis may occur at the injection site. The lesion is infiltrated with lymphocytes and macrophages, although neutrophils are present in the first hours of the reaction (Fig. 33.1).

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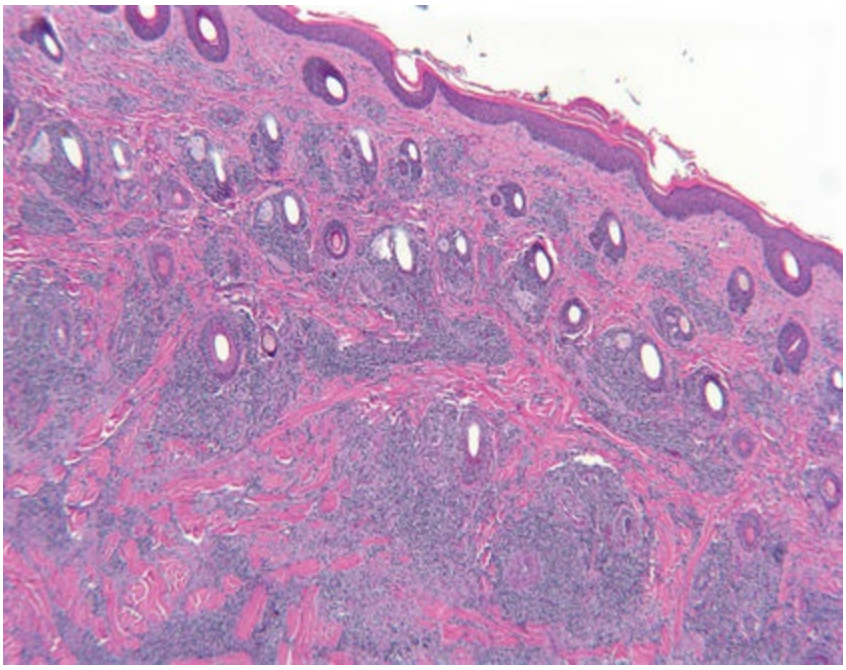


FIG. 33.1 A histological section of a positive tuberculin reaction in bovine skin. Note the perivascular mononuclear cell infiltration as well as the lack of neutrophils or edema. (Courtesy Dr. G. Adams.)

The tuberculin reaction is mediated by T cells. When an animal is infected with *Mycobacterium tuberculosis,* the organisms are readily phagocytosed by macrophages. Some of this mycobacterial antigen triggers a Th1 response and generates memory cells. These memory T cells will respond to injected mycobacterial antigens such as tuberculin. Since a positive tuberculin test can be elicited many years after exposure to an antigen, some of these memory T cells must be very long lived.

When tuberculin is injected intradermally, it is taken up by Langerhans cells, which then migrate to the draining lymph node (Fig. 33.2). Here they present antigen to memory T cells that respond by generating Th1 effector cells. The Th1 cells recognize the antigen when they encounter it in the skin and accumulate around the antigen deposit. By 12 hours the injection site is infiltrated with T cells. In humans and mice, α/β T cells tend to predominate, whereas in sheep and cattle, γ/δ WC1 T cells predominate. There are no B cells in the lesion.

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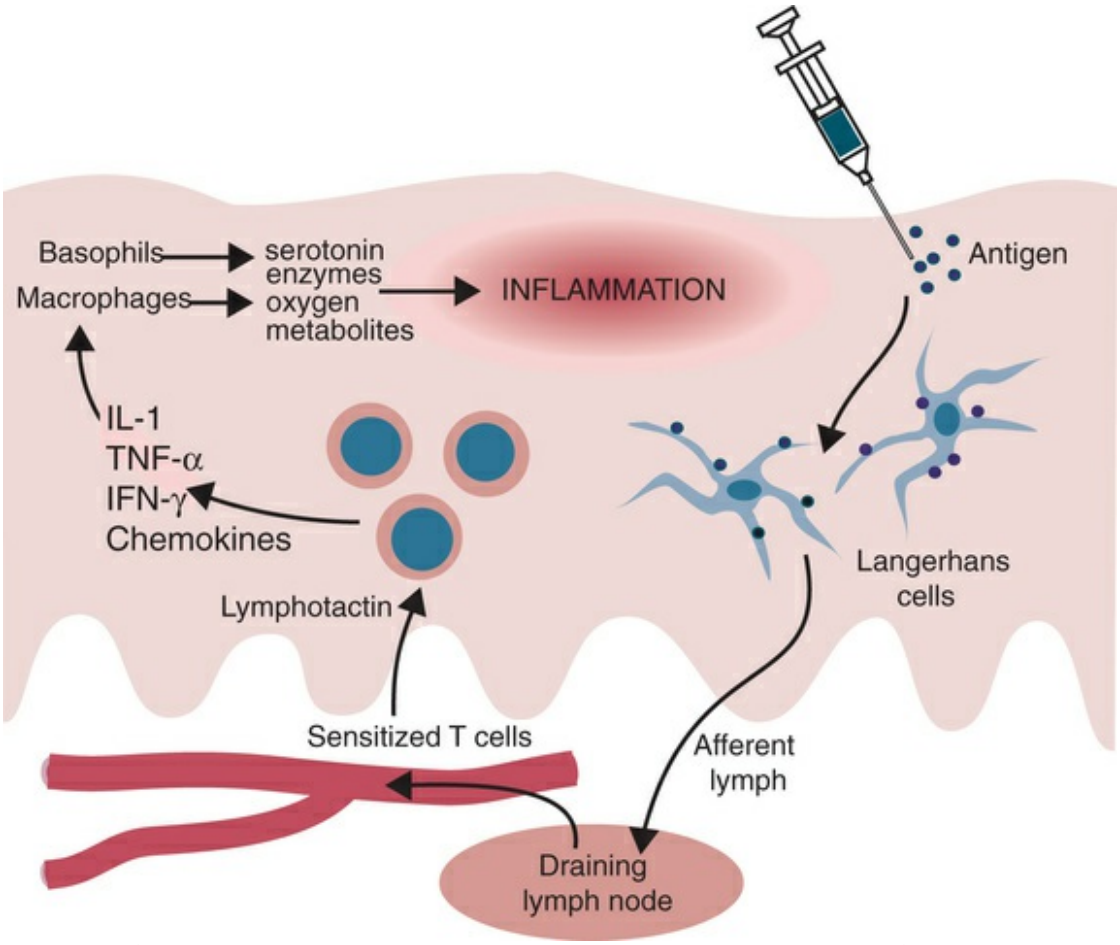


FIG. 33.2 A schematic diagram depicting the mechanism of a delayed hypersensitivity reaction.

The γ/δ T cells recruit other Th1 lymphocytes and macrophages to the site. The Th1 cells secrete interferon-γ (IFN-γ), interleukin-2 (IL-2), and IL-16. The first two act on endothelial cells to increase expression of adherence molecules. IL-2 stimulates production of chemokines that attract and activate more T cells. IL-16 attracts $CD4^+$ T cells. These macrophages also release serotonin and chemokines that attract basophils. Basophil-derived serotonin (in rodents) or histamine (in humans) causes yet more inflammation and enhances migration of mononuclear cells into the lesion. The T cell–derived chemokines can also induce mast cell degranulation, whereas some $CD4^+$ T cells activate mast cells directly through MHC class II–bound antigen.

T cell–derived chemokines cause inflammation and attract even more T cells. Most of these new T cells are not specifically sensitized to the inducing antigen. Only a very small proportion, perhaps 5%, of the lymphocytes seen in a delayed hypersensitivity

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reaction, are specific for the antigen. Most are attracted nonspecifically by XCL1 (lymphotactin). By 60 to 72 hours, the predominant lymphocytes are α/β^+, $CD4^+$, and $CD8^+$. Macrophages accumulate in the lesion attracted by CXCL8 and may be activated by IFN-γ. The tissue damage in intense delayed hypersensitivity reactions may be due to the release of proteases and oxidants from these activated macrophages. The macrophages ingest and eventually destroy the injected antigen. This, plus the migration of regulatory cells into the lesion, permits the tissues to return eventually to normal.

Cutaneous Basophil Hypersensitivity

Feeding ticks can trigger a delayed inflammatory response in the skin where basophils and mast cells predominate (Fig. 33.3). This type of reaction is called cutaneous basophil hypersensitivity (CBH). Parasite-derived antigens are taken up by dendritic cells and presented to Th2 cells. This triggers local IgE and IgG responses that recruit and activate basophils. These basophils accumulate around the tick mouth parts and contribute to local tissue defenses. Similar reactions occur in chickens in response to intradermal Rous sarcoma virus, in rabbits in response to schistosomes, and in humans with allergic contact dermatitis and renal allograft rejection. CBH reactions may also contribute to the development of flea allergy dermatitis in dogs.

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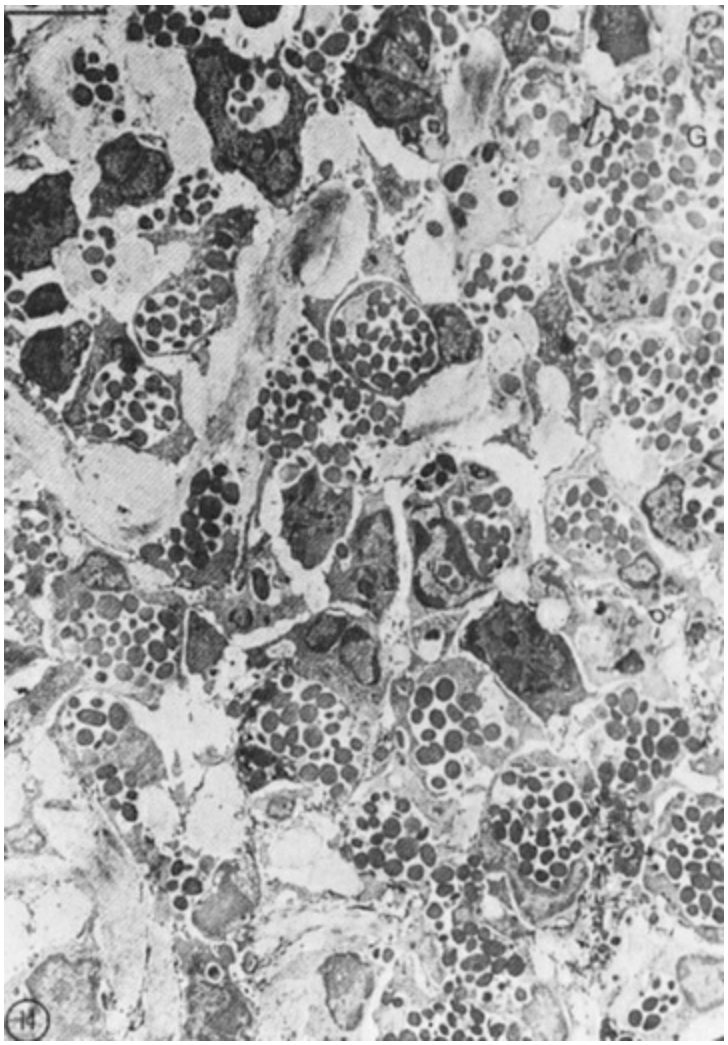


FIG. 33.3 A section of guinea pig skin 18 hours after attachment of a tick in an animal sensitized by prior infestation with tick larvae. The skin is infiltrated with large numbers of basophils. (From McLaren D, Worms MJ, Askenase PW: Cutaneous basophil associated resistance to ectoparasites (ticks). Electron microscopy of *Rhipicephalus appendiculatus* larval feeding sites in actively sensitised guinea pigs and recipients of immune serum, *J Pathol* 139:289, 1983.)

Tuberculin Reactions in Cattle

Because a positive tuberculin reaction occurs only in animals that have, or have had, tuberculosis, skin testing may be used to identify animals affected by this disease. Indeed, the tuberculin test has provided the basis for all tuberculosis eradication schemes that involve the detection and subsequent elimination of infected animals.

Skin testing of cattle may be performed in several ways (Table 33.1). The simplest is the single intradermal (SID) test. In this test,

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0.1 mL of PPD tuberculin derived from *M. tuberculosis* or *Mycobacterium bovis* is injected into one caudal fold (the folds of skin underneath the tail), and the injection site is examined 72 to 96 hours later. A comparison is easily made between the injected and the uninjected folds, and a positive reaction consisting of a firm lump or marked discoloration at the injection site is readily detected.

TABLE 33.1
Tuberculin Tests Used in Cattle

Test	Use	Advantages	Disadvantages
Single intradermal	Routine testing	Simple	Prone to false positives Poor sensitivity
Comparative	When avian TB or Johne's disease is prevalent	More specific than SID	More complex than SID
Short thermal	Use in postpartum animals and in infected animals	High efficiency	Time consuming Risk for anaphylaxis
Stormont	Use in postpartum animals and in advanced cases	Very sensitive and accurate	Three visits required May sensitize an animal

In the United States, two separate tests are performed. Thus two injections of tuberculin are made. One into the mucocutaneous junction of the vulva and the other into a caudal fold; in other countries, tuberculin is normally injected into the skin on the side of the neck. The neck site is more sensitive than the caudal folds, but restraint of the animal may be more difficult, and good injection technique is critical.

The advantage of the SID test is its simplicity and low cost, but it has significant limitations. Interpretation of results may be inconsistent, plus the need for a second visit and low accuracy. Its main disadvantage is that because of poor specificity, it cannot distinguish between tuberculosis and infection by related mycobacteria such as *Mycobacterium avium* and *Mycobacterium avium paratuberculosis,* or the *Nocardia* group of organisms. A second disadvantage is that some animals react positively to the test but on necropsy do not have detectable tuberculosis lesions. The reasons for this are unclear but these false positives probably result from sensitization by environmental mycobacteria such as *Mycobacterium phlei.*

False-negative SID tests may occur in animals with advanced tuberculosis, in animals with very early infection, in animals that

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have calved within the preceding 4 to 6 weeks, in very old cows, and in animals tested during the preceding 1 to 10 weeks. The lack of reaction (anergy) seen in advanced cases of tuberculosis is also seen in clinical Johne's disease and appears to be due to the presence of an IgG antibody that prevents T cells from reacting with antigen. There is also evidence for suppression by regulatory cells. Repeated short-interval tuberculin testing leads to desensitization associated with elevated IL-10 and decreased IL-1β responses. (It does not influence IFN-γ responses.) It also results in the induction of antibodies against some *M. bovis* antigens such as HSP 70 and HSP 83.

Because of these defects in the SID, several modifications of this test have been developed. The comparative cervical test, for example, involves intradermal inoculation of both avian and bovine tuberculins. Each tuberculin is injected into the side of the neck at separate sites, and these sites are examined 72 hours later. In general, if the avian tuberculin site shows the greatest reaction, the animal is considered to be infected with *M. avium* or *M. avium paratuberculosis.* On the other hand, if the *M. bovis* site shows a significantly greater reaction, then it is believed that the animal is infected with *M. bovis* or *M. tuberculosis.* This test is useful when a high prevalence of avian tuberculosis or Johne's disease is anticipated. PPD from *M. bovis* is more specific in cattle than *M. tuberculosis,* giving less cross-reaction with *M. avium* as well as being more appropriate for use in cattle, and is therefore preferred. In practice, the comparative test has a sensitivity of 90% (10% false negatives) and a specificity of greater than 99% (<1% false positives); however, this depends on the criteria used to read the results.

Another modified tuberculin test is the short thermal test, in which a large volume of tuberculin solution is given subcutaneously, and the animal is examined for a rise in temperature between 4 and 8 hours later. (Presumably the tuberculin acts on T cells that then provoke the release of IL-1 and other cytokines from macrophages.) The Stormont test relies on the increased sensitivity of a test site, which occurs after a single injection; it is performed by giving two doses of tuberculin at the same injection site 7 days apart. Both tests are relatively sensitive.

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As a result, they may be used in postpartum cows as well as for the testing of heavily infected animals.

Tuberculin Reactions in Other Animals

Tuberculin skin testing has never been a widely employed procedure in domestic animals other than cattle, so information on these is scant. Nevertheless, it appears that the ability of different species to mount a classic tuberculin reaction varies greatly. In pigs and cats, for example, the tuberculin test is positive for only a short period following infection. In pigs and dogs, the best test is an SID test given in the skin behind the ear, whereas in cats, the short thermal test is probably best. In sheep and goats, the antigen is usually given in the caudal fold, but the results are usually unreliable in these species as well. Horses appear to be unusually sensitive to tuberculin, and the dose used must be reduced accordingly. Nevertheless, the results obtained do not always correlate well with the disease status of the animal. In birds, good reactions may be obtained by inoculating tuberculin into the wattle or wing web.

Johnin Reactions

Animals infected with *M. avium paratuberculosis,* the cause of Johne's disease, develop a delayed hypersensitivity reaction following intradermal inoculation of an extract of this organism, called *johnin.* The injection site contains a mixed mononuclear cell infiltrate, possibly with multinucleate giant cells or plasma cells. Sheep with paucibacillary intestinal lesions tend to have greater induration and greater cellular infiltration than animals with multibacillary lesions. Johnin can be used in a single intradermal test but, like tuberculin, it may be negative in animals with clinical disease. An intravenous johnin test will be positive in these cases and may be a preferable alternative to the SID test. In this test the antigen is administered intravenously, and the animal's temperature is noted 6 hours later. A rise in temperature of 1° C or neutrophilia is considered a positive result. These tests are probably of limited usefulness in individual animals but may help identify infected herds.

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Other Skin Tests

Positive delayed hypersensitivity skin reactions may be obtained in any infectious disease in which T cell–mediated immunity has a significant role. Thus extracts of *Brucella abortus* have been used in attempts to diagnose brucellosis. These include brucellin, a filtrate of a 20-day broth culture, and brucellergen, a nucleoprotein extract. Because these extracts may induce antibodies to *Brucella,* they cannot be employed in areas where eradication is monitored by serological tests. In glanders of horses, a culture filtrate of the organism *Burkholderia mallei,* termed *mallein,* is used for skin testing. Mallein can be used in either a short thermal test or an ophthalmic test. An ophthalmic test, also occasionally employed in tuberculosis, is performed by dropping the antigen solution into an eye. Transient conjunctivitis develops if the test is positive. Another method of testing for glanders is the intrapalpebral test. In this test, mallein is injected into the skin of the lower eyelid, where a positive reaction results in swelling and ophthalmia.

Intradermal skin testing with microbial extracts is also employed in the diagnosis of many fungal diseases; thus histoplasmin is used for histoplasmosis, coccidioidin for coccidioidomycosis, and so on. In these cases, the tests are not very specific, and the test procedure may effectively sensitize the tested animal, causing it to become serologically positive. This problem also arises when toxoplasmin is used in attempts to diagnose toxoplasmosis (Chapter 28).

Tuberculosis Serology

It must not be assumed that *M. bovis* stimulates only T cells. As infection progresses, antibodies are produced. These are directed against the immunodominant antigens MPB83 and MPB70 released by *M. bovis* in advanced infections. The highest and earliest immune response is directed against MPB83. These antibodies can be detected using ELISA tests or lateral flow assays (Chapter 42). These assays are useful in the identification of infected animals missed by intradermal skin testing. Their sensitivity is often low, but they are simple, rapid and inexpensive.

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Pathological Consequences of Type IV Hypersensitivity

Tubercle Formation

Although the tuberculin reaction induced by intradermal inoculation is artificial in that antigen is administered by injection, a similar inflammatory response occurs if living tubercle bacilli lodge in tissues and sensitize an animal. However, *M. tuberculosis* is resistant to intracellular destruction until M1 macrophages are activated by Th1 cells (Chapter 18), and dead organisms are very slowly removed because they contain large quantities of poorly metabolized waxes. As a result, the reaction to whole organisms is prolonged, and macrophages accumulate in very large numbers. Many of these macrophages ingest the bacteria but fail to prevent its growth and so die. Other macrophages fuse to form multinucleated giant cells. After 4 to 5 weeks of infection, microscopic granulomas enlarge and coalesce. The lesion that develops around invading tubercle bacilli therefore consists of a mass of caseous (cheesy!) debris containing both living and dead organisms surrounded by a layer of fibroblasts, lymphocytes, and macrophages, which in this location are called epithelioid cells (Chapter 6). The entire lesion is a type I granuloma called a tubercle (Fig. 33.4). The mycobacteria are unable to multiply within the necrotic tissue because of its low pH and lack of oxygen. Nevertheless, some bacteria may survive in a dormant state. If the host mounts an adequate immune response of the correct (Th1) type, this may be sufficient to control the infection. However, if immunity is insufficient or inappropriate (e.g., a Th2 response), the organisms may escape from the tubercle and spread to local lymph nodes and nearby tissues. When the response is inadequate, the multiplying organisms continue to spread, and the resulting lung damage, together with liquefaction of the caseous center of the tubercle, leads to rapidly progressive disease.

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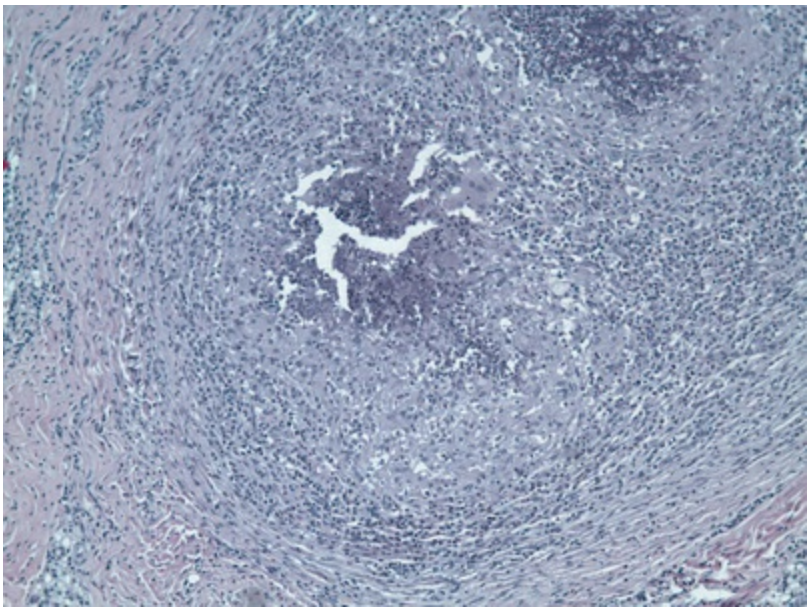


FIG. 33.4 A histological section from the lymph node of a cow infected with *Mycobacterium bovis* showing a small tubercle. The dark central mass is caseous material. It is surrounded by layers of macrophages and lymphocytes and walled off by fibroblasts.
(Courtesy Dr. J. Edwards.)

During the early stages of granuloma formation, macrophages are highly motile and provide the pathogen with fresh cells to infect. These infected macrophages die but then recruit uninfected macrophages to the site of infection. They phagocytose old macrophages and their bacterial contents. This process leads to the efficient spread and expansion of the bacterial population. Thus virulent mycobacteria exploit the process by which macrophages promote tissue repair.

Allergic Contact Dermatitis

If certain reactive chemicals are painted onto the skin, they may trigger inflammation mediated through PRRs and inflammasomes. For example, TLR4 is a receptor for multiple contact sensitizers such as nickel or trinitrochlorobenzene. They therefore trigger inflammation by releasing cytokines such as IL-1β and TNF-α. Additionally, if chemically reactive, they may bind to skin proteins such as keratin and act as strong haptens. The resulting hapten-protein complexes are captured by Langerhans cells in the dermis (Fig. 33.5). The Langerhans cells migrate to draining lymph nodes and present the antigen to T cells. Repeated exposure will increase

the degree of sensitization. Subsequent exposure to the sensitizing chemical may elicit a T cell–mediated inflammatory response called allergic contact dermatitis. Thus when encountering these hapten-protein complexes again, Langerhans cells secrete large amounts of IL-12, IL-18, and IL-23 that activate both Th1 and Th17 cells. These hapten-specific helper cells produce large amounts of IFN-γ and IL-17 and promote the activities of cytotoxic T cells. Following exposure to a sensitizing chemical, macrophages and $CD8^+$ lymphocytes infiltrate the dermis, taking about 24 hours to generate a response. Eventually, these cytotoxic T cells kill the altered cells, resulting in the development of intraepithelial vesicles. This destructive reaction together with the production of IL-33 and accompanying inflammation presents as an intensely pruritic skin reaction (Fig. 33.6). In addition to α/β T cells, other cell types, such as γ/δ T cells, B-1 cells, mast cells, and NKT cells, may contribute to the reaction. The inflammation is eventually moderated by IL-10 and TGF-β from Treg cells.

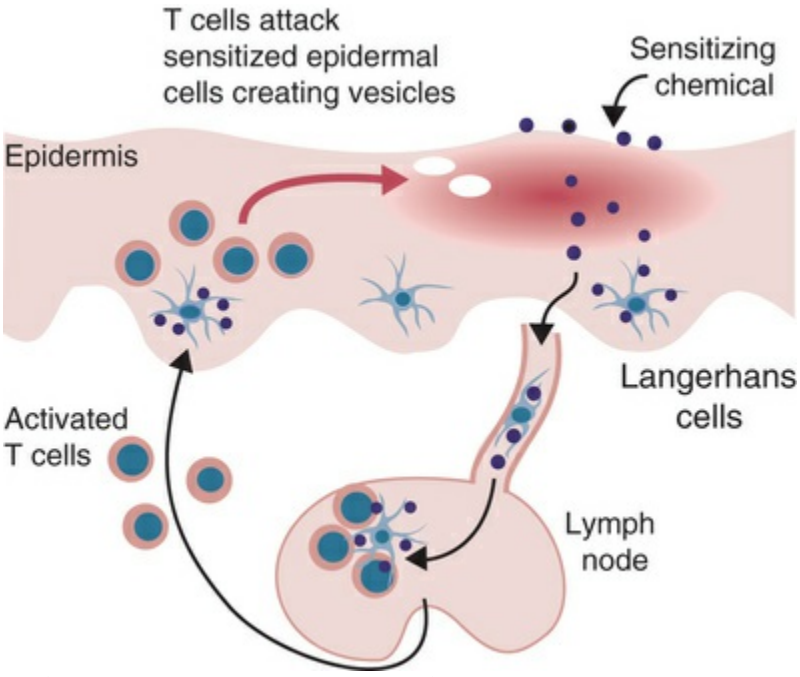


FIG. 33.5 The pathogenesis of allergic contact dermatitis.

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FIG. 33.6 A severe case of an allergic contact dermatitis in a Great Dane. The contact sensitizer was not identified but it appeared to originate in the concrete surrounding a swimming pool. Chloride ions appear to release a contact sensitizer from concrete. (Courtesy Dr. R. Kennis.)

Recent studies have demonstrated that some forms of contact dermatitis can be readily induced in mice that lack all types of lymphocytes except NK cells! In addition, contact dermatitis appears to be antigen-specific insofar as primed animals mount a much stronger response than unprimed animals. This appears to be a property of a subpopulation of NK cells. These NK cells can survive for at least 28 days in mice and form a "memory" cell population. These results clearly are at variance with our previous ideas about the antigenic specificity of NK cells and their role in immunity. It is also of interest to note that contact dermatitis will not occur in skin that lacks functional nerve fibers. Clearly, allergic dermatitis has a complex and poorly understood etiology.

The chemicals that induce allergic contact dermatitis are usually highly reactive molecules that combine chemically with skin

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proteins to act as haptens; they include formaldehyde, picric acid, aniline dyes, plant resins and oils, organophosphates, some topical medications such as neomycin, and salts of metals such as nickel and beryllium (Fig. 33.7). Thus allergic contact dermatitis can occur on pathologists' fingers as a result of exposure to formaldehyde; on the ears of dogs treated with neomycin for otitis externa; on the foot pads, scrotum, and ventral abdomen of dogs on exposure to some carpet dyes and deodorizers; on parts of the body exposed to the oils (urushiols) of the poison ivy plant *(Rhus radicans);* and around the neck of animals as a result of exposure to dichlorvos (2,2-dichlorovinyldimethylphosphate) in flea collars (Box 33.1). Severe lesions may develop on the teats of dairy cattle as a result of a contact dermatitis to a component of milking machine rubber (N-isopropyl-N-phenyl diamine). Calcium cyanamide ($CaCN_2$) is used to reduce *Escherichia coli* levels in bedding. Dairy cattle have developed severe dermatitis on contact with calcium cyanamide spread on the floor of a cattle shed to prevent mastitis.

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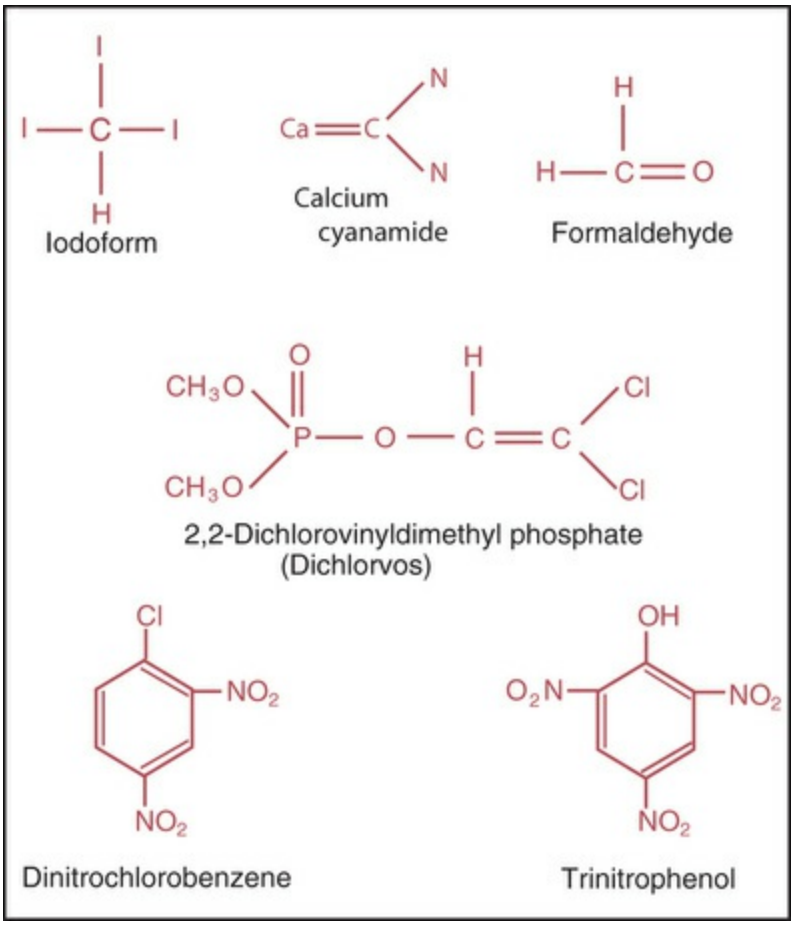


FIG. 33.7 Some of the simple chemicals that can cause allergic contact dermatitis.

Box 33.1

Sources of Contact Allergens in Animals

Insecticides in flea collars

In sprays

In dips

Wood preservatives

Floor waxes

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Carpet dyes

Some pollens

Dermatological drugs (creams, ointments)

Leather products

Paints

House plants

Allergic contact dermatitis involving the muzzle of dogs has been reported to result from sensitivity to plastics in food bowls. Some dogs, instead of developing the more usual type I hypersensitivity to pollen proteins, experience an allergic contact dermatitis as a result of a type IV hypersensitivity to pollen resins. It is unusual for allergic contact dermatitis to affect the haired areas of the skin unless the allergen is in a liquid. Thus allergic contact dermatitis to shampoo components may result in total-body involvement. The period required for sensitization ranges from 6 months to several years.

The lesions of allergic contact dermatitis range from a mild erythema to severe erythematous vesiculation. However, because of the intense pruritus, self-trauma, excoriation, ulceration, and secondary staphylococcal pyoderma often mask the true nature of the lesion. If the exposure to the allergen persists, hyperkeratosis, acanthosis, and dermal fibrosis may eventually occur. Histologically, the lesion is marked by a mononuclear cell infiltration and vacuolation of skin cells under attack by cytotoxic T cells (Table 33.2).

TABLE 33.2

Comparison of the Major Forms of Allergic Dermatitis

	Atopic Dermatitis	Allergic Contact Dermatitis
Pathogenesis	Type I hypersensitivity	Type IV hypersensitivity
Clinical signs	Hyperemia, urticaria, pruritus	Hyperemia, vesiculation, alopecia, erythema
Distribution	Face, nose, eyes, feet, perineum	Hairless areas, usually ventral abdomen and feet
Major allergens	Foods and pollens, fleas, inhaled allergens	Reactive chemicals, dyes in contact with skin
Diagnosis	Intradermal testing, immediate response	Delayed response on patch testing

Pathology	Eosinophilic infiltration, edema	Mononuclear cell infiltration, vesiculation
Treatment	Steroids, antihistamines, hyposensitization	Steroids

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Allergic contact dermatitis is diagnosed by removal of the suspected antigen and by patch testing. In "closed" patch tests, suspected allergens are used to impregnate gauze swabs that are then attached to the shaved skin with tape. After 48 to 72 hours the dressing is removed and the areas in contact with the swabs examined. A positive reaction is indicated by local erythema and vesiculation. Closed-patch tests may be impractical for some dogs and cats. An "open" patch test may therefore be employed. In this procedure, a solution of the suspected allergen is applied to shaved normal skin and the area examined daily for up to 5 days. Identification of the offending allergen and its avoidance by the animal is the optimal therapy for allergic contact dermatitis. Hyposensitization therapy is not effective. Steroids are used to treat acute cases, with antibiotics to control secondary infections.

Mucocutaneous Diseases

Three related mucocutaneous disorders—erythema multiforme, Stevens-Johnson syndrome, and toxic epidermal necrolysis—are well recognized in humans and have been diagnosed in dogs and cats. The three diseases are characterized by skin loss of increasing severity. Erythema multiforme is characterized by patchy skin loss and low morbidity; Stevens-Johnson syndrome is more severe but involves less than 10% of the body surface; toxic epidermal necrolysis is much more serious, with affected individuals losing more than 30% of their epidermis. Mortality is high. The three conditions, however, overlap considerably. Stevens-Johnson syndrome and toxic epidermal necrolysis are believed to involve a T cell-mediated hypersensitivity to drugs. Erythema multiforme is not associated with drug administration. Affected animals develop vesicles, shed large areas of epidermis, and develop skin ulcers as a result of widespread keratinocyte apoptosis. The apoptosis is believed to result from drugs or their metabolites binding to the epidermal cells and upregulating CD95L expression as well as the production of soluble CD95L and granulysin, triggering their destruction by cytotoxic T cells. (Intradermal inoculation of granulysin solutions in mice at a concentration found in blister fluid

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results in the development of lesions mimicking Stevens-Johnson syndrome.) The skin lesions are infiltrated mainly by $CD8^+$ T cells and fewer $CD4^+$ cells. Many different drugs may trigger these responses, but common inducers in dogs include trimethoprim-potentiated sulfonamides, β-lactam antibiotics, penicillin, and cephalexin. Beginning about 14 days after drug exposure, the skin begins to blister and slough. Animals develop generalized illness, including dyspnea, vomiting, fever, and weight loss. In dogs, sloughing of the epidermis occurs over the nasal planum, the footpads, and the oral, pharyngeal, nasal, conjunctival, and preputial mucosa. Fluid loss leads to electrolyte imbalances, whereas life-threatening secondary infections are common. Biopsy specimens show extensive epidermal cell death.

Treatment involves immediate withdrawal of the offending drug followed by symptomatic treatment, including fluid replacement. Corticosteroids should be avoided since they increase the animal's susceptibility to skin infections and worsen the prognosis. Antibiotics should only be administered if skin infections occur. Intravenous administration of high doses of human immunoglobulins have been used successfully to treat this disease in dogs. It is believed that these immunoglobulins block CD95/CD95-ligand interactions and prevent keratinocyte apoptosis (Chapter 41).

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Measurement of Cell-Mediated Immunity

Although diagnostic immunology is based largely on the detection of serum antibodies, measurement of cell-mediated immune responsiveness in animals may be desirable under some circumstances. For example, in determining the effectiveness of a vaccine, one must take into account that serum antibody levels may not truly reflect the degree of immunity possessed by an animal. Animals without detectable antibodies may possess significant cell-mediated immunity. The term cell-mediated immunity encompasses a diverse set of mechanisms that employ T cells and macrophages for protection. Currently, both *in vivo* and *in vitro* techniques are used for this purpose.

In Vivo Techniques

The simplest *in vivo* test of cell-mediated immunity is an intradermal skin test such as the tuberculin test. The inflammation and swelling that occur in response to intradermally injected antigens may be considered cell mediated, provided that it has the characteristic time course and histological features of a type IV reaction. Intradermal skin tests are not always convenient, they are difficult to quantitate, and injection of an antigen may sensitize an animal, thus preventing further testing.

It is sometimes useful to measure the ability of an animal to mount cell-mediated immune responses in general rather than the response to one specific antigen. One way to do this is to give the animal a small skin allograft and measure its survival time. A much simpler technique is to paint a small area of the animal's skin with a contact sensitizer such as dinitrochlorobenzene. The intensity of the resulting allergic contact dermatitis provides a rough estimate of the animal's ability to mount a cell-mediated immune response.

If the T cell–stimulating lectin phytohemagglutinin is injected intradermally, it provokes a local tissue reaction with many features of a delayed hypersensitivity response. In pigs, for example, this

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reaction is characterized by infiltration with γ/δ^+, $CD4^-$, and $CD8^-$ T cells. This is a very convenient and rapid method of assessing an animal's ability to mount a cell-mediated response without the need for first sensitizing the animal to an antigen. However, the response to phytohemagglutinin is nonspecific, and its interpretation may be difficult.

In Vitro Techniques

In vitro tests are designed to measure the antigen-specific activation and proliferation of T cells. These also include their cytotoxic activities and their production of cytokines. All of these tests require that T cells be grown in cell culture; therefore, few are useful for use in the field.

To measure T cell proliferation in response to an antigen, a suspension of purified peripheral blood lymphocytes from the animal to be tested is mixed with the antigen and cultured for 48 to 96 hours (Fig. 33.8). Twelve hours before harvesting, thymidine labeled with the radioactive isotope tritium is added to the cultures. Normal, nondividing lymphocytes do not take up thymidine, but dividing cells that are actively synthesizing DNA do. Thus if the T cells are proliferating, they will take up the tritiated thymidine. Their radioactivity will provide a measure of this proliferation. The greater their proliferation in response of the cells to an antigen, the greater will be their radioactivity. The ratio of the radioactivity in the stimulated cultures to the radioactivity in the controls is called the stimulation index. A related technique is to measure the proliferation of lymphocytes in response to mitogenic lectins such as concanavalin A (see Box 13.2). The intensity of the lymphocyte proliferative response, as measured by tritiated thymidine uptake, provides an estimate of the reactivity of an animal's lymphocytes.

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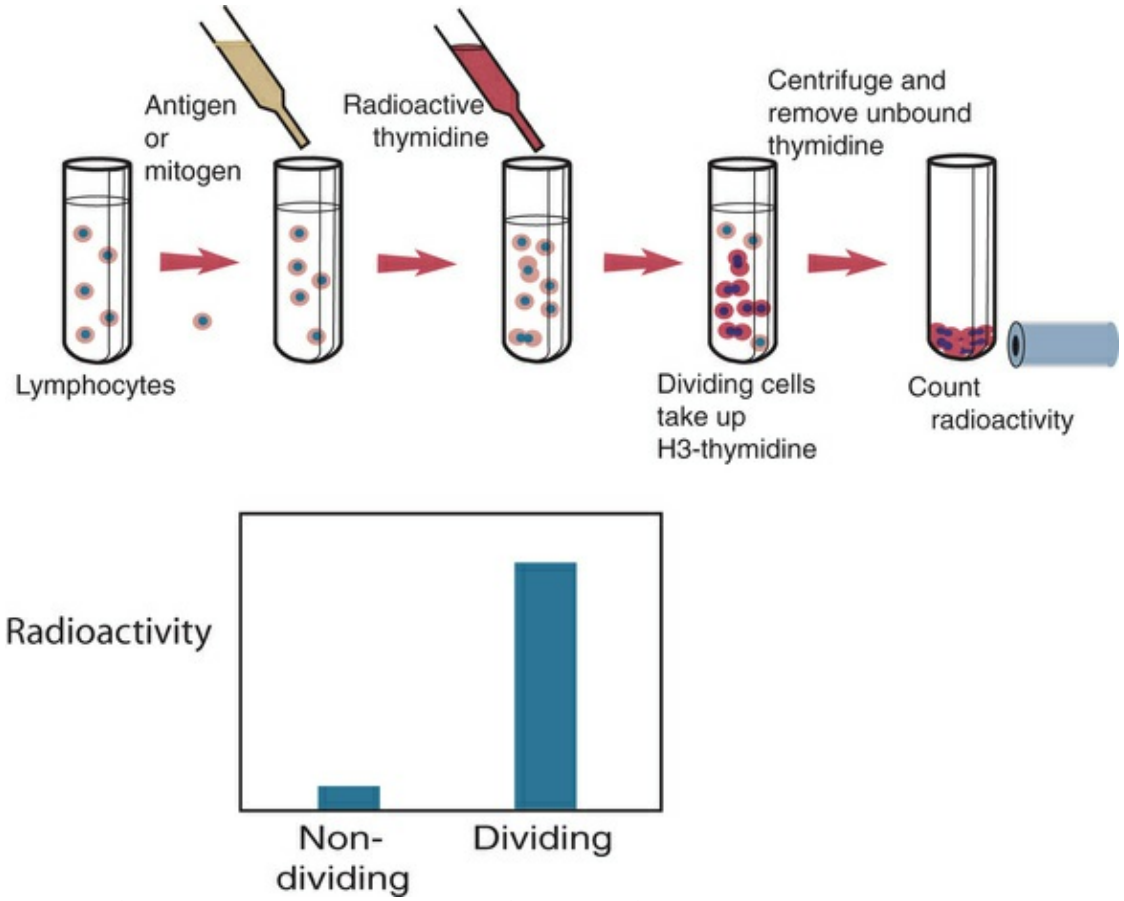


FIG. 33.8 The measurement of cell proliferation by detecting the uptake of tritiated thymidine. Cells are stimulated to divide by specific antigen or a mitogen. The thymidine is incorporated into the DNA of the dividing cells. The uptake is simply measured by the radioactivity of the cells.

Radioactive tritium may be replaced in proliferation assays by a simple colorimetric enzyme assay. Methylthiazoldiphenyltetrazolium bromide (MTT) is a pale yellow compound that serves as a substrate for active mitochondrial enzymes. The enzymes change the MTT color to dark blue. The intensity of this color change is a measure of the number of living cells in a culture. In proliferation assays, the number of living cells increases, and this can be measured colorimetrically. The test is sufficiently sensitive to quantify the increase in T cell numbers triggered by antigen or mitogens.

To measure T cell–mediated cytotoxicity, it is necessary to have a simple method of measuring cell death. This is usually based on the fact that living cells take up and retain chromium ions. When a cell dies, the chromium is released into the extracellular fluid. Radioactive sodium chromate (^{51}Cr) may be used to label target

cells (Fig. 33.9). Lymphocytes from an immune animal are mixed in an appropriate ratio with ^{51}Cr-labeled target cells. The mixture is then incubated for 4 to 24 hours at 37° C. At the end of this time the cell suspension is centrifuged and the presence of free ^{51}Cr in the supernatant measured. The amount of chromium released is related directly to the number of target cells killed. The amount of chromium released in the absence of cytotoxic cells must also be measured and subtracted from that released in the presence of cytotoxic cells to obtain a true reading.

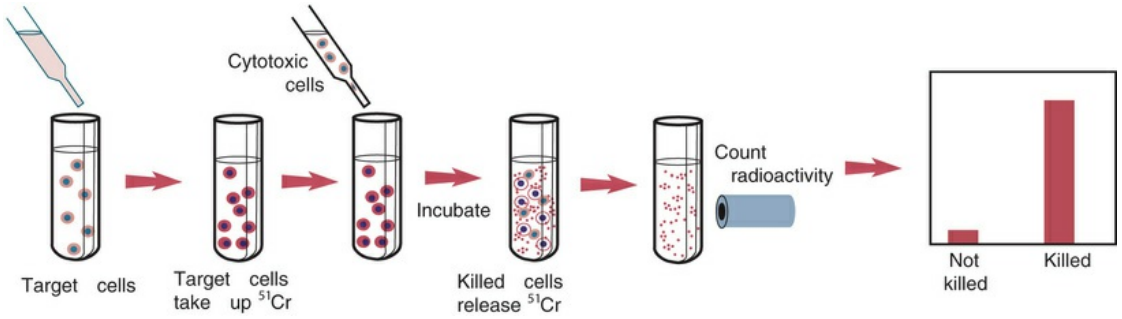


FIG. 33.9 The measurement of cell death by detecting the release of chromium-51 by dying cells. This release may be triggered by cytotoxic T cells or NK cells.

A third in vitro assay is the measurement of cytokine release by T cells. One important example of this technique involves measuring the release of IFN-γ by peripheral blood lymphocytes following exposure to tuberculin or purified mycobacterial antigens (Fig. 33.10). This method has been developed as an alternative or supplement to the tuberculin test for the diagnosis of tuberculosis in cattle and deer. It involves adding tuberculin PPD to heparinized blood and incubating the mixture for 24 to 48 hours at 37° C. The plasma is then removed and assayed for any interferon produced, either by means of a simple bioassay or preferably by use of a sandwich enzyme-linked immunosorbent assay (ELISA) employing monoclonal antibodies. Three "antigens" are commonly used: no antigen (negative control), *M. bovis* PPD, and *M. avium* PPD. The *M. avium* PPD is used to detect false-positive cross-reactions. Purified, recombinant mycobacterial proteins such as ESAT-6 can reduce the incidence of false-positive results even further. This technique has advantages over conventional tuberculin tests in that it does not

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sensitize the animal under testing by injection of antigen. In addition, the animal does not have to be held for several days for the test to be read. It is also much simpler than other in vitro tests for cell-mediated immunity. The assay is at least as sensitive as the single intradermal test and, if purified recombinant mycobacterial proteins are employed, is highly specific. (Its sensitivity is about 85%, and its specificity is as high as 90% to 99%.) Positive results are obtained earlier than by skin testing. However, it does appear to detect tuberculosis in a slightly different population of animals than the skin test. It has also been successfully used to diagnose Johne's disease in sheep as well as tuberculosis in cats and dogs.

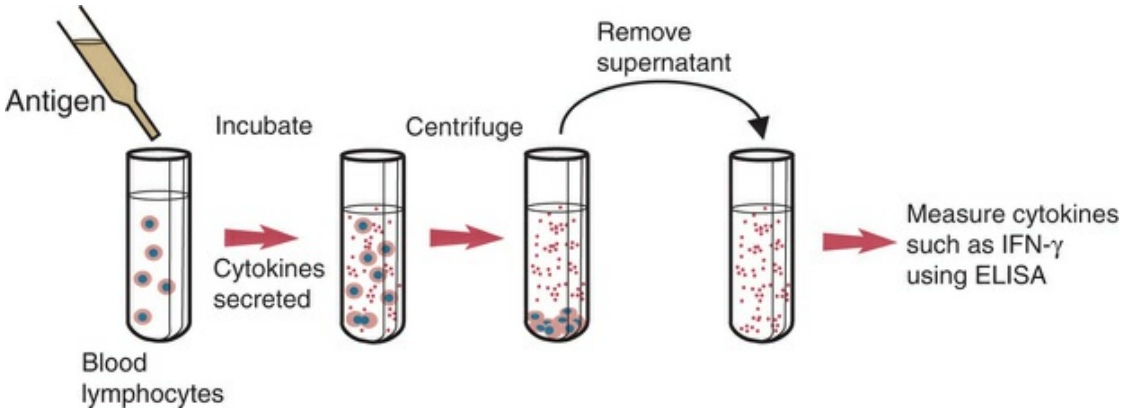


FIG. 33.10 The release of IFN-γ by peripheral blood lymphocytes following exposure to tuberculin or to purified mycobacterial antigens. This technique can be used for the diagnosis of tuberculosis in cattle and deer. Tuberculin PPD is added to blood, and the mixture is incubated for 24 to 48 hours. The plasma is then removed and assayed for any interferon produced.

It is possible to use a variation of a sandwich ELISA assay (Chapter 42) to determine the frequency of cytokine-secreting cells (Fig. 33.11). In the Enzyme-Linked ImmunoSpot (ELISpot) assay, a capture-antibody directed against the cytokine of interest coats the bottom of plastic tissue culture wells. The cells to be tested are cultured on this surface and exposed to the antigen of interest. If the cells secrete the cytokine of interest, it will bind to nearby capture-antibodies. Once the culture period is completed, the presence of this bound cytokine can be detected by a conventional sandwich ELISA using specific detection antibody and enzyme-labeled antiglobulin. This results in the development of a pattern of colored

spots that each correspond to the location of a cytokine-secreting cell. These spots can be counted and the frequency of specific cytokine-producing cells determined. This assay can also be used to quantitate cytotoxic cells by detecting granzyme or perforin production.

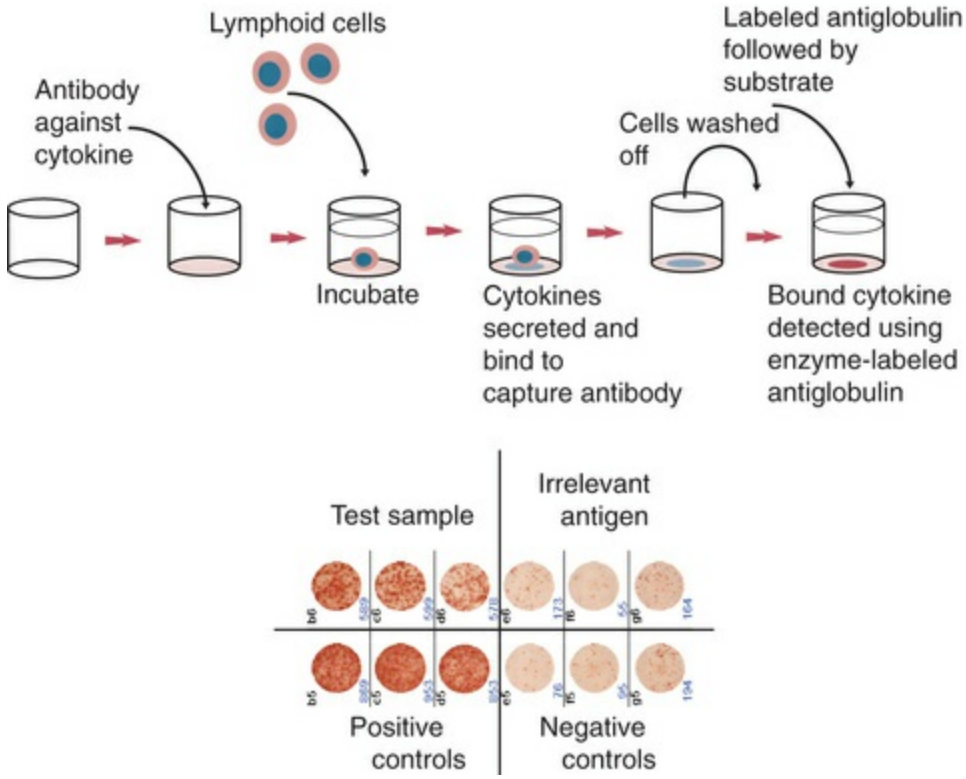


FIG. 33.11 The principles of the ELISpot assay. The photograph shows the IFN-γ response of bovine peripheral blood mononuclear cells exposed to a defined *Anaplasma marginale* antigen. (Courtesy Dr. W. Mwangi.)

Although all of the assays described previously can be used to measure at least some aspects of cell-mediated immunity, none provides a complete picture. The investigator may of course simply be interested in the response to a single antigen or organism. In these cases, either a skin test or an in vitro assay may be appropriate. This is best exemplified by the tests available for the diagnosis of tuberculosis. *In vitro* tests are also useful if the time course of a cell-mediated immune response is to be examined. Repeated testing can be performed simply by obtaining more lymphocytes. If, on the other hand, an investigator wishes to obtain an overview of an animal's abilities in this area, one of the

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nonspecific *in vivo* assays may be more appropriate. These can be useful, for example, in assessing immune function in young animals thought to be immunodeficient. However, it is important to point out that in these animals, a complete hematological examination should be performed before more complex assays are considered. It is also prudent to measure the important lymphocyte subpopulations by flow cytometry. An animal that has no T cells is unlikely to mount any sort of cell-mediated response.

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34

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Organ Graft Rejection and Pregnancy

CHAPTER OUTLINE

Grafting of Organs, 377
Allograft Rejection, 378
Histocompatibility Antigens, 378
Renal Allografts, 379
Pathogenesis of Allograft Rejection, 380
Innate Mechanisms, 380
Adaptive Mechanisms, 380
Graft Destruction, 381
Prevention of Allograft Rejection, 382
Skin Allografts, 382
Liver Allografts, 382
Cardiac Allografts, 382
Corneal Allografts, 382
Bone Allografts, 383
Bone Marrow Allografts, 383
Graft-versus-Host Disease, 383
Xenografts, 384
Allografts and the Reproductive System, 385
Sperm, 385

VetBooks.ir

Pregnancy, 385

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Define allografts, isografts, and xenografts.
- Explain how allografts are rejected.
- Describe how the response to donor histocompatibility antigens causes acute rejection.
- Understand why chronic rejection and rejection directed against donor blood groups are mainly antibody mediated.
- Explain how bone marrow stem cell allografts given to immunosuppressed recipients may cause graft-versus-host disease.
- Describe the basic processes occurring in graft-versus-host disease.
- Explain why some allografts, such as those from the cornea, are not readily rejected.
- Describe the mechanisms that ensure that the fetal allograft is not rejected.
- Describe how xenografts trigger very strong rejection responses.
- List the major drugs employed to combat allograft rejection.

Although the immune responses first attracted the attention of scientists because of their ability to fight infections, the observation that animals reject foreign organ grafts led to a much broader view of the immune system in that it indicated that the immune system had a surveillance function. The rejection of a foreign organ graft simply reflects the role of the immune system in identifying and destroying "abnormal" cells.

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Grafting of Organs

Advances in surgery have permitted the transfer of many tissues or organs between different parts of the body or between different individuals. When moved to a different part of an animal's own body, such transplants do not trigger an immune response. This type of graft within an individual is called an autograft (Fig. 34.1). Examples of autografting include the use of skin to cover a burn in plastic surgery and the use of a segment of vein to bypass blocked cardiac arteries. Since autografts do not express foreign antigens, they do not trigger an immune response.

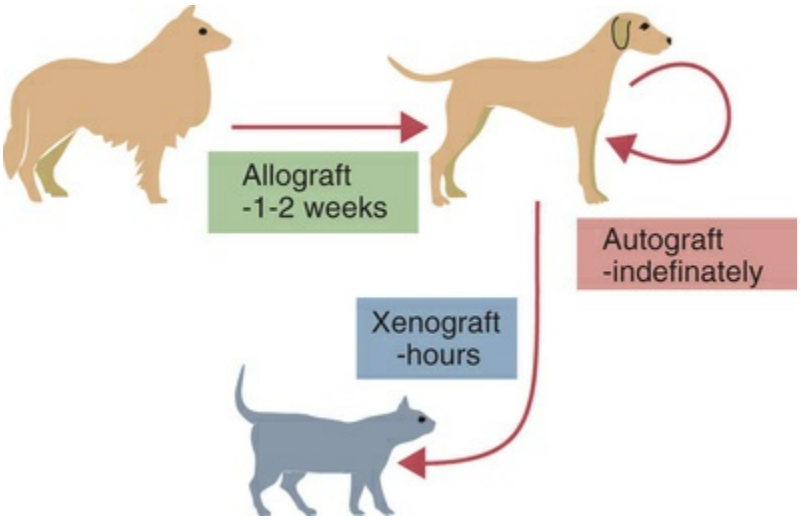


FIG. 34.1 The differences among autografts, allografts, and xenografts.

Isografts are grafts transplanted between two genetically identical individuals. Thus a graft between identical (monozygotic) twins is an isograft. Similarly, grafts between two inbred mice of the same strain are isografts and present no immunological difficulties. Since the animals are identical, the immune system of the recipient cannot differentiate between the graft and normal body cells.

Allografts are transplanted between genetically different members of the same species. Most grafts performed on animals or humans for therapeutic reasons are of this type because tissues are

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obtained from a donor who is usually unrelated to the graft recipient. Because the major histocompatibility complex (MHC) and blood group molecules on the allograft are different from those of their host, allografts induce a strong immune response that causes graft rejection. This rejection process must be suppressed if the grafted organ is to survive.

Xenografts are organ grafts transplanted between animals of different species. Thus the transplant of a pig liver into a human is a xenograft. Xenografted tissues differ from their host both biochemically and immunologically. As a result, they provoke a rapid, intense rejection response that is very difficult to suppress.

Clinical grafting in domestic animals is a recent procedure. However, renal allografting is now routine in dogs and cats, and bone marrow allografts promise to be very useful in some forms of tumor therapy. Most current organ grafts are obtained from healthy donor animals. This raises ethical issues as to whether it is appropriate to subject a donor animal to major surgery in order to provide an organ for another animal. Although the benefits of allografting to the recipient are obvious, it is unclear how the donor animal might benefit. Unlike human donors driven by altruism, an animal donor is given no choice in the matter. It is possible, however, to justify organ donation if thereby an animal would be saved from inevitable euthanasia and if the donor could be provided with a good home. For this reason, many animal transplantation centers require that the donor animal be adopted and cared for by the owner of the recipient animal.

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Allograft Rejection

The identification and destruction of foreign molecules are central to the body's defense. Allografted organs represent a major source of these foreign molecules. They contain not only antigens such as the foreign blood group glycoproteins and MHC molecules expressed on the grafted cells, but also endogenous antigens on the MHC class I molecules of these same cells. The mechanisms of allograft rejection are basically the same irrespective of the organ grafted, and both antibodies and T cells participate in the rejection of allografts.

Histocompatibility Antigens

When an organ is transplanted into a genetically dissimilar animal, the recipient will mount an immune response against many different antigens in and on the cells of the allograft. These are called histocompatibility antigens. Three types of histocompatibility antigens are of importance in stimulating graft rejection. These are the MHC class I molecules, the MHC class II molecules, and the major blood group molecules. All are expressed on the surface of the graft cells, but their distribution varies. MHC class I antigens are found on almost all nucleated cells. The major blood group antigens are found both on red cells and nucleated cells. MHC class II antigens, in contrast, have a restricted distribution that varies among mammals (Chapter 11). For example, in rats and mice, MHC class II molecules are expressed only on the professional antigen-presenting cells: macrophages, dendritic cells, and B cells. In other species, such as humans and pigs, MHC class II molecules are also expressed on the endothelium of renal arteries and glomeruli, the sites where host cells first make contact with the graft. These MHC class II molecules are recognized as foreign and trigger the rejection process. It is important to note that, as a result of these differences, it is much easier to prolong renal allograft survival in laboratory rodents than in humans or pigs.

As would be expected, grafts that differ minimally from the recipient will generally survive longer than grafts that are highly

incompatible. When blood group A-O-compatible pigs are given renal allografts, median survival is about 12 days for MHC–unmatched grafts, 25 days for grafts compatible for MHC class I alone, 32 days for grafts compatible for MHC class II alone, and 80 days for grafts compatible for both class I and class II (Fig. 34.2). When dogs are given MHC–unmatched renal allografts, the grafts survive for about 10 days. Completely matched allografts in dogs survive for about 40 days. A more impressive result is obtained with canine liver grafts, which survive for about 8 days in unmatched animals and for 200 to 300 days in DLA–matched recipients.

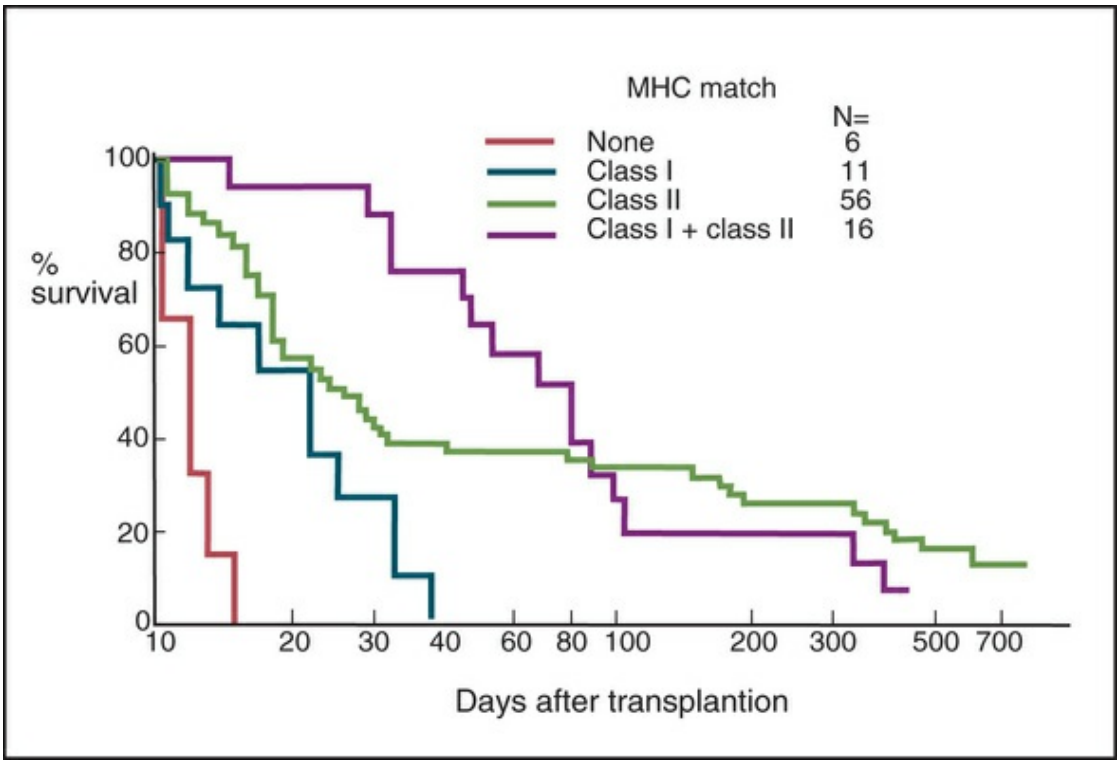


FIG. 34.2 Survival time of renal allografts between SLA-incompatible minipigs clearly depends on the degree of MHC compatibility between donor and host. (From Pescovitz MD, Thistlethwaite JR Jr, Auchincloss H Jr, Ildstad ST, Sharp TG, Terrill R, Sachs DH. Effect of class II antigen matching on renal allograft survival in miniature swine. *J Exp Med* 1160:1495-1508, 1984.)

The failure of MHC and blood group–compatible grafts to survive indefinitely is a result of the cumulative effects of many other minor antigenic differences. For example, skin grafts from male donors placed on histocompatible females are usually

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rejected, although the reverse is not the case. This is because male cells carry an antigen coded for by genes on the Y chromosome, called the H-Y antigen.

During the rejection process, the grafted tissue gradually becomes infiltrated with cytotoxic T cells, which cause progressive damage to the endothelial cells lining small blood vessels (Fig. 34.3). The T cells roll along the endothelial surface and bind using leukocyte function-associated antigen-1 (LFA-1). T cell–mediated damage releases chemokines that attract more T cells into the graft. Cellular destruction, stoppage of blood flow, hemorrhage, and death of the grafted organ follow thrombosis of these vessels. The blood vessels of second organ grafts become blocked even more rapidly as a result of the action of antibodies and complement on the vascular endothelium. This secondary reaction is specific for any graft from the original donor. It is not restricted to any particular site or to any specific organ since MHC and blood group molecules are present on most nucleated cells.

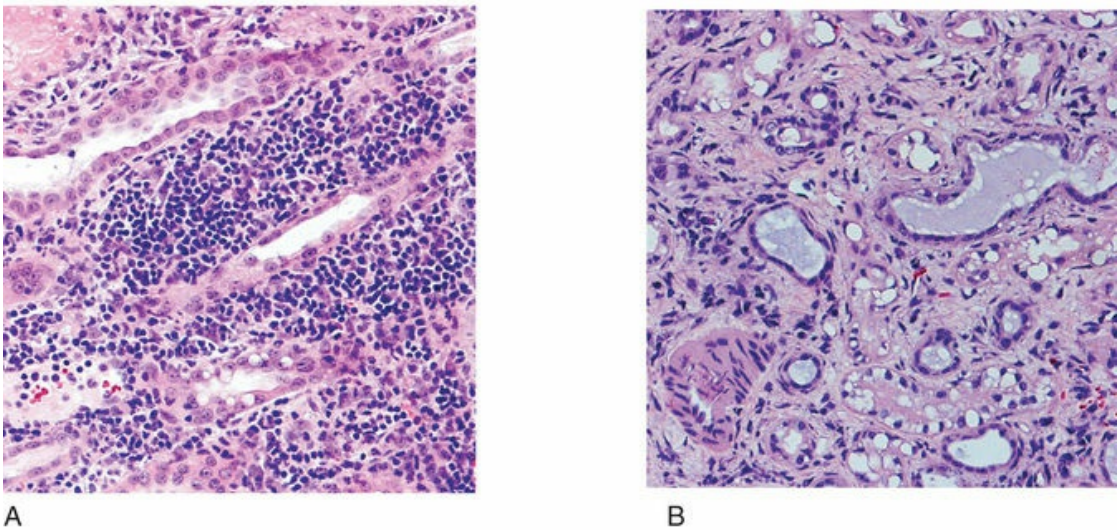


FIG. 34.3 **A,** Section of a canine kidney that had been acutely rejected and as a consequence is densely infiltrated with lymphocytes. **B,** Section of a kidney that has undergone chronic allograft rejection. In this case the section shows interstitial fibrosis with tubular atrophy and a mild lymphocytic infiltration. (Courtesy Dr. A.E. Kyles.)

In practice, it is usually not difficult to ensure that the donor and

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recipient have identical major blood group antigens. MHC compatibility is much harder to achieve because MHC polymorphism ensures that individuals differ widely in their MHC haplotype. In general, the more closely donor and recipient are related, the less will be their MHC difference. For this reason it is preferable that grafts be obtained from a recipient's parents or siblings. If this is not possible, a donor must be selected at random and the inevitable rejection responses suppressed by drugs such as cyclosporine or tacrolimus (Chapter 41).

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Renal Allografts

Renal allograft rejection is of major clinical importance in humans and has been widely studied in animals. It therefore serves as a good example of the allograft response. Rejection may occur at any time after transplantation. In humans, in whom a great deal of experience with transplantation has been gained, four distinct clinical rejection syndromes are recognized. *Hyperacute rejection* occurs within 48 hours after grafting. Rejection occurring up to 7 days after grafting is called *accelerated rejection.* Rejection after 7 days is called *acute rejection. Chronic rejection* develops several months or years after grafting. It is unclear whether a similar classification is useful in animals.

When kidneys are allografted, the blood supply to the transplanted kidney is established at the time of transplantation. The graft and host cells come into contact almost immediately. In an unsensitized host, a primary immune response is mounted, and renal allografts are only rejected after at least 10 days and possibly much longer. In sensitized animals in which the immune system is already primed, hyperacute rejection occurs, and the graft is destroyed within days or even hours without ever becoming functional. Acute rejection should be suspected when the recipient shows a rapidly rising blood creatinine associated with an enlarged, painful kidney accompanied by signs of depression, anorexia, vomiting, proteinuria, hematuria, and ultrasonography showing an enlarged, hypoechoic kidney. In contrast, chronic rejection should be suspected if the creatinine and urea levels rise gradually, and this is associated with proteinuria, microscopic hematuria, and a small, hyperechoic kidney. This is also associated with a slow loss of renal function and tends to be related to interstitial fibrosis and proliferation of vascular endothelium. Renal biopsy is necessary to confirm rejection. Interestingly, a significant number of feline renal allograft recipients may also develop retroperitoneal fibrosis that results in ureteral obstruction.

Pathogenesis of Allograft Rejection

The allograft rejection process is directed against the dominant antigens on the cells of the graft. The MHC molecules tend to trigger a T cell–mediated rejection response, whereas the blood group antigens tend to trigger antibody formation. The rejection process may be divided into two phases. First, the host's lymphocytes encounter the antigens of the graft and trigger a response. Second, cytotoxic T cells and antibodies from the host enter the graft and destroy graft cells (Fig. 34.4).

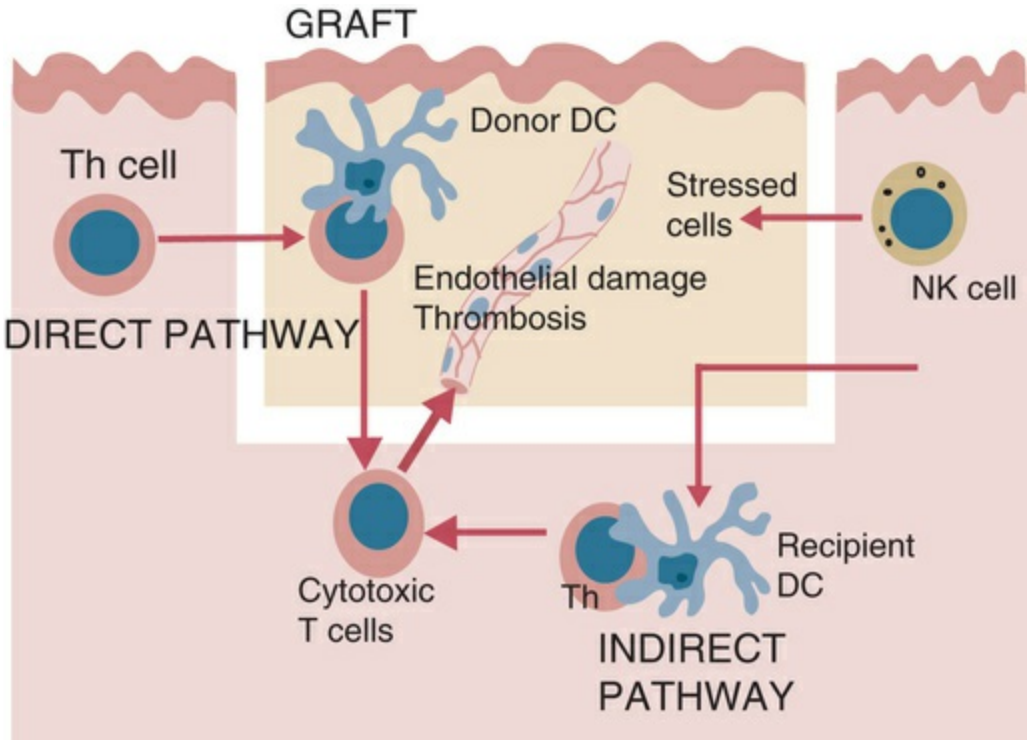


FIG. 34.4 Some of the mechanisms involved in the rejection of an allograft (see text for details).

Innate Mechanisms

Damage to the graft as a result of surgical trauma and ischemia followed by reperfusion upregulates MHC expression and generates cytokines and inflammatory mediators that attract neutrophils and macrophages into the graft. If large quantities of damage-associated molecular patterns (DAMPs) such as high mobility group box protein-1 (HMGB1) are generated, they will activate toll-like receptors and other pattern-recognition receptors. An increase in expression of the stress protein MIC-A on graft

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endothelial cells can activate NK cells. Complement components such as C5a and C3a may also activate antigen-presenting cells (APCs) within the graft.

Adaptive Mechanisms

Donor antigens are presented to the T cells of the recipient by APCs. The graft recipient may be sensitized by a direct pathway in which recipient T cells circulating through the graft encounter antigens presented by donor APCs. These donor APCs may also carry antigens to draining lymph nodes and the spleen. Alternatively, recipients may be sensitized when their own APCs enter the graft and encounter and process donor antigens (the indirect pathway). The direct pathway operates early in the rejection process but is replaced by the indirect pathway once donor APCs have been destroyed. In humans, the direct pathway is responsible for the vigorous immune response that occurs in acute rejection, whereas the indirect pathway is more important in chronic rejection. Although macrophages and dendritic cells are important APCs, donor B cells and tubular epithelial and endothelial cells can also process antigen, and they too can activate recipient T cells.

In laboratory rodents, MHC class II molecules are expressed on professional APCs. In these species, the intensity of graft rejection is related to the number of donor B cells, macrophages, and dendritic cells transplanted within the graft. Removal of these cells by careful flushing of the graft before surgery or by pretreatment of the donor with cytotoxic drugs greatly reduces the intensity of the rejection process. In other mammals in which MHC class II molecules are also expressed on vascular endothelial cells, these “passenger” cells are of less significance. Additionally, shed exosomes from the graft may enter the recipient and be captured by the host's APCs. These “cross-dressed” APCs may then be presented to recipient T cells.

The APCs that process donor MHC molecules migrate to the draining lymph node and activate T cells. The paracortical regions of lymph nodes draining a graft therefore contain dividing lymphocytes. The number of these cells is greatest about six days after grafting and declines rapidly once the graft has been rejected. In addition to these signs of an active T cell–mediated response,

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germinal center formation occurs in the cortex, and plasma cells accumulate in the medulla, indicating that antibody formation is also occurring. In a conventional immune response, only one cell in 10^5 to 10^6 T cells can respond to a specific antigen. In graft rejection, however, there may be 1% to 10% of responding T cells since these cells have low activation thresholds for foreign MHC molecules.

The activated Th1 cells produce interleukin-2 (IL-2) and interferon-γ (IFN-γ) and so turn on both NK cells and cytotoxic T cells. The NK cells produce more IFN-γ and tumor necrosis factor-α (TNF-α) that activates macrophages and additional NK cells. The cytotoxic T cells recognize the foreign peptides bound to recipient MHC class I molecules and kill any target cell they encounter. Donor MHC class II molecules trigger an immune response in two ways. First, because they are foreign proteins, they are processed as endogenous antigens. Second, they may directly bind to recipient T cell receptors (TCRs) and trigger cytotoxicity. IL-2 and IFN-γ not only promote cytotoxic T cell activity but also enhance the expression of MHC molecules on the cells of the graft. During allograft rejection, therefore, MHC expression is increased, and the graft becomes an even more attractive target for cytotoxic T cells.

Although cytotoxic T cells are of major importance in acute allograft rejection, antibodies, eosinophils, NK cells, and macrophages also play a significant role in hyperacute and chronic rejection (Fig. 34.5). Hyperacute rejection occurs when the recipient has preexisting antibodies to graft MHC or blood groups. These bind to graft vascular endothelial cells, activate complement by the classical pathway, and cause endothelial cell lysis. The damaged endothelial cells trigger platelet deposition as well generating multiple chemokines and cytokines. These also attract leukocytes and the damage results in thrombosis and infarction. Anti–MHC antibodies also play a major role in secondary rejection, since they activate the classical complement pathway and mediate antibody-dependent cytotoxic cell activity.

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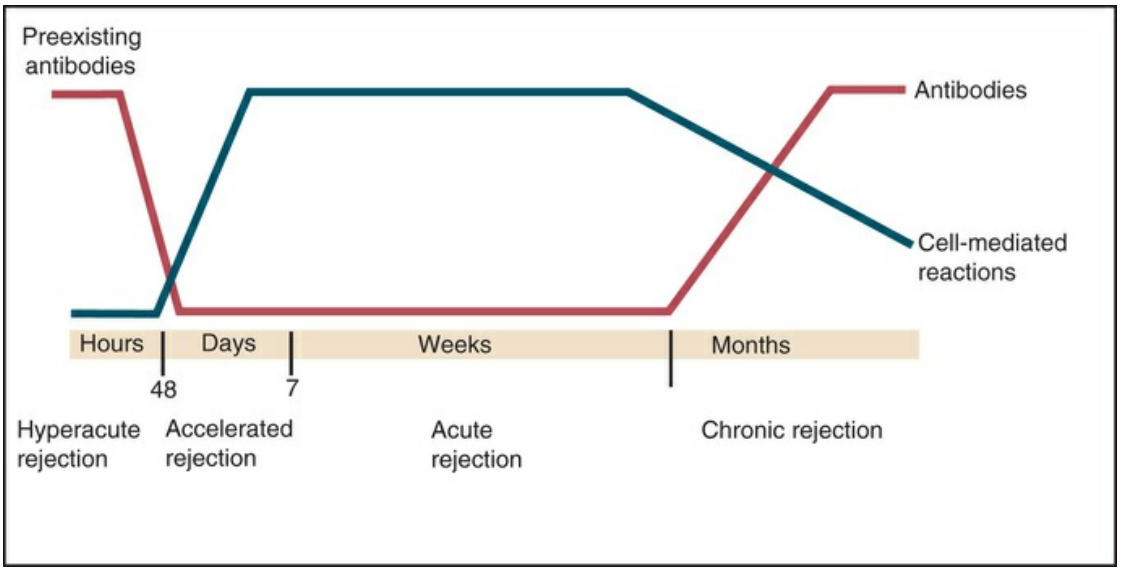


FIG. 34.5 Role of antibodies and cell-mediated immunity in different allograft rejection syndromes.

Graft Destruction

Once activated, cytotoxic T cells bind and destroy vascular endothelium and other accessible cells through caspase-mediated apoptosis. As a result, hemorrhage, platelet aggregation, thrombosis, and stoppage of blood flow occur. The grafted tissue dies because of the failure of its blood supply. $CD4^+$ T cells that enter the graft may release cytotoxic cytokines such as TNF-α. This triggers apoptosis in endothelial cells. Invading cytotoxic T cells can also cross the basement membrane and cause apoptosis of renal tubular cells. Activated macrophages releasing proinflammatory cytokines impair graft function and intensify T cell–mediated rejection.

Prevention of Allograft Rejection

In preventing allograft rejection, the transplantation surgeon seeks to induce the minimal level of immunosuppression to prevent rejection while not making the recipient more susceptible than necessary to infection. Dogs mount very strong allograft responses, and kidney allografts are rejected in 6 to 14 days in untreated animals. Unrelated dogs with renal allografts show about 50% 1-year survival when treated with azathioprine, prednisolone, and cyclosporine. Survival is considerably enhanced by a simultaneous

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bone marrow allograft from the donor animal or by treatment with rabbit anti-dog thymocyte serum. In practice, median survival times of 8 months can be achieved, with some animals surviving for longer than 5 years. However, this varies greatly between transplant centers, and much depends upon appropriate selection of recipients. Dogs have significant perioperative mortality. Thromboembolic complications are common and many experience recurrent acute infections, especially respiratory tract infections with *Bordetella bronchiseptica* as well as urinary tract infections. Newer immunosuppressive agents such as leflunomide show promise of improving the prognosis for canine renal allografting (Chapter 41).

Cats that receive renal allografts without immunosuppression die in 8 to 34 days. Immunosuppressive therapy involves the use of prednisolone and cyclosporine possibly supplemented with ketoconazole. (The ketoconazole suppresses cyclosporine metabolism in the liver and prolongs its half-life.) The therapy can begin 2 days before surgery so that cyclosporine levels are optimal when the graft is introduced. Six-month survival of treated cats ranges from 59% to 70%, whereas 3-year survival ranges from 40% to 50%. The longest survival time reported for cats receiving renal allografts is 8 years. These figures are gradually improving as experience grows. Long-term complications include acute or chronic rejection and opportunistic infections. (Infection is the second most important cause of death or euthanasia after acute rejection.) Acute rejection can occur at any time, especially if cyclosporine levels fall below the therapeutic range. Chronic allograft rejection (graft vascular disease) due to progressive arterial arteriosclerosis may cause ischemic graft destruction. It is not responsive to immunosuppressive therapy.

In some circumstances, such as when a dog has maintained functioning renal allografts for several years, immunosuppressive therapy may be reduced gradually and eventually discontinued as graft acceptance becomes complete. It is probable that the immunosuppressive drugs gradually eliminate antigen-sensitive cells. Once their numbers are sufficiently low, the large mass of grafted tissue may be sufficient to establish and maintain tolerance.

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Skin Allografts

Although the mechanisms of rejection are similar among different tissues, minor differences are observed in the process. For example, if a skin graft is placed on an animal, it takes several days for blood vessels and lymphatic connections to be established between the graft and the host. Only when these connections are made can host cells enter the graft and commence the rejection process. The first sign of rejection is a transient neutrophil accumulation around the blood vessels at the base of the graft. This is followed by infiltration with mononuclear cells (lymphocytes and macrophages) that eventually extends throughout the grafted skin. The first signs of tissue damage are observed in the capillaries of the graft, whose endothelium is destroyed. As a result, the blood clots, blood flow stops, and tissue death follows. The presence of Langerhans cells in the epidermis significantly enhances the antigenicity of skin allografts. In a secondary reaction, host blood vessels usually do not have time to grow into a skin graft since a destructive mononuclear cell and neutrophil infiltration rapidly develops in the graft bed.

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Liver Allografts

It was originally reported that a high percentage of liver allografts between outbred pigs were accepted without immunosuppression. However, these pigs were not genetically defined, and the degree of MHC mismatching was unclear. When liver allografts are made between genetically defined miniature pigs with known MHC differences, it is found that their rejection rate is similar to that observed with kidney or skin allografts. Liver graft rejection in dogs tends to occur fairly slowly. This inhibition of liver allograft rejection appears to be due to the production of indoleamine 2,3-dioxygenase (IDO) by hepatocytes. IDO destroys the amino acid tryptophan (Chapter 20). Since tryptophan is essential for Th1 responses, its absence within the grafted liver is highly immunosuppressive.

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Cardiac Allografts

Acute rejection of canine heart allografts is associated with massive lymphocytic infiltration and myocyte damage leading to rapid graft destruction. If, however, the rejection process is slowed for some reason, the pathological process in the chronically rejected organ changes. In these cases, lymphocytes and antibodies directed against vascular smooth muscle cells stimulate vascular rejection. T cells and macrophages release a chemokine cascade that activates vascular smooth muscle and endothelial cells. The resulting smooth muscle cell growth and inflammation lead to obliteration of the blood vessel lumen and eventually cardiac failure. This graft arteriosclerosis (or graft vascular disease) results from the growth-stimulating effects of both T cell–derived cytokines and antibodies. A similar lesion is seen in renal allografts undergoing chronic rejection.

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Corneal Allografts

Certain areas of the body, such as the anterior chamber of the eye, the cornea, the thymus, the testes, and the brain, are immune-privileged sites. As a result, grafts made into these sites may not be rejected. In humans, for example, 90% of first-time corneal allografts survive without tissue typing or immunosuppressive drugs. These sites are privileged because the body rigorously controls inflammation in these critical tissues. Several mechanisms are involved in this. They have an impermeable blood-tissue barrier, lack dendritic cells, contain suppressor cells, express low levels of MHC class I and II molecules, and may contain high levels of immunosuppressive molecules such as IDO, transforming growth factor-β (eyes and testes), neuropeptides (eyes), complement inhibitors (eyes), and corticosteroids (testes). Molecules found in normal aqueous humor also interfere with innate immune mechanisms. They block NK cell lysis, inhibit neutrophil activation by CD95L, suppress nitric oxide production by activated macrophages, and interfere with alternative complement activation. The eye and testes are also unique in that they express very high levels of CD95L. As a result, any $CD95^+$ T cells that enter these organs will bind to CD95L and be killed by apoptosis.

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Bone Allografts

Bone cortical allografts are used to repair severe non-reconstructible diaphyseal fractures as well as to reconstruct defects created by the resection of tumors. Rejection of bone allografts is rarely a problem, probably because of the absence of soft tissues in the graft. Unfortunately, long-term bone allografts have a high incidence of mechanical failure because the graft is resorbed before it is replaced. Joints may be successfully transplanted in horses provided that such joints have been previously frozen.

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Bone Marrow Allografts

High-dose, total-body irradiation may be administered to dogs to completely destroy tumors such as leukemias. Unfortunately, such treatment also destroys bone marrow stem cells, and these must be replaced by a bone marrow allograft. The new hematopoietic stem cells restore bone marrow function (Fig. 34.6). The recipient dog must first be conditioned by total-body irradiation or chemotherapy with cyclophosphamide. This creates space for the growing transplanted cells, reduces the intensity of the rejection process, and, in leukemic animals, destroys all tumor cells. Marrow is aspirated from the long bones of the donor and administered intravenously to the recipient. Hematopoietic stem cells migrate from the blood to the bone marrow. An optimal dose is about 2×10^8 allogeneic bone marrow cells per kilogram of body weight for matched recipients. The success of this procedure is relatively low ranging from 20% in untreated mismatched canine marrow allografts to 90% using treated matched allografts. In a successfully engrafted dog, it takes about 30 days for the granulocytes to return to normal, but the lymphocytes take about 200 days to recover. Marrow survival is not generally enhanced by treatment with single immunosuppressive agents, but combinations such as mycophenolate mofetil plus cyclosporine or methotrexate plus cyclosporine can result in the development of stable canine bone marrow chimeras.

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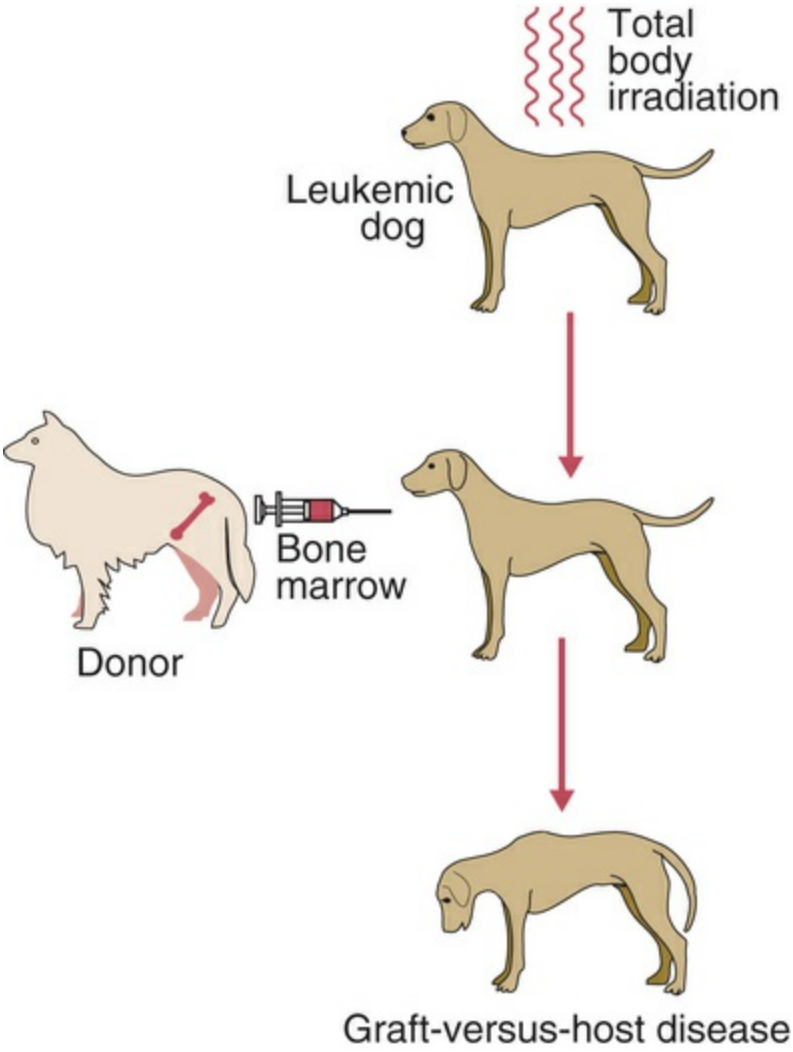


FIG. 34.6 Induction of graft-versus-host disease in dogs that have received a bone marrow allograft.

Graft-versus-Host Disease

If healthy lymphocytes are injected into the skin of an allogeneic recipient, the lymphocytes attack the host cells and cause local inflammation. Provided the recipient has a functioning immune system, this graft-versus-host (GVH) reaction is not serious because the recipient can destroy the foreign lymphocytes and thus terminate the reaction. If, however, the recipient cannot reject the grafted lymphocytes because it has been immunosuppressed or is otherwise immunodeficient, the grafted cells may cause uncontrolled destruction of the host's tissues and, eventually, death. Thus GVH disease occurs in bone marrow allograft recipients who have been effectively immunosuppressed by total-body irradiation

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or cyclophosphamide treatment.

The lesions generated in GVH disease depend on the MHC differences between donor and recipient. When they differ only in the MHC class I molecules, the disease is mainly caused by cytotoxic T cells attacking the nucleated cells of the host. This leads to a wasting syndrome characterized by bone marrow destruction leading to pancytopenia, aplastic anemia, loss of recipient T and B cells, and hypogammaglobulinemia. Th17 and NK cells also participate in this process while Tregs are lost. Lymphocytes infiltrate the intestine, skin, and liver and secrete TNF-α, IL-1 and IL-6 causing inflammation, mucosal destruction and diarrhea, skin and mouth ulcers, liver destruction, and jaundice.

If donor and recipient differ in MHC II, both graft and host $CD4^+$ T helper cells may be stimulated. The production of Th2-derived cytokines may lead to immunostimulation, autoantibody formation, and even a syndrome resembling systemic lupus erythematosus and polyarthritis (Chapter 38). (This is called autoimmune GVH disease.)

In practice, pure class I or class II disparities rarely occur naturally. Thus, in dogs, GVH disease can either be an acute disease causing death within 4 weeks of transplantation or prolonged and chronic. The major target organs are the skin, liver, gastrointestinal tract, and lymphoid system. The first clinical signs are exudative ear lesions, scleral injection, hyperkeratosis, alopecia, skin atrophy, and generalized erythema seen by 10 days (Fig. 34.7). Jaundice and diarrhea frequently occur, as does inflammation of the eyes, nose, and oral mucous membranes. An antiglobulin-positive hemolytic anemia may also develop. The immunosuppressive drug methotrexate, together with monoclonal anti-lymphocyte antibodies, may be used to suppress GVH disease.

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FIG. 34.7 Very severe cutaneous erythematous lesions on the face of a dog suffering from graft-versus-host disease as a result of a bone marrow allograft. (From Harris CK, Beck ER, Gasper PW: Bone marrow transplantation in the dog, *Compend Contin Educ Pract Vet* 8:337-344, 1986.)

The intestinal microbiota also influence the severity of GVH disease following allogeneic bone marrow transplantation— at least in mice. Thus recipient mice that developed the disease had a dramatic loss of bacterial diversity and a different composition compared to recipient mice that did not develop GVH disease. The changes were associated with an increase in Lactobacilli and a decrease in Clostridia. It may be that the reduction in disease severity is a result of the Lactobacilli preventing an increase in Enterococci. Similar changes in the gut commensal microflora occur in humans receiving bone marrow allografts.

It is of interest to note that bone marrow transplantation in cats that previously received immunosuppressive irradiation or cyclosporine is a very successful procedure and that GVH disease is not a major problem in this species.

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Xenografts

Although humans currently receive organs from dead human donors, the demand for organ grafts greatly exceeds the supply. It is possible that xenografting from nonhuman donors would eliminate this shortage. Unfortunately, xenografts are usually rejected within a few hours. The pathology of hyperacute xenograft rejection includes extensive hemorrhage and thrombosis brought about by massive destruction of endothelial cells. This allows blood cells to escape while exposing the underlying basement membrane to platelets.

Concordant xenografts are those between two closely related species such as between a chimpanzee and a human. In these cases, rejection is largely mediated by cellular reactions. In discordant xenografts (those between unrelated mammals such as from a pig to a human), rejection is mediated largely by humoral mechanisms. In practice, concordant human xenografting from other primates such as chimpanzees or baboons is impractical because of the difficulty in providing large numbers of donor animals. Pigs, however, may be more practical sources of organs. They breed rapidly, and their organs are of an appropriate size. Unfortunately pig organs trigger a severe discordant xenograft rejection response in humans mediated by natural anticarbohydrate antibodies. Humans and Old World monkeys lack the enzyme α1,3-galactosyltransferase and therefore do not make carbohydrates or glycoproteins with the α1,3-galactosyl linkage (Gal α1-3Gal). Because humans are exposed to this structure on many bacteria, we make high levels of antibodies to the Gal α1-3Gal epitope. Indeed, more than 2% of total human IgM and IgG consists of antibodies to this epitope. The αGal epitope, on the other hand, is found in pig glycoproteins. If a pig organ is grafted into a human, these antibodies bind to graft cells, activate the classical complement pathway, and lyse the pig cells. A second mechanism that contributes to hyperacute rejection is activation of the alternate complement pathway as a result of the failure of human complement factor H to prevent assembly of the alternate C3 convertase on the surface of pig cells. A third mechanism results

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from the species-specific activity of complement control proteins. Thus the natural inhibitors of complement such as CD46, CD55, and CD59 found on pig cells cannot control the activation of human complement. Transgenic pigs have been produced that express these human complement inhibitors on their cells and do not trigger hyperacute rejection on grafting. Should the xenograft survive attack by these natural antibodies and complement, it is still susceptible to delayed attack from induced antibodies and from antibody-dependent, cell-mediated cytotoxicity mediated through NK cells and monocytes. Many barriers are yet to be overcome if pig organs are ever to be routinely used as human organ transplants.

One other point relevant to xenografting is that donor animals may carry viruses that could cause disease in a severely immunosuppressed recipient or, even worse, recombine with human viruses to create new and potentially hazardous pathogens. These xenograft-derived infections (xenozoonoses) are of special concern if primates are used as organ donors. These animals are known to carry viruses such as simian immunodeficiency virus and herpes B virus that can infect humans. Pigs possess an endogenous retrovirus that has the ability to infect some human cell lines in tissue culture, although it is not known to cause human disease.

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Allografts and the Reproductive System

Sperm

Allogeneic sperm can successfully and repeatedly penetrate the female reproductive tract without provoking graft rejection. One reason for this is that seminal plasma is immunosuppressive. Sperm exposed to this fluid are nonimmunogenic, even after washing. Prostatic fluid, one of the immunosuppressive components of seminal plasma, also inhibits complement-mediated hemolysis. Seminal plasma promotes the expansion of Treg cells that subsequently migrate to the endometrium and promote tolerance to paternal alloantigens. Not only does seminal plasma cause the recruitment of monocytes to the vaginal wall, it also modulates the development of monocyte-derived dendritic cells and directs them to differentiate toward a regulatory subset. These regulatory cells are not triggered to mature by LPS, TNF-α, or CD40L. They may promote fertility by inducing tolerance to paternal alloantigens but they may also increases susceptibility to infectious agents. Nevertheless, occasional cases of infertility resulting from the production of antisperm antibodies in the uterus and vagina do occur.

Pregnancy

When mammals became viviparous and the fetus developed inside its mother's uterus, a significant immunological problem had to be overcome. The fetus could not be rejected like an allograft even though it possesses paternal MHC molecules and its trophoblast lodges deep in the uterine wall. In a normal pregnancy the fetus establishes and maintains itself despite MHC incompatibility. The uterus is not a privileged site since grafts from other tissues, such as skin, implanted in the uterine wall are readily rejected. Likewise, a mother may make antibodies against fetal blood group antigens, and these can destroy fetal red blood cells either *in utero,* as in primates, or following ingestion of colostrum, as occurs in other

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mammals (Chapter 31). Nevertheless, fetal allograft rejection does not occur.

The initial steps in pregnancy include implantation of the fertilized ovum in the uterine wall and its acceptance by the mother's immune system. This implantation is regulated by communication between the conceptus and the uterus. This involves hormones such as progesterone and interferons, especially IFN-τ and IFN-γ (Chapter 27).

In many species, pregnancy is associated with a strong skewing of the mother's immune system in favor of Th2 responses and a reduction in Th1 responses. (This raises the interesting concept that infections that promote a strong Th1 response might reverse this skewing, compromise pregnancy, and lead to abortion. This would certainly apply to protozoan infections such as toxoplasmosis, *Neospora caninum* infection, as well as brucellosis.)

The immunological destruction of the fetus and its trophoblast is prevented by the combined activities of many different immunoregulatory mechanisms acting at the maternal-fetal interface (Fig. 34.8).

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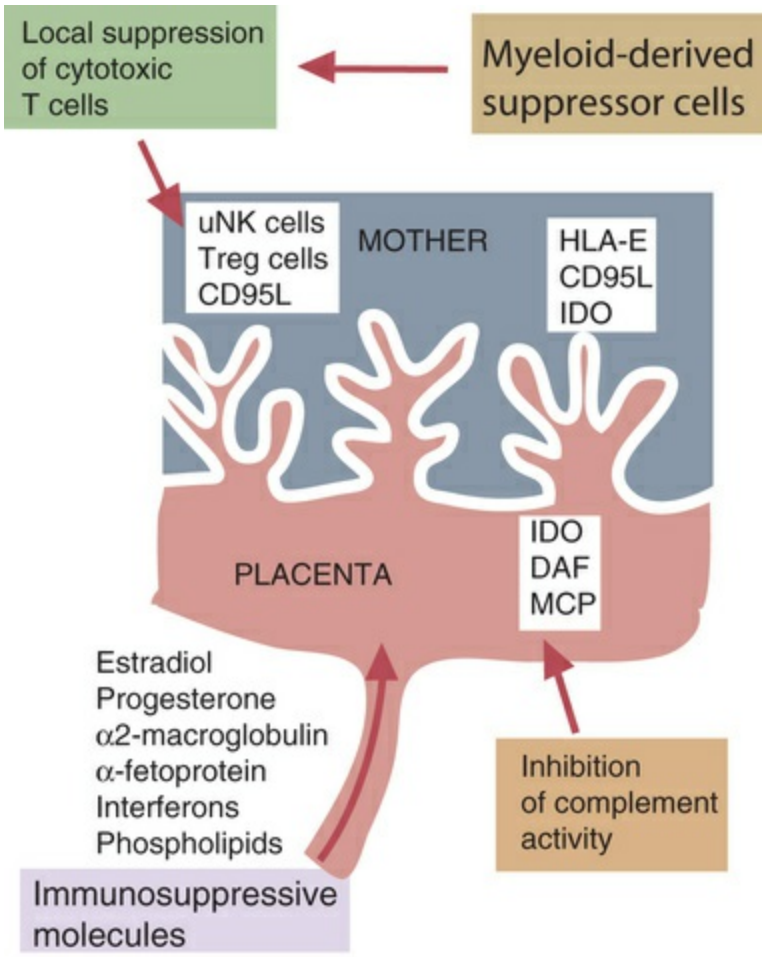


FIG. 34.8 Some of the immunosuppressive factors and processes that prevent rejection of the fetus by the mother's immune system.

First, no polymorphic (classical) MHC molecules are expressed on preimplantation embryos or oocytes. Likewise, polymorphic MHC class Ia or class II molecules are not expressed on the cell layer of the trophoblast in contact with maternal tissues. Cytokines that usually enhance MHC expression, such as IFN-γ, have no effect on trophoblast cells. The absence of classical MHC molecules should permit trophoblast cells to avoid destruction by cytotoxic T cells but make them susceptible to attack by NK cells. This is prevented, however, by expression of the nonpolymorphic MHC class Ib molecules HLA-C, HLA-G, and HLA-E. These molecules inhibit NK cell cytotoxicity while the NK cells control invasion of the uterine wall by the trophoblast. Thus there is a balance between MHC class Ib expression on the trophoblast and NK cells in the uterine wall that together regulates trophoblast growth and invasion. Bovine classical MHC class I molecules are not expressed

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on the trophoblast before day 120 gestation. Equine trophoblast cells also appear to have reduced class I expression.

NK cells predominate in the placenta, especially early in pregnancy. Over time, they gradually decline and are replaced by T cells that predominate at term. These NK cells belong to a distinct uterine subtype called uNK cells. uNK cells, also called endometrial gland cells, have been described in rodents, bats, pigs, and horses. They are not usually cytotoxic but they secrete large amounts of chemokines and cytokines. These NK cells also promote immune tolerance by suppressing Th17 cells by releasing IFN-γ.

In the hemochorial placenta found in humans and other primates, the uNK cells release angiogenic factors that remodel the spiral arteries located within the uterus to increase their diameter, and thus increase placental blood flow as the fetus grows. If they fail to do this, the blood flow to the fetus is insufficient and results in preeclampsia or miscarriage.

Treg cells play an important role in preventing fetal rejection. Estrogen treatment and pregnancy both induce FoxP3 expression, as does seminal plasma, and help promote Treg production. Trophoblast cells also secrete IDO, which blocks Th1 and Th17 responses and promotes apoptosis of cytotoxic T cells. Inhibitors of IDO permit maternal rejection of allogeneic fetuses in mice. Treg cells upregulate IDO expression in dendritic cells. In addition, IDO induces trophoblast HLA-G expression, suggesting that these molecules interact to maintain pregnancy. In humans, substantial numbers of maternal T cells cross the placenta to reside in fetal lymph nodes. These induce Treg cells that suppress maternal responses to paternal antigens probably through IL-10 production while at the same time suppressing production of Th1, Th2, and Th17 cells.

A population of profoundly immunosuppressive cells called myeloid-derived suppressor cells (MDSCs) accumulates in the uterus of pregnant mice and women. They suppress T cell activation and function. The presence of these cells down-regulates T cell L-selectin expression and so reduces the ability of naïve T cells to enter lymph nodes and become activated. They appear to be of critical importance in maintaining pregnancy in mice. MSDCs are an important cause of the immunosuppression that occurs in some

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cancer patients (Chapter 35).

Galectins regulate immune responses by binding to cell surface carbohydrates (glycans). One subfamily of these galectins are specifically expressed in the placenta of primates, especially in the syncytiotrophoblast, the placental layer that contacts the fetal cells. These specific galectins induce apoptosis in T cells. Thus they serve as local immunosuppressive agents that reduce the danger of maternal immune attacks on the fetus. The trophoblast cells of the human fetal placenta also induce M2 macrophages that produce IL-10.

Although these mechanisms minimize maternal sensitization by allogeneic fetal cells, cytotoxic T cells or antibodies do develop during pregnancy. We know that a pregnant female may mount an immune response to her fetus. For example, in the pregnant mare, placental cells invade the uterine wall to form structures called endometrial cups under the influence of IL-22. These in turn stimulate a strong immune response against paternal MHC antigens around day 60 of gestation. As a result, the cups are surrounded by large numbers of $CD4^+$ and $CD8^+$ T cells, macrophages, and plasma cells. This eventually leads to degeneration of the endometrial cups around 120 days of pregnancy. Despite these responses, the pregnancy is unaffected. It is possible that these are Th2 and Treg responses dominated by IL-10 production that does not threaten the pregnancy rather than Th1 and Th17 responses that may lead to fetal rejection (Box 34.1).

Box 34.1

T Cell Memory and Pregnancy

As pointed out in the text, pregnant animals do get sensitized to fetal antigens. Maternal CD8+ T cells directed against fetal antigens proliferate but do not express effector functions. However, these primed cells do develop into memory T cells and persist in the mother until the next pregnancy. These persistent memory T cells have no apparent effect on a second pregnancy. They do, however, sensitize the mother so that she is able to rapidly reject skin allografts bearing the same fetal antigens. Thus fetal antigen exposure results in production of a long-lived CD8+ T cell

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population. However, these cells are selectively dysfunctional. They reject skin grafts but not the fetus. In effect they retain the ability to protect the body while protecting the fetus.

Barton BM, et al. Pregnancy promotes tolerance to future offspring by programming selective dysfunction in long-lived maternal T cells, *J Leukoc Biol* 101:975-987, 2017.

Up to 90% of pregnant mares make antibodies to foal MHC class I molecules. Similar antibodies develop in multiparous sheep and cattle. In some mouse strains, up to 95% of pregnant animals make antibodies against fetal MHC molecules. Up to 40% of women make antibodies to fetal MHC molecules after giving birth. The presence of these antibodies has no adverse effect on the course of the pregnancy. On the contrary, the maternal immune response may actually stimulate placental function. In mice, hybrid placentas are larger than the placentas of inbred animals, and females tolerant to paternal antigens have smaller placentas than intolerant females. Other studies show that mothers sensitized to paternal MHC molecules have better fetal survival. This may be due to the stimulatory effect of IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) from maternal T cells on trophoblast growth. It is of interest to note that in cattle there is a clear association between retention of the placenta and its MHC class I haplotype. MHC class I compatibility between a mother and her calf increases the risk of a retained placenta, whereas MHC class II compatibility has no effect. It has been suggested that the expulsion of the placenta after birth may be due, at least in part, to an allograft response.

Some antibodies made by the mother against fetal antigens may coat placental cells, preventing their destruction by maternal T cells. This blocking antibody can be eluted from the placenta and shown to suppress other cell-mediated immune reactions against paternal antigens, such as graft rejection. Absence of this blocking antibody accounts for some cases of recurrent abortion in women. Nevertheless, it can also be shown that totally immunodeficient mice can have successful pregnancies.

The fetus does not depend entirely on maternal mechanisms for its protection. The placenta is a source of many immunosuppressive factors, including estradiol and progesterone and possibly also

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chorionic gonadotropin. In addition, some pregnancy-associated glycoproteins, including α_2-macroglobulin, α-fetoprotein, the major protein in fetal serum and placental interferons, have immunosuppressive properties. In mammals, unique interferons (IFN-ω in humans, horses, and dogs; IFN-τ in ruminants; and IFN-γ and IFN-δ in pigs) from the embryonic trophoblast act as signaling proteins between the embryo and mother during early development. These interferons may also inhibit lymphocyte activities. Amniotic fluid is rich in immunosuppressive phospholipids.

Despite the previous discussion, if the antigenic differences between the mother and her fetus are very great, then pregnancy may not go to completion. Studies on xenogeneic hybridization of two different mouse species show that the embryos develop until midgestation and are then attacked and destroyed by maternal lymphocytes. Similarly, donkey embryos transferred to horse mares are destroyed by large numbers of maternal lymphocytes.

Mild immunosuppression is a consistent feature of late pregnancy and the early postpartum period. Pregnant animals may have minor deficiencies in cell-mediated immune reactivity to nonfetal antigens. Dairy cows experience a periparturient depression in neutrophil function and reduced T cell cytotoxicity and cytokine production. This suppression appears to be due to multiple causes including the stress of parturition, the production of glucocorticoids, the loss of immunoglobulins into colostrum, and a negative energy balance. In mares, blood lymphocyte responses to mitogens drop from 4 weeks before to 5 weeks after parturition. NK cell activity in pigs drops at the end of gestation to reach a low point 2 to 3 weeks after parturition. Ewes in late pregnancy may show a reduction of some immunoglobulin classes such as IgG1. This may be due to alterations in helper T cell function or, more plausibly, to diversion of the IgG1 into the mammary gland to produce colostrum. This suppression may be significant in parasitized animals, in which the immune response barely controls the parasite. Similarly, immunosuppression may permit *Demodex* mite populations to rise in pregnant or lactating bitches and aid in the transmission of mites to their puppies.

Parturition can be considered to be mediated in part by a sterile

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inflammatory response following invasion of the uterus by large numbers of leukocytes, especially macrophages, neutrophils, and T cells, late in pregnancy. These cells release a mixture of cytokines, proteases (especially collagenases), prostanoids, and chemokines. The cytokines stimulate uterine stromal cells to amplify the process, and the collagenases remodel collagen, weakening fetal membranes; soften the cervix; and increase the contractility of the myometrium. All this leads to fetal expulsion. After delivery, the uterus involutes as most of the leukocytes leave or are destroyed.

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35

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Cancer Immunology and Immunotherapy

CHAPTER OUTLINE

Tumors as Allografts? 389
- Tumor Neoantigens, 389
- Inflammation and Tumors, 390

Cellular Defenses, 390
- Natural Killer Cells, 390
- Cytotoxic T Cells, 391
- Macrophage-Mediated Immunity, 391
- Antibody-Mediated Immunity, 392

Failure of Immunity to Cancer Cells, 392
- Cancer Cell Selection, 392
- Immunosuppression, 392
- *T Cell Dysfunction, 393*
- *CD95 Ligand, 393*
- Regulatory Cells, 393
- Myeloid-Derived Suppressor Cells, 393
- Blocking Antibodies, 393

Tumor Immunotherapy, 393

VetBooks.ir

Active Immunotherapy, 394
Passive Immunotherapy, 394
Cytokine Therapy, 394
Adoptive Cell Transfer, 394
Immune Checkpoint Therapy, 395
Antibody Therapy, 395
Immunoprevention, 395
Some Selected Tumors, 396
Injection Site Sarcomas, 396
Canine Transmissible VenerealSarcoma, **397**
Devil Facial Tumor Disease, 398
Papillomas, 398
Equine Sarcoids, 398
Ocular Squamous Cell Carcinomas, 398
Swine Melanomas, 398
Lymphoid Tumors, 399
Bovine Lymphosarcoma, 399
Lymphomas in Other Species, **400**
Avian Lymphoid Tumors, **400**

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

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- Understand and explain how cancer cells may trigger an immune response.
- Describe how cancer cells that survive may be selected for their lack of antigenicity.
- Describe the importance of inflammation in cancer immunotherapy.
- Outline how one important mechanism involves killing by natural killer (NK) cells.
- Explain how cytotoxic T cells, activated macrophages, or antibodies may also attack cancer cells.
- Understand how failure of antitumor immunity involves not only tumor cell selection but also the activities of regulatory T cells and blocking antibodies.
- Explain how T cell cytotoxicity is regulated by molecules such as PD-1 and CTLA-4.
- Understand how immune checkpoint therapy can induce long-term cancer remissions.
- Discuss why many tumors are profoundly immunosuppressive.
- Understand the prevention, diagnosis, and treatment of injection site sarcomas in cats.
- Define tumor antigen, checkpoint therapy, neoantigens, and tumor-associated antigens.

Normal cellular functions depend on careful regulation of cell division. When cells multiply, it is essential that they do so only as and when required. Unfortunately, as a result of mutations triggered by chemicals, radiation, or viruses, cells may break free of these constraints. A cell that is proliferating in an uncontrolled fashion will give rise to a growing clone of cells that eventually develops into a tumor. If these cells remain clustered together at a single site, the tumor is said to be benign. Benign tumors can usually be removed by surgery. In some cases, however, tumor cells break off from the main tumor mass and are carried by the blood or lymph to distant sites where they lodge and continue to grow. This form of tumor is said to be malignant. The secondary tumors that arise in these distant sites are called metastases. Surgical treatment of malignant tumors may be very difficult because it may be impossible to remove all metastases. Malignant tumors are subdivided according to their tissue of origin. Tumors arising from epithelial cells are called carcinomas; those arising from

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mesenchymal cells, such as muscle, lymphoid, or connective tissue cells, are called sarcomas. A leukemia is a tumor derived from hematopoietic stem cells.

The essential difference between a normal cell and a cancer cell is a loss of control of cell growth as a result of multiple mutations. These mutations may also result in cancer cells expressing abnormal proteins on their surface.

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Tumors as Allografts?

When organ transplantation became a common procedure as a result of the development of potent immunosuppressive drugs, it was found that patients with prolonged graft survival were also more likely to develop certain cancers than non-immunosuppressed individuals. It was therefore suggested that the immune system was responsible for the prevention of cancer. From this suggestion, the concept of immune surveillance emerged. This theory held that cell mutation is a common event. When mutation occurs, the structure of the protein coded by the mutated gene is altered. This alteration may make the protein antigenic. The recognition of these new antigens by cytotoxic T cells results in the elimination of abnormal cells, especially tumors. Thus in a healthy individual the immune system rapidly recognizes and eliminates abnormal cells. The theory suggested that cancer would only result if cancer cells somehow evaded destruction by T cells.

This immune surveillance theory soon ran into problems. Common human cancers such as those of the lung or breast do not develop more frequently in immunodeficient individuals. Likewise, nude *(nu/nu)* mice, although T cell–deficient, are no more susceptible than normal mice to chemically induced or spontaneous tumors (Chapter 39). Many tumor antigens induce tolerance in a manner similar to normal self-antigens. Thus most evidence has failed to support the idea that the immune system readily distinguishes between cancer cells and normal, healthy cells.

Notwithstanding this, there are situations in which the immune system does recognize and kill cancer cells. For example, some immunodeficient mouse strains, which are "cleaner" subjects than nude mice, show an increased prevalence of spontaneous cancer. (Nude mice have some persistent T and B cell function and intact innate defenses.) These include recombinase-activating gene *(RAG)* knockout mice that cannot produce functional T or B cells and *STAT-1* knockout mice that are unresponsive to interferon-γ (IFN-γ). *RAG* knockout mice suffer from an increased prevalence of spontaneous tumors of the intestinal epithelium, whereas *RAG/STAT-1* knockout mice develop mammary cancers.

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Most humans who develop "spontaneous" cancer have a normal immune system. Immunosuppressed individuals such as allograft recipients and AIDS patients develop a different spectrum of cancers from that of the general population. The only cancers to which they are at greater risk are lymphoid tumors and those caused by viruses, such as Kaposi's sarcoma. Immunosuppressed humans are at no more likely than the general population to develop the common cancers such as those of breast, lung, or colon. The situation is the same in animals. For example, in a study of 111 cats that received kidney allografts, 25 of these developed cancer. The most common tumor was a lymphoma that developed in 14 cats. All the lymphomas that were characterized were mid- to high-grade large B cell lymphomas. This is also the most common type of tumor in human transplant recipients.

Tumor Neoantigens

Cancer cells develop as a result of multiple mutations. These mutations may generate molecules that are unique to the cancer cells (tumor-specific antigens) or, more commonly, abnormal or unusual molecules (tumor-associated antigens). To distinguish between normal and cancer cells, host T cells must recognize these new antigens. Five major types of tumor antigen have been identified. First, there are differentiation antigens associated with specific stages in the development of a cell type. For example, some cancer cells express the products of developmental genes that are turned off in adult cells and are only expressed early in an individual's development. These proteins are called oncofetal antigens. Examples include tumors of the gastrointestinal tract that produce a glycoprotein called carcinoembryonic antigen (CEA; also called CD66e), normally found only in the fetal intestine. The presence of CEA in serum may indicate the presence of a colon or rectal adenocarcinoma. α-fetoprotein produced by hepatoma cells is normally found only in the fetal liver. Likewise, squamous cell carcinoma cells may possess antigens normally restricted to fetal liver and skin. These oncofetal antigens are usually poor immunogens and do not provoke protective immunity. However, their detection may be useful for diagnosis and for monitoring the

progress of the tumor.

Second, there are mutated forms of normal cellular proteins. For example, melanoma cells may express the products of mutated oncogenes on their surface (Fig. 35.1). Some tumor antigens are recognized because they are abnormally glycosylated. Chemically induced tumors may express surface antigens unique to the tumor and not to the inducing chemical. Because carcinogenic chemicals can produce many different mutations, tumors induced by a single chemical in different animals may be antigenically different. Even within a single chemically induced tumor mass, distinct subpopulations of cells exist. As a result, immunity to one chemically induced tumor does not prevent growth of a second tumor caused by the same chemical.

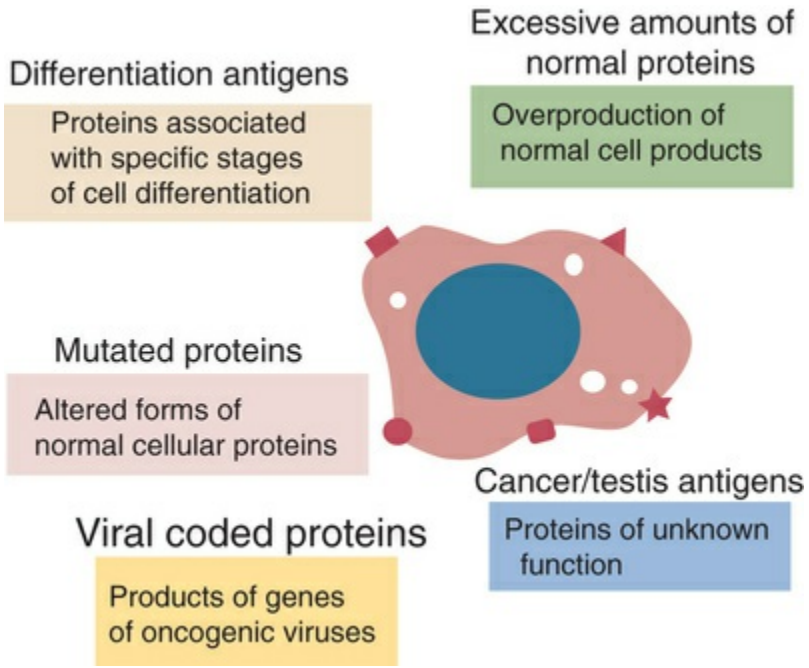


FIG. 35.1 Some of the great variety of new antigens that may appear on the surface of tumor cells and provoke an immune response.

Third, normal proteins are produced in excessive amounts. A good example is the production of prostate-specific antigen (PSA) by prostate carcinomas of humans. PSA is exclusively produced by the prostate epithelium. Increased blood levels of this protein indicate excessive prostate activity. One cause of this is the growth of a carcinoma.

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Fourth, cancer/testis (CT) antigens are a group of tumor antigens only expressed in the testes and in various malignancies. Their function is unknown.

Fifth, tumors caused by viruses may express antigens characteristic of the inducing virus. These antigens, although coded for by a viral genome, are not part of a virion. Examples include the FOCMA antigens found on the neoplastic lymphoid cells of cats infected with feline leukemia virus and Marek's cancer-specific antigens found on Marek's disease tumor cells in chickens. (Both of these are virus-induced, naturally occurring, T cell tumors.)

Studies on these mutations and the neoantigens they generate suggest that they are not widely shared but are tumor specific. They depend on the genome of an individual tumor. Their production also differs between tumor types. Thus neoantigen expression is very high in melanomas and lung, stomach, and colorectal tumors, and low in many leukemias. The vast majority of mutations do not generate antigens that can be recognized by T cells.

Inflammation and Tumors

The tumor microenvironment often determines the development and fate of cancer cells. The cancer cells communicate with nearby cells, especially fibroblasts and inflammatory cells. As a result, the elimination of cancer cells by immune mechanisms is determined in part by the presence of inflammation. Chronic inflammatory diseases increase the risk for developing many types of cancer; conversely, the use of nonsteroidal antiinflammatory drugs reduces tumor susceptibility (Fig. 35.2).

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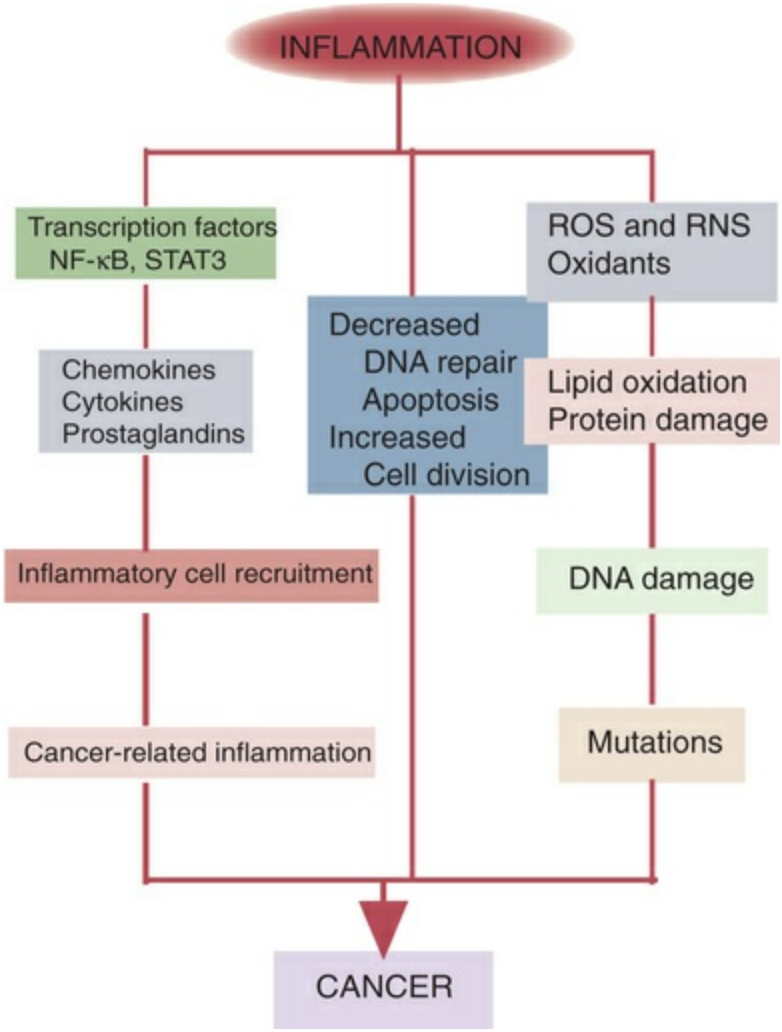


FIG. 35.2 The mechanisms by which inflammation can promote tumor formation.

Cancer cells exploit signals from their microenvironment. Stromal cells such as fibroblasts, macrophages, and endothelial cells can all generate IL-6, a cytokine that promotes tumor growth and angiogenesis. Inflammatory cells such as macrophages, mast cells, and tumor-infiltrating lymphocytes can promote tumor growth by remodeling tissues, stimulating angiogenesis, and suppressing immune responses. This polarization may result from the activities of regulatory T (Treg) cells within the tumors secreting transforming growth factor-β (TGF-β) and IL-10.

Blocking of inflammatory mediators, key transcription factors, or inflammatory cells decreases the incidence and spread of cancer. Conversely, adoptive transfer of inflammatory cells may promote tumor development. As a result, more than half of the mass of a tumor may consist of supporting cells, including fibroblasts,

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macrophages, and vascular endothelial cells. Cancers cannot spread and metastasize without the support of these cells. Inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and macrophages are often required for tumor development and spread.

If a metastasizing tumor does not invade lymphoid organs, it may escape immune surveillance. On the other hand, tumors that invade lymph nodes can be divided into strongly and weakly immunogenic types. Strongly immunogenic tumors elicit a strong T cell response following processing by dendritic cells. Weakly immunogenic tumors tend to grow as walled-off nodules that may not be processed in sufficient amounts to trigger their destruction. These are the most common tumors in humans. Inflammatory tumor cells trigger dendritic cell activation and processing. On the other hand, tumors that fail to generate inflammation may simply be ignored by the immune system.

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Cellular Defenses

Natural Killer Cells

The cytotoxic innate lymphoid cells known as natural killer (NK) cells are discussed in Chapter 19. NK cells can detect and kill cancer cells and infected or damaged cells without prior priming. They have two major types of receptors: inhibitory receptors that can recognize the absence of MHC class I molecules on a cell surface and in so doing are triggered to kill abnormal cells; and activating receptors that can recognize the presence of stress-induced proteins on cell surfaces and as a result will kill stressed cells. Thus NK cells effectively kill two types of cellular targets: cells that fail to express MHC class I molecules and cells that express certain stress-related proteins. Both conditions commonly apply to cancer cells. As a result, NK cells play a significant role in the destruction of tumors.

Cytotoxic T Cells

T cells have the potential to destroy cancer cells. They possess receptors that can recognize antigen-MHC complexes expressed on all nucleated cells. The immense size of the T cell antigen receptor repertoire enables it to identify and respond to new antigens made by cancer cells.

Until recently, evidence that T cells could control tumor growth came only from mice and human melanomas. For example, T cell stimulation by IL-2 benefited a subset of melanoma patients. Subsequent studies with the checkpoint inhibitor ipilimumab that interferes with T cell CTLA-4 also showed great benefit to melanoma patients. Infusion of tumor-infiltrating lymphocytes, some of which are cytotoxic, may also benefit some melanoma patients.

Tumor-infiltrating CD8+ cytotoxic T cells are found in many cancers. Their presence tends to be associated with a better prognosis. Some success has been achieved in humans by isolating these cells from excised tumors and growing them in tissue culture. These cultured cells are then transfused into the patient and may

induce remission. Similar results may be obtained with lymphokine-activated T killer (T-LAK) cells. T-LAK cells are generated by taking the patient's peripheral blood lymphocytes, culturing and activating them *in vitro* with interleukin-2, and then returning them intravenously to the patient. This procedure has been shown to be effective in some dogs (Fig. 35.3).

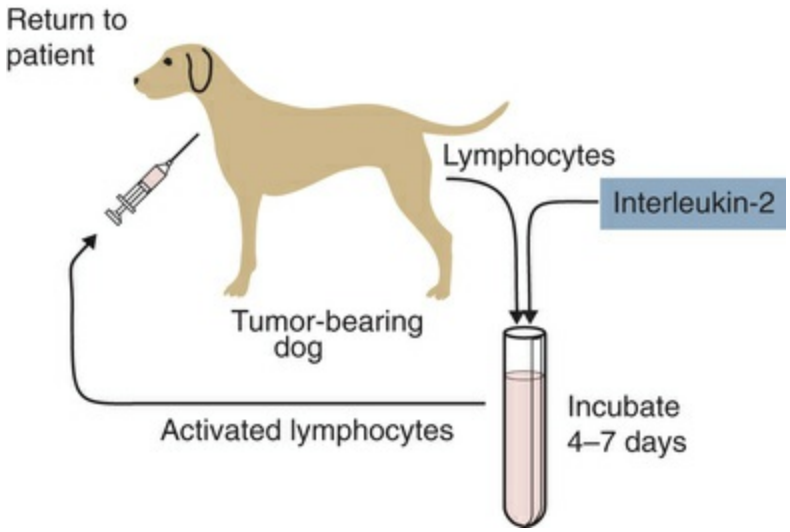


FIG. 35.3 The production of lymphokine-activated killer (T-LAK) cells by incubation of blood lymphocytes in the presence of IL-2 for 4 to 7 days.

The products of mutated genes or abnormally expressed cellular proteins may be processed and presented to T cells. As a result, lymphocytes from some tumor-bearing animals may kill cancer cells cultured *in vitro.* For example, a protein called cyclin B1 that regulates mitosis is barely expressed in normal cells but overexpressed in many tumors, where it can stimulate T cell cytotoxicity.

Checkpoint inhibitors have shown activity against many different tumor types, and it is believed that their effectiveness is due to enhanced cytotoxic T cell activity overcoming inhibitory receptor induced tolerance (Box 35.1). Implicit in this is the fact that these T cells must recognize some peptides presented by the MHC I of malignant cells. These antigens may be normal cellular proteins to which tolerance is weak, or, alternatively, they may be neoantigens —peptides that are not present in normal animals and created by tumor specific mutations.

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Box 35.1

The Role of the Microbiota in Cancer Surveillance

When cancer patients are treated with modern immunotherapy, some respond much more effectively than others. One reason for these differences is ascribed to their microbiota. Anticancer immune T cells are shaped by the microbiota. This may be due to cross-reactivity between microbial and tumor antigens, or microbial products stimulating PRRs that then influence the type and intensity of immune responses. Thus the effectiveness of anti–CTLA-4 therapy in mice depends upon the presence of *Bacteroides fragilis* in their intestine. Anti–PD-1 therapy was more effective in the presence of *Bifidobacterium.* Some investigators have developed a "cancer hygiene hypothesis" suggesting that recent increases in the prevalence of some cancers may be due to dysbiosis or underexposure to certain bacterial species.

Zitvogel L, Ayyoub M, Routy B, Kroemer G: Microbiome and anti-cancer immunosurveillance, *Cell* 165:276-287, 2016.

Macrophage-Mediated Immunity

Solid tumors may be infiltrated by macrophages attracted to the tumor by proinflammatory cytokines and prostaglandins. One of their roles is to promote and regulate angiogenesis. Their presence and activities may determine progression to malignancy. Macrophages may promote cancer cell proliferation and metastasis by releasing growth factors such as TGF-β, PDGF, and FGF. Cancer cells expressing the receptor for colony-stimulating factor-1 (CSF-1) tend to be more aggressive and metastasize more than those that lack this receptor. M1 macrophages may have antitumor activities, and secrete cytotoxic molecules, including potent oxidants. Nonspecific activation of macrophages by bacillus Calmette-Guérin (BCG) or *Propionibacterium acnes* results in enhanced production of IL-1 or TNF-α and subsequent enhancement of helper T cell and NK cell activity. IL-1 has a cytostatic effect on some tumors, and TNF-α may have potent antitumor activity. Unfortunately, malignant tumors inhibit macrophage activation, and tumor-

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associated macrophages are usually of the M2 phenotype. This may be due to IL-10 produced by Treg cells or from factors such as monocyte chemotactic protein (MCP-1 or CCL2) secreted by the cancer cells themselves. These M2 macrophages may enhance tumor growth, suppress T and NK cell responses, promote angiogenesis, and enhance cancer cell infiltration.

Antibody-Mediated Immunity

Antibodies to cancer cells are found in many tumor-bearing animals; for instance, about 50% of dogs with lymphosarcomas have antitumor antibodies. These antibodies may, together with complement, lyse free cancer cells within the bloodstream. Antibodies are not effective in destroying solid cancers.

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Failure of Immunity to Cancer Cells

The fact that tumors are so readily induced and are so relatively common testifies to the inadequacies of the immunological protective mechanisms. Studies of tumor-bearing animals have indicated several mechanisms by which immune systems fail to reject tumors.

Cancer Cell Selection

Cells do not usually become malignant in a single step. Rather, they gradually become malignant over a long period, going from benign to malignant in a process called tumor progression. The process occurs through a series of mutations that switch genes on and off. These mutations do not necessarily alter the immunogenicity of cancer cells, or do so in small steps. Immunogenicity may not alter until the cells are irreversibly committed to malignancy. Thus there are two selection mechanisms by which cancer cells can evade the host's immune response and so enhance their own survival. One is "sneaking through," the process by which malignant cells may not trigger an immune response until the tumor has reached a size at which it cannot be controlled by the host. Thus in experimental tumors, small numbers of cancer cells may grow after subcutaneous inoculation, although large numbers may not. It may be that the cancer cells may not reach lymph nodes and trigger an immune response until the tumor burden is too large to be controlled. Even a very small tumor may contain an enormous number of cells. For example, a 10-mm tumor contains about 10^9 cells. The second mechanism, cancer cell selection, reflects the fact that cancer cells that have mutated in such a way as to be antigenically different from the host will induce a strong immune response and be eliminated without leading to disease. Cancer cells survive because of their lack of antigenicity and their inability to stimulate an immune response. To this extent, therefore, tumors that do develop have, by definition, already beaten the immune system.

Immunosuppression

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It is commonly observed that tumor-bearing animals are immunosuppressed (Fig. 35.4). This is most clearly seen in animals with lymphoid tumors; for example, B cell tumors tend to suppress antibody formation, whereas T cell tumors suppress cell-mediated immune responses and NK cell activity. This immunosuppression can include defects in antigen recognition, in co-stimulation, and in cytokine production. Immunosuppression in animals with chemically induced tumors is due in part to production of immunosuppressive molecules such as prostaglandin E_2 by tumor cells or tumor-associated macrophages. The presence of actively growing cancer cells represents a severe protein drain on an animal. This protein loss may also be immunosuppressive. Some tumors contain areas of cellular necrosis. This necrosis releases potassium ions that suppress T effector function.

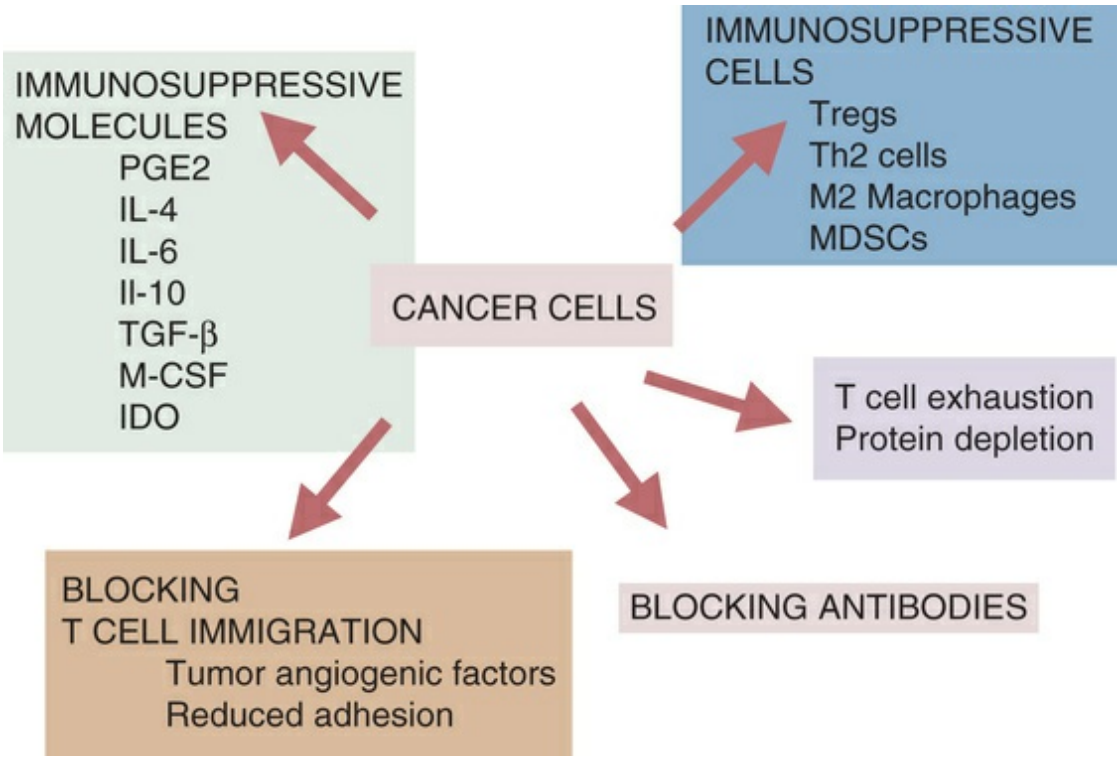


FIG. 35.4 Some of the immunosuppressive effects of cancers.

Some tumor-derived molecules may redirect macrophage activities so that they promote tumor growth. Thus tumor-derived IL-4, IL-6, IL-10, TGF-β, prostaglandin E_2, and macrophage colony-stimulating factor can deactivate or suppress the activation of

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macrophages and Th1 responses. TGF-β can convert antitumor effector cells into Treg cells. Many tumors produce indolamine 2,3-dioxygenase (IDO), a potent immunosuppressive agent and a suppressor of NK cell function (Chapter 20).

Cancer cells can suppress macrophage cytokine production and circumvent macrophage cytotoxicity. Tumors may also evade T cell responses as a result of their failure to trigger inflammation and other innate responses. Interferon-induced signaling is impaired in the B and T cells of many patients with cancer. Once tumors have effectively immunosuppressed the host, they enter the escape phase during which their growth is uncontrolled.

T Cell Dysfunction

Within the tumor microenvironment, numerous factors act together to suppress T cell function. A combination of hypoxia, nutrient shortage, acidic pH, suppressive cytokines such as TGF-β and IL-10, lipids like PGE_2, as well as other metabolites, suppress the activities of tumor infiltrating T cells. Cytotoxic T cells must be able to contact cancer cells in order to kill them. The tumor microenvironment may block T cell recruitment by degrading chemokines. The T cells may be unable to reach the cancer cells by changes in the vasculature or by trapping in the extracellular matrix. T cell cytotoxicity may be blocked by upregulating PD-L1 expression as a result of hypoxia, by IDO production suppressing T cell functions, or by excessive regulatory activities. Within tumors, tumor-derived angiogenic factors such as vascular endothelial growth factor (VEGF) and endothelin-1 can block the expression of the endothelial cell adhesion molecules so that T cells may be unable to leave blood vessels. Tumor vascular endothelium may express immunosuppressive molecules such as IDO and PGE_2. Tumor endothelium can also express ligands such as TRAIL that may kill T cells as they cross the vascular endothelium (Chapter 3).

CD95 Ligand

CD95 ligand (CD95L) is normally expressed on cytotoxic T cells and NK cells. When it binds to the death receptor CD95 on target cells, it triggers their apoptosis. CD95L has also been detected on some

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leukemic T cells and NK cells, colon adenocarcinoma cells, melanomas, and hepatocellular carcinomas. Since cytotoxic T cells may also express CD95, cytotoxicity may work in reverse and $CD95L^+$ tumor cells may kill the T cells. At the same time, these cancer cells may downregulate their own CD95 so that they become resistant to cell-mediated cytotoxicity. Some cancer cells such as those in lung carcinomas may secrete decoy receptors for CD95L. These decoy receptors bind CD95L and prevent it from binding to CD95. Cancer cells that downregulate CD95 while producing decoy receptors may be resistant to T cell cytotoxicity.

Regulatory Cells

Much of the immunosuppression in tumor-bearing individuals may be due to regulatory cells. These may be $CD8^+$ Treg cells, IL-10–secreting Th2 cells, M2 macrophages, or even B cells. Enhanced regulatory cell activity can be detected in many tumor-bearing animals. Thus in normal dogs, $FoxP3^+$ Treg cells constitute about 5% of blood T cells and 10% of lymph node T cells. In tumor-bearing dogs, however, they may constitute as many as 7.5% in blood and 17% in tumor-draining lymph nodes. Dogs with osteosarcomas have elevated Treg and fewer CD8 cells in blood, lymph nodes, and tumors. The CD8/Treg ratio is significantly lower in tumor-bearing dogs, and dogs with the greatest decrease have a shorter survival time. Treg numbers also rise in dogs with carcinomas.

Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSCs) are immature myeloid cells that normally develop into macrophages, granulocytes, and dendritic cells. They are generated by soluble factors produced by cancer cells and are attracted by chemokines or hypoxia to the tumors. Once inside tumors, MDSCs suppress cytotoxic T cell responses by expressing the inhibitory surface marker PD-L1, and by secreting immunosuppressive mediators such as arginase, IL-10, TGF-β, reactive oxygen species, nitric oxide, and peroxynitrite. Peroxynitrite causes nitrate addition to TCRs and thus inactivates them. MDSCs also produce arginase that impairs T cell function by

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reducing expression of CD3ζ. VEGF promotes MDSC production by blocking dendritic cell maturation. IL-1β also promotes MDSC production. Some MDSCs promote the production of Treg cells. MDSCs may also enhance cancer cell survival by promoting a switch from M1 to M2 cells. Collectively, therefore, these cells effectively suppress immune defenses against tumors. Myeloid MDSCs have been identified in tumor-bearing dogs where they suppress lymphocyte proliferation. For example, immunosuppression has been well documented in dogs with mammary carcinomas. Affected dogs had normal numbers of T cells but the proportion of Treg cells was increased as were the number of MDSCs. These cells increased significantly in late-stage disease and in dogs with confirmed metastases. It is of interest to note that MDSCs are also present in corneal allografts, where they promote graft survival as well as in the placenta where they prevent fetal rejection (Chapter 34).

Blocking Antibodies

Passively administered serum from tumor-bearing animals may permit the tumors in recipient animals to grow even faster, a phenomenon called enhancement. This serum may also inhibit T cell cytotoxicity. Many tumors release soluble antigens into the bloodstream, and these may bind to cytotoxic T cells, saturating their antigen receptors and blocking their ability to bind to target cells. Alternatively, blocking antibodies may be produced. These are non-complement–activating, anti-tumor antibodies that mask tumor antigens on cell surfaces and protect them from attack by cytotoxic T cells. In general, the presence or absence of blocking antibodies correlates well with the state of progression of a tumor.

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Tumor Immunotherapy

Immunotherapy may be either active or passive. In active immunotherapy, the patient's own immune system is stimulated to respond to the tumor. In passive immunotherapy, immune cells or their products are administered.

Active Immunotherapy

Three general approaches have been used in attempts to cure or modify tumor growth through immunotherapy (Box 35.2). The simplest is to stimulate the immune system nonspecifically (Fig. 35.5). Any improvement in an animal's immune abilities will tend to enhance its resistance to tumors, although a cure may be expected only if the tumor mass is small or is surgically excised. The most widely used immune stimulant is the attenuated strain of *Mycobacterium bovis*, BCG. This organism activates macrophages and stimulates cytokine release, thus promoting T cell activity. It may be given systemically or injected directly into the tumor mass. Most positive results from the use of BCG have come from studies on human patients with melanomas or bladder cancer. Direct injection of BCG into skin melanomas may cause regression, not only of the injected lesion but also, occasionally, of uninjected skin metastases. However, visceral metastases usually remain unaffected. BCG enhances survival or remission in some leukemias, and its direct intravesicular application in human bladder cancer gives response rates of up to 70%. However, BCG can cause severe lesions at the site of injection and, occasionally, systemic hypersensitivity.

Box 35.2

Some Approaches to Tumor Immunotherapy

Nonspecific Immune Stimulation

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Microbial products (e.g., bacille Calmette-Guérin, *Propionibacterium acnes*, yeast glucans, levamisole)

Complex carbohydrates (glycans)

Cytokines (interferons, tumor necrosis factor, interleukin-2, interleukin-4)

Lymphokine-activated killers (natural killer cells, T cells, tumor-infiltrating lymphocytes)

Checkpoint inhibitors

Passive Immunization

Monoclonal antibodies against tumor antigens (alone or conjugated to toxins)

Active Immunization

Chemically modified tumor cells

DNA vaccination against related antigens in another species

Vaccination against oncogenic viruses (feline leukemia, Marek's disease)

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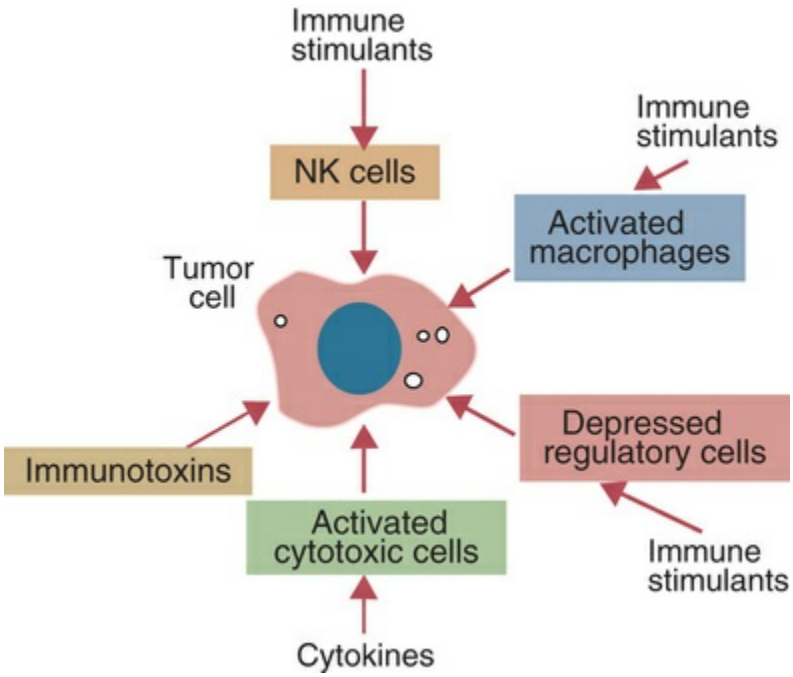


FIG. 35.5 The major cell types that participate in cancer cell destruction and methods of stimulating that activity to enhance tumor cell destruction.

Many investigators have also studied the effects of vaccinating a patient with tumor cells or antigens. This approach has worked best in human melanoma patients. Because many tumors can evade the immune response, it is usual to treat the tumor cells in an attempt to enhance their antigenicity. Thus irradiated, neuraminidase-treated, or glutaraldehyde-treated cancer cells have been used in experimental tumor vaccines.

Passive Immunotherapy

Cytokine Therapy

Many attempts have been made to treat human cancer patients with individual cytokines but with limited success. Interferons, for example, are effective only against selected tumors. Thus 70% to 90% of patients with hairy cell leukemia treated with IFN-α show complete or partial remission. On the other hand, administration of IL-2 to melanoma and renal cell cancer patients induces partial or complete remission in only 15% to 20% of cases. Low doses of recombinant human IL-2 when injected locally into papillomas or carcinomas of the vulva in cattle induced remissions in 83% of treated animals. Some complete regressions were observed. IL-2

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therapy produced 63% complete remissions in cattle with ocular squamous cell carcinomas.

Adoptive Cell Transfer

In humans, tumors have been surgically removed from cancer-bearing patients, then the lymphocytes within these tumors were cultured in the presence of IL-2 for 4 to 6 weeks so that their numbers grew significantly. These tumor-infiltrating lymphocytes (TILs) recognize and infiltrate only the tumors from which they come. Returned with IL-2 to the donor patients, they have produced remissions in about one-third of patients. The most encouraging results have been obtained in patients with melanomas and some leukemias.

Limited success has been obtained in treating melanoma in gray horses by immunotherapy. For example, administration of a plasmid expressing IL-13 directly into metastases produced significant regression in 60% of cases and appeared to be safe. In dogs with melanomas, plasmids containing DNA coding for the herpesvirus thymidine kinase suicide gene were able to sensitize transfected cells to ganciclovir. (Ganciclovir is a potent antiherpesvirus drug.) The treatment induced substantial regression.

Immune Checkpoint Therapy

The most important defense pathway against tumors uses cytotoxic CD8 T cells. Cancer cells may be killed by these T cells provided that the T cells can recognize the cancer cell neoantigens and respond accordingly. However, some cancer cells avoid destruction by sending "blocking" signals to the T cells. These signals are mediated by checkpoint proteins such as the protein PD-1 (programmed death-1, CD279) and its ligands PD-L1 (CD274) and PD-L2 (CD273). PD-1 is expressed on activated T cells and its normal function is to reduce collateral damage since it prevents T cells from generating cytotoxic signals and damaging surrounding tissues. Signaling through the PD-1 pathway suppresses T cell cytotoxicity and triggers T cell apoptosis. Thus if cancer cells can produce PD-1 ligands, they will not be attacked by cytotoxic T cells. Additionally, some cancer cells induce PD1 expression on activated

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T cells. This also results in a loss of T cell function and eventual T cell "exhaustion."

Checkpoint inhibitors can block these proteins, reverse T cell exhaustion, and so permit T cell tumor destruction to proceed (Fig. 35.6). These inhibitors are monoclonal antibodies whose targets include programmed cell death protein 1 (PD-1) and its ligand, PD-L1 (pembrolizumab and nivolumab). A second T cell inhibitory pathway uses the T cell receptor cytotoxic T-lymphocyte–associated protein 4 (CTLA-4). CTLA-4 is normally expressed on T cell surfaces following activation (Chapter 14). Its ligands are CD80 and 86 expressed on antigen-presenting cells, and its role is to prevent uncontrolled T cell activation. CTLA-4 expressed on cancer cells will prevent their destruction. Monoclonal antibodies against CTLA-4 (ipilimumab) will block this suppressive pathway and permit cancer cell destruction. Checkpoint inhibitors have achieved remarkable success in the treatment of metastatic melanomas, kidney and lung tumors, and hematologic cancers. A combination of anti–PD-1 and anti–CTLA-4 appears to be exceptionally effective in permitting T cell cytotoxicity to proceed and results in long-term remissions.

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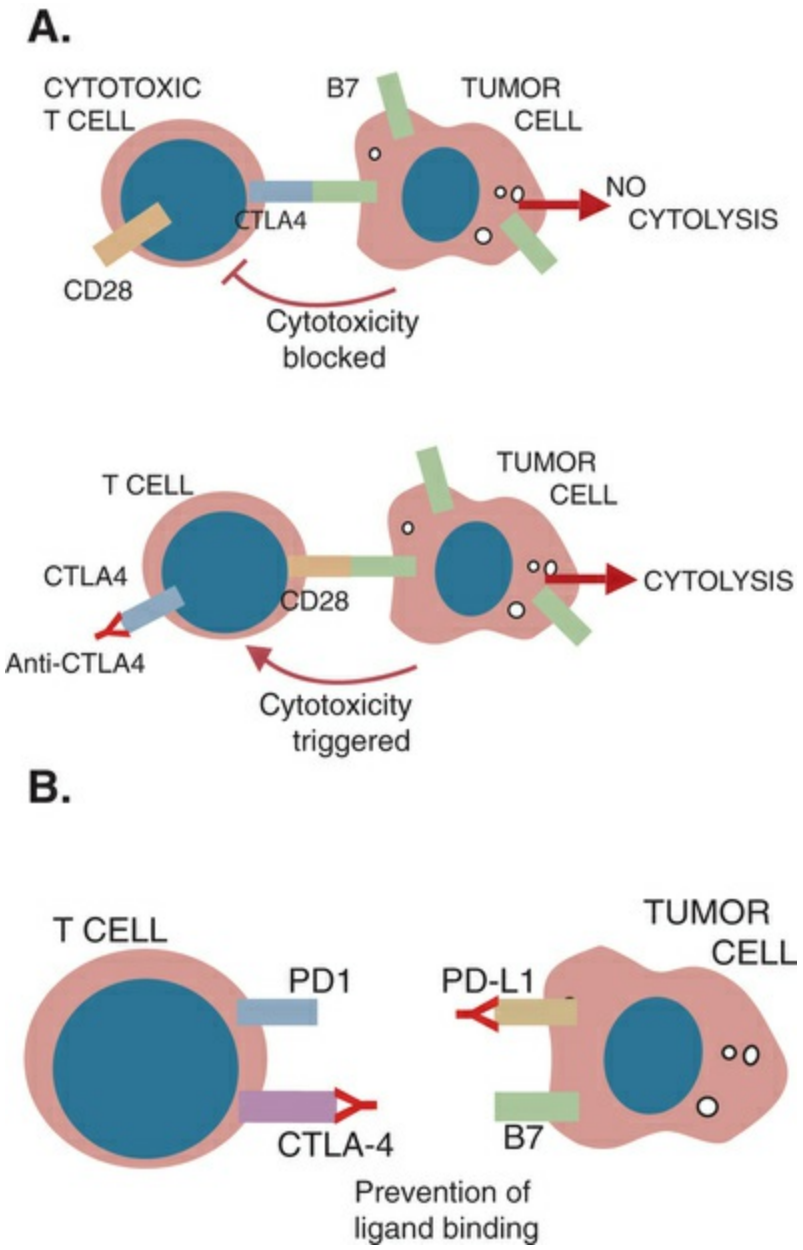


FIG. 35.6 The principles of immune checkpoint therapy. **A.** CTLA-4 is an inhibitory receptor expressed on T cells. Its ligand is B7 expressed on cancer cells blocking of CTLA-4 by antibodies permit cytotoxic T cells to exercise their full activity against tumor cells. **B.** Alternatively (or additionally), PD1 blocks tumor cell apoptosis by binding to its ligand PD-L1. Blockage of this interaction allows cytotoxicity and apoptosis to proceed.

Unfortunately, not all cancers respond to checkpoint inhibitors. Some T cells may be irreversibly exhausted or so exhausted that their function may only be restored temporarily, and the disease relapses after an early response. Checkpoint inhibitors can also be used in combination with other chemotherapeutic agents such as tyrosine kinase inhibitors or therapeutic vaccines to enhance their

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effectiveness.

Antibody Therapy

Monoclonal antibodies can be used to destroy tumors, either when given alone or when complexed to highly cytotoxic drugs or potent radioisotopes, which they carry directly to the tumor cells. One of the first such monoclonal antibodies to be produced was directed against canine T cells (CL/MAb231) and produced encouraging results when used to manage lymphomas in dogs. It apparently worked by inducing ADCC and complement-mediated lysis. New monoclonal antibodies against lymphomas in dogs are currently undergoing clinical trials. One is directed against CD20 in B cells and another is directed against CD52 on T cells. Other potential animal tumor targets include CD47 in B cell lymphomas, epithelial cell growth factor receptor in mammary carcinomas, and carcinoembryonic antigen for colon carcinomas.

Immunoprevention

In contrast to the techniques described previously, most of which have met with only limited success, there are established successful techniques for vaccination against tumor viruses. These include such effective vaccines against viral antigens such as hepatitis B and human papillomavirus, the causes of hepatocellular carcinoma and cervical cancer, respectively. The most important of these in veterinary medicine are the vaccines against feline leukemia. These vaccines usually contain high concentrations of the major viral antigens, and immunity is almost entirely directed against viral glycoproteins. Other important vaccines are those directed against Marek's disease, a T cell tumor of chickens caused by a herpesvirus. The immune response evoked by these vaccines has two components. First, humoral and cell-mediated responses act directly on the virus to reduce the quantity available to infect cells. Second, an immune response is provoked against virus-coded antigens on the surface of tumor cells. Both the antiviral and antitumor immune responses act synergistically to protect the birds.

A vaccine designed to enhance survival in recurrent melanoma is available for use in dogs. This vaccine consists of an *Escherichia coli*

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expression plasmid engineered to encode the human tyrosinase gene. Four doses are administered transdermally by a needle-free device and boosted at six-monthly intervals. The plasmid contains a cytomegalovirus promoter and a kanamycin resistance selection marker. The plasmid encodes the human tyrosinase gene, and as a result promotes the synthesis of this enzyme in the recipient animal. Vaccinated dogs then mount an immune response against this foreign tyrosinase. Tyrosinase catalyzes the hydroxylation of tyrosine to dihydroxy-phenylalanine, which is a key step in melanin production. The immune response to the tyrosinase induces both antibodies and cytotoxic T cells against the dog's melanoma cells, and this may prevent tumor recurrence.

The USDA has also approved a DNA vaccine directed against canine B cell lymphomas. The antigenic target is CD20. This is expressed in immature B cells and is present in about 70% of B cell lymphomas.

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Some Selected Tumors

Injection Site Sarcomas

When cats are vaccinated, any inflammation at the injection site usually resolves rapidly and completely. In some cats, however, sarcomas develop at these sites many months or years after vaccination (Fig. 35.7). These tumors are mainly fibrosarcomas, malignant histiocytomas, and osteosarcomas. Less common forms include rhabdomyosarcomas, hemangiosarcomas, chondrosarcomas, liposarcomas, and lymphosarcomas. These tumors are highly invasive. Successful treatment requires a combination of radical surgical excision and adjunctive therapy, including radiation, immunotherapy (such as IL-2 treatment), and chemotherapy, but recurrence is common.

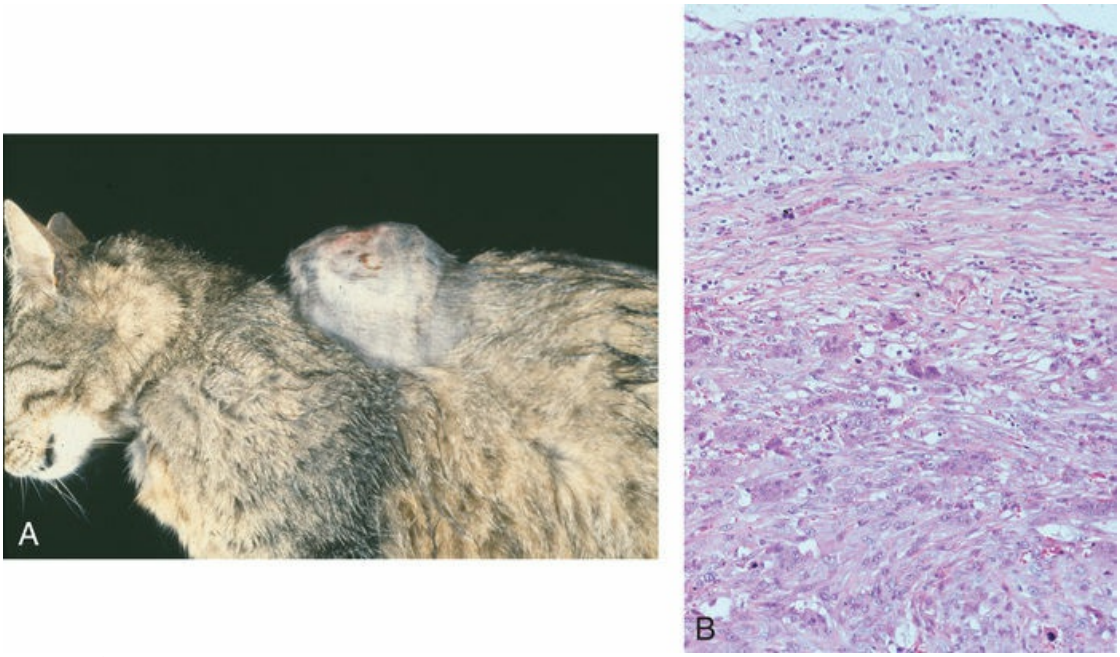


FIG. 35.7 **A,** Post-vaccinal sarcoma in a cat. Note its characteristic position over the scapulae, where the vaccine has been administered subcutaneously. **B,** A histological section of a post-vaccinal sarcoma. This is a fibrosarcoma showing long interwoven bundles of spindle cells (H&E stain). (Courtesy Dr. M.J. Hendrick.)

These tumors were first noticed following the introduction of potent, inactivated, adjuvanted vaccines such as those directed

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against rabies and feline leukemia. Cats with sarcomas occurring at sites where vaccines are currently administered were compared with cats that developed sarcomas at non-vaccine–injection sites. Cats receiving FeLV vaccine were 5.5 times more likely to develop a sarcoma at the injection site than cats that had not received a vaccine. There was a twofold increase in risk with rabies vaccination. However, the risk was not enormously high. It has been calculated that 1 to 3.6 sarcomas develop per 10,000 vaccinated cats in the US. The risk increase with the number of doses of vaccine administered; a 50% increase following one dose, a 127% increase following two doses, and a 175% increase following three or four vaccines given simultaneously. Vaccine-associated sarcomas tend to occur in younger animals and are larger and more aggressive than sarcomas arising at other sites. They metastasize in 25% to 70% of cases. In one study, injection site sarcomas developed on average 26 months after rabies vaccination and 11 months after FeLV vaccination. Global, web-based surveys suggest a somewhat lower prevalence of sarcomas (0.63 sarcomas/10,000 cats or 0.32 sarcomas/10,000 doses of all vaccines, or one sarcoma from 31,000 doses administered). It must be pointed out, therefore, that the chances of developing a sarcoma are considerably smaller than the disease risks incurred by unvaccinated cats. In addition to rabies and FeLV vaccines, sarcomas have also been associated with vaccines against feline panleukopenia, feline herpesvirus, and feline calicivirus. Similar vaccination-related injection site sarcomas have been reported in ferrets, dogs, and a horse.

The pathogenesis of these sarcomas is unclear, but it is assumed that carcinogenesis occurs through multiple steps associated with prolonged inflammation or tissue damage. When first reported, it was assumed that tumor development resulted from the presence of potent adjuvants. Tumor development has, however, also been associated with the use of non-adjuvanted vaccines and even with injection of substances other than vaccines, including penicillin, glucocorticoids, lufenuron, cisplatin and meloxicam, as well as the presence of persistent suture material, a retained surgical swab, or implanted microchips. There is no evidence that feline sarcoma virus, feline immunodeficiency virus, or feline leukemia viruses cause these tumors.

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Prolonged irritation will increase the activation state of the cells involved in inflammation and tissue repair. The repair process involves stem cells that can differentiate to replace damaged ones. These stem cells are long lived and so have plenty of opportunities to accumulate mutations. Signaling pathways in stem cells will promote cellular self-renewal. Chronic, prolonged irritation leads to an increase in local stem cells and the possibility that some may mutate. During chronic inflammation, macrophages secrete growth factors and angiogenic factors that enhance cell growth. These factors upregulate NF-κB activity in affected tissues. Oxidants released from activated macrophages may act as carcinogens, especially in rapidly dividing cells. Although the mechanisms are unclear, NF-κB promotes both malignant transformation and metastases and may inhibit apoptosis of premalignant cells.

Fibroblasts proliferate at sites of chronic inflammation and wound healing. In some of these fibroblasts, the *sis* oncogene may be activated, whereas in others, there are mutations in the gene coding for the tumor suppressor factor *p53.* The *sis* oncogene codes for the platelet-derived growth factor (PDGF) receptor, and vaccine-associated sarcomas have been shown to express both PDGF and its receptor. In contrast, non-vaccine–associated tumors and normal cat lymphocytes are PDGF negative. It has been suggested, therefore, that lymphocytes within the vaccine-associated sarcomas secrete PDGF, which then serves as a growth factor for the fibroblasts. This combination of abnormalities could result in the loss of growth control in the fibroblasts engaged in the chronic inflammatory process.

The tumor suppressor gene *p53* codes for a nuclear protein that regulates the cell cycle. Wild-type *p53* increases in response to cell damage. This delays the cell's progress through the cell cycle and permits DNA repair before the cell divides. If the cell is severely damaged, *p53* triggers apoptosis and prevents cellular damage being transmitted to the next generation. Damaged cells in which *p53* is absent or mutated can continue to divide, giving rise to abnormal and possibly malignant cells. As many as 60% of injection site sarcomas may express mutated *p53.* It is also apparent that they contain many cells with broken DNA strands.

Notwithstanding the above, there is no evidence to prove that

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injecting less irritating products can reduce the incidence of sarcomas. No specific brands of vaccine, no specific manufacturers, and no other vaccination-associated factors have been associated with an increased prevalence of sarcomas.

To assess the risks of tumors developing at vaccination sites, it has been recommended that vaccines be administered at standardized sites on cats. For example, current recommendations are to inject rabies vaccine into the right pelvic limb and FeLV vaccine into the left pelvic limb ("rabies, right; leukemia, left"). Vaccines should be administered as distally as possible to permit amputation if required. The site of vaccine administration and the product used should be recorded for each vaccine to help in assessing risk factors. Clients should be instructed to monitor injection sites for swellings or lumps so that any developing tumors are detected and excised as early as possible.

Canine Transmissible Venereal Sarcoma

Transmissible venereal sarcoma is a cancer transmitted between dogs during copulation. It results from transplantation of neoplastic cells. (In effect, the cancer cells are infectious agents.) To colonize a new host, these cells must establish themselves in allogeneic hosts. This is not always successful, and after an initial 1 to 3 month growth phase, the tumor eventually enters a stationary phase and then regresses and is eliminated. Nevertheless, lethal metastases do occur in up to 7% of puppies and immunosuppressed dogs. The tumor persists in the face of an allograft response as a result of multiple somatic mutations. When growing aggressively, these cancer cells fail to express β_2-microglobulin, and, as a result, MHC class I antigens are not assembled on the cell surface. Exposed dogs, whether or not they develop progressive tumors, develop antibodies to tumor cells, although the serum of dogs with regressive cancers is most effective in inhibiting tumor growth. Thirty to forty percent of cells in the regressive phase express MHC classes I and II. Dogs whose cancers regress also develop cytotoxic T cells. If recipient dogs are immunosuppressed, the tendency to malignant growth is enhanced. The tumor cells appear to secrete a cytotoxic factor that kills B cells. Genetic analysis of these tumor

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cells suggests that they originate from a clone of myeloid cells that arose in a wolf or East Asian breed of dog about 10,000 years ago.

Devil Facial Tumor Disease

The large carnivorous marsupial, the Tasmanian devil *(Sarcophilus harrissii)*, is on the brink of extinction as a result of devil facial tumor disease, a transmissible cancer. This disease first appeared in 1996 and has spread across Tasmania. It has reduced some devil populations by as much as 90%. Tumor cells are transmitted when devils bite each other around the face, a common behavior. Animals die within 3 to 6 months of acquiring the cancer since they are unable to mount an immune response to the foreign cells. The tumor cells grow and form a large mass that is eventually lethal (Fig. 35.8). Almost very devil "infected" with these tumor cells dies of cancer. Although devils have a functioning immune system, their limited MHC diversity prevents them from recognizing the tumor cells as foreign. (The tumor cells originated from Schwann cells from a female devil in the early-1990s but are continuing to evolve.) A second, genetically distinct facial tumor lineage (DFT2) has also been recognized in devils. Facial tumor cells do not express surface MHC class I due to down-regulation of their β2-microglobulin and *TAP* genes. This downregulation is a result of epigenetic deacetylation of histones. Thus there is no histocompatibility barrier to tumor growth. Although devils have functioning NK cells these cannot kill the tumor cells for unknown reasons. MHC expression can be restored by exposing facial tumor cells to recombinant devil IFN-γ and subsequent activation of the MHC class II transactivator, a critical transcription factor. Blood mononuclear cells activated by mitogens *in vitro* can also kill devil tumor cells. Encouraging results have also been obtained by vaccinating animals with killed adjuvanted tumor cells. It also appears that resistance to this cancer is emerging in some wild populations.

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FIG. 35.8 A Tasmanian Devil with facial tumor disease. (Courtesy Dr. D. Phalen.)

Papillomas

Warts are self-limited tumors of epidermal cells induced by papillomaviruses. The wart virus invades epidermal cells in the basal cell layer of the skin, but these cells do not express viral antigen. Since no viral antigen is expressed in this area where the blood supply is good, the cells are not attacked by lymphocytes. As the infected cells move away from the basal layer toward the skin surface, they also move away from blood vessels, and the chances of immunological attack are minimized. Increasing amounts of virus are shed as the cells move toward the surface into a region devoid of antibodies or lymphocytes. Wart vaccines containing inactivated papillomavirus are available.

Equine Sarcoids

Equine sarcoids are locally aggressive fibroblastic cancers of horse skin associated with abortive infection by bovine papillomavirus and host genetic susceptibility. They are persistent and often recur following surgery. Nevertheless, they may be amenable to immunotherapy. Sarcoids are characteristically infiltrated with CD8+ and CD4+ T cells. If BCG vaccine is infiltrated into the tissues

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between the tumor and normal skin, regression occurs in about two-thirds of cases. The rate of regression depends on the size of the tumor (surgical debulking is required to remove most of the tumor mass), and multiple treatments are usually necessary for a complete cure. Mycobacterial cell walls may also be used to treat this tumor. They possess the advantage of not rendering an animal tuberculin positive. Sarcoids are also responsive to other immunostimulants such as killed *P. acnes* and the antiviral drug acyclovir.

Ocular Squamous Cell Carcinomas

Ocular squamous cell carcinoma is a common and economically important cancer of cattle that responds to immunotherapy. One successful treatment involves inoculation of affected animals with a phenol-saline extract of allogeneic carcinoma cells. This suggests that these cells possess tumor-associated antigens. Indeed, sera from affected cattle can react with tumor cells (but not normal cells) obtained from the eyes of other cattle. It is also of interest to note that sera from some cattle with ocular squamous cell carcinoma also react with equine sarcoid and bovine papilloma cells, implying that all three may have a common cause.

Swine Melanomas

The Sinclair melanoma-bearing swine is an inbred line that spontaneously develops melanomas. Most such tumors are benign and regress spontaneously; however, some are malignant and lethal. The tumor regression seen in most of these pigs is immunologically mediated. The tumors are invaded by macrophages, and at the same time the animals generate non-MHC–restricted cytotoxic $CD4^-$, $CD8^-$, γ/δ^+ T cells. Recovering pigs may also produce antibodies against melanoma antigens.

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Lymphoid Tumors

Adaptive immunity requires that antigen-sensitive cells stimulated by exposure to antigen respond by division and differentiation. Much of the complexity of the immune system is due to the need to control this response. A failure may result in uncontrolled lymphoid cell proliferation and the development of lymphoid tumors. The surveillance theory was originally proposed when it was observed that immunosuppressed animals and humans had an increased prevalence of cancers. However, an unusually high proportion of these are of lymphoid origin. Therefore it is likely that some of the lymphoid neoplasia that develop in immunosuppressed individuals result from a failure of immunological regulation rather than from a failure of surveillance.

Normal immune responses, whether antibody or cell mediated, involve a burst of rapid proliferation in lymphocytes. This burst of proliferation must be carefully controlled (Chapter 18). Although uncontrolled lymphocyte function may induce autoimmunity, uncontrolled lymphocyte proliferation may result in the development of a lymphoma or lymphosarcoma. It is no accident that animals with autoimmune disease are more likely than normal animals to develop lymphoid cell tumors (Table 35.1).

TABLE 35.1

Immunosuppressive Effects of Lymphoid Tumors

Tumor	Cell Type	Evidence for Immunosuppression	Mechanisms
Feline leukemia	T cell	Lymphopenia Prolonged skin grafts Increased susceptibility to infection Lack of response to mitogens	Suppressive viral protein, pI5E Suppressor cells
Marek's disease	T cell	Lack of response to mitogens Depressed cell-mediated cytotoxicity Depressed IgG production	Suppressor macrophages
Avian lymphoid leucosis	B cell	Increased susceptibility to infection	Suppressor lymphocytes
Bovine leucosis	B cell	Depressed serum IgM	Soluble suppressor factor
Myeloma	B cell	Increased susceptibility to infection	Soluble tumor-cell factor Negative feedback
Canine malignant lymphoma	B cell	Predisposition to infection associated with autoimmune disorders	Unknown
Equine lymphosarcoma	T cell	Increased susceptibility to infection	Tumor of suppressor cells

Several important viruses stimulate nonspecific lymphocyte

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proliferation. These include the maedi-visna virus, the Aleutian disease parvovirus, and the herpesvirus responsible for malignant catarrhal fever (MCF). MCF is a fatal lymphoproliferative disease of cattle and sheep characterized by lymphadenopathy with widespread tissue accumulations of lymphocytes. Lymphocytes from MCF-infected animals show prolonged growth in tissue culture.

Neoplastic transformation may occur in lymphoid cells of both branches of the immune system. Providing that the tumor cells have not dedifferentiated as a result of very rapid growth (as in acute lymphatic leukemia of calves), it is possible to identify the origin of the cells present in lymphoid tumors by their surface antigens. For example, the presence of cell surface immunoglobulin is characteristic of B cells, whereas the presence of CD3 or CD2 is an identifying feature of T cells.

Bovine Lymphosarcoma

Bovine lymphosarcoma is one of the most common cancers of cattle. It occurs in two main forms: an enzootic form and a sporadic form. The enzootic form is caused by bovine leukemia virus (BLV), a delta retrovirus. BLV is transmitted by infected lymphocytes and can be spread by contaminated instruments, by vaccines containing blood, or by biting flies; or calves may be infected *in utero*. The primary target of the virus is the pre-B cell, although a specific BLV receptor has yet to be identified. Early in infection the proportion of B cells in peripheral blood increases before there is a significant increase in the number of blood lymphocytes. Eventually some infected animals develop a persistent lymphocytosis (PL) with lymphocyte counts in the range of 20,000 to 80,000/μL. Not all BLV-infected cattle develop PL, although 95% of cattle with this condition are infected with BLV. These lymphocytes may be enlarged, are $CD5^+$, express increased levels of IgM, and altered glycosylation. Cells in PL are not malignant and can occasionally return to the normal state. BLV becomes stably integrated into these B cells. Some T cells may also contain the BLV provirus. About 1% to 5% of BLV-infected cattle develop a multicentric lymphosarcoma between 1 and 8 years after infection. Susceptibility to cancer

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development differs among species. Sheep are very sensitive, cattle have intermediate sensitivity, and goats are the least sensitive. Animals that develop these cancers die within 3 to 6 months. If there is bone marrow involvement, lymphocyte counts can reach 100,000/μL.

BLV is essential for neoplastic transformation but not for the continued growth of cancer cells. The mechanism by which BLV initiates tumor development is unclear since there is no rearrangement of any known oncogenes. A viral gene called *Tax* appears to initiate tumorigenesis. *Tax* encodes a transactivating protein that can turn on many different cellular genes and that deregulates many different regulatory pathways rather than a single key pathway. Animals with advanced clinical bovine leukosis may be immunosuppressed as a result of the presence in their serum of a suppressor factor. This suppression is reflected by reduced numbers of T cells, less responsive B cells, lowered serum IgM and IgG2 responses and reduced responses to many vaccines. Circulating Foxp3 Tregs are also increased. Occasionally the neoplastic cells in bovine leukosis may be sufficiently differentiated to secrete immunoglobulin as in myelomas. The cells in the sporadic form of bovine leukosis are predominantly T cells, but some originating from pre-B cells have also been identified.

Lymphomas in Other Species

In sheep, lymphomas are divided fairly evenly between T and B cells, and about 15% are unclassifiable (null cells). Some of these may be due to BLV infection. A B cell lymphoma inherited as an autosomal recessive condition is recognized in swine. Equine lymphomas are predominantly T cell in origin although some equine B cell lymphomas are T cell rich.

Horses with lymphosarcomas are commonly immunosuppressed. This usually involves T cells, but B cell function may also be impaired. Equine B cell neoplasms generally cause a monoclonal gammopathy. A case of a horse with a lymphosarcoma with suppressor cell activity has been described. The animal presented with signs of immunodeficiency and was found to be deficient in IgM. The cancer cells grew in the presence

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of IL-2, possessed many T cell markers, and were noncytotoxic. Equine T cell lymphomas have been associated with immune-mediated anemia and thrombocytopenia.

In dogs, leukemias may be classified on the basis of the cell type involved (lymphoid or myeloid) and on the basis of the clinical course and cytology (acute or chronic). Chronic lymphoid leukemia (CLL) is most frequently diagnosed. It is characterized by the presence of large numbers of mature lymphocytes in the blood. Animals may be asymptomatic, and the course of the disease is slow. About 70% of these cases involve $CD3^+$T cells, and most are large granular lymphocytes (LGLs). Of these LGLs, about 65% are α/β T cells, and the remainder are γ/δ T cells. The non-LGL T cell CLL cases involve α/β T cells. $CD21^+$, $CD79a^+$ malignant B cells account for about 30% of canine CLL cases. B cell CLL is over-represented in small breed dogs. Chronic myeloid leukemias are extremely rare in dogs.

Acute leukemias, which are less common in dogs, may be of B cell origin (20%) or myeloid origin (70%). The rest of these acute leukemias are difficult to classify and are considered undifferentiated. Many of these tumor cells, both myeloid and lymphoid, express CD34. The prognosis of these acute leukemias is usually very poor.

Lymphomas account for 5% to 7% of canine malignancies. There is no evidence to suggest that these tumors are virus induced. They may be classified according to their apparent site of origin (e.g., multicentric, alimentary, or anterior mediastinal) or, alternatively, by their cell type (e.g., histiocytic, lymphocytic, lymphoblastic, or plasmacytic). The lymphocytic forms are usually of T cell origin. These T cell lymphomas are quite heterogeneous and while usually aggressive, some, such as T-zone lymphomas are slowly growing. Immunophenotyping can be a useful feature in classifying these T cell lymphomas. $CD4^+$ is the commonest subtype. Thus T-zone lymphomas are $CD45^-$. Aggressive T cell lymphomas may be $CD45^+$. Low levels of MHC class II expression on canine B cell lymphomas carry a poor prognosis. In many cases of canine lymphoma, affected dogs produce antibodies against crude tumor antigens. These antigens are not found on normal lymphoid cells. Cutaneous $CD3^+$T cell lymphomas (mycosis fungoides) are common

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in old dogs. Eighty percent are $CD8^+$, whereas the remainder are double-negative. Most (70%) have γ/δ TCRs, especially if the tumor is confined to the epidermis.

Avian Lymphoid Tumors

Marek's disease herpesvirus induces T cell tumors and severe immunosuppression in chickens. Birds with this disease are usually immunosuppressed. Thus their antibody responses, rejection of allografts, and delayed hypersensitivity responses are all depressed. This depression results from several factors, including virus-induced lymphoid destruction and the development of suppressor macrophages. These macrophages restrict the replication of the tumor cells, but in doing so they suppress the resistance of birds to other infections. Late immunosuppression can occur in the absence of tumors. Lymphoid leukosis is a tumor of B cell origin. Affected birds normally have a depressed antibody response and reduced responses to mitogens. Nevertheless, some cases of this disease may present with hypergammaglobulinemia.

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36

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Autoimmunity

General Principles

CHAPTER OUTLINE

Induction of Autoimmunity, 402
Normal Immune Responses, 402
Antigens Hidden in Cells or Tissues (Cryptic Antigens), 402
Antigens Generated by Molecular Changes, 403
Receptor Editing, 403
Abnormal Immune Responses, 403
Failure of Regulatory Control, 403
Infection-Induced Autoimmunity, 403
Molecular Mimicry, 404
Epitope Spreading, 405
Bystander Activation, 405
Microchimerism, 405
Predisposing Factors, 406
Genetic Predisposition, 406
Breed Predispositions, 406

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Intestinal Microbiota, 407
Mechanisms of Tissue Damage in Autoimmunity, 407
Type I Hypersensitivity, 407
Type II Hypersensitivity, 407
Type III Hypersensitivity, 408
Type IV Hypersensitivity, 408

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Explain why autoimmunity is an inescapable consequence of the way in which the adaptive immune system has evolved.
- Explain that not all autoimmune responses are pathological.
- Describe how most autoimmune diseases result from a failure to ensure that tolerance is maintained against self-antigens.
- Summarize the evidence that there is a strong genetic predisposition to develop autoimmunity.
- Explain how some autoimmune diseases are triggered by immune stimulants such as virus infections and drugs.
- Understand how the lesions that develop in autoimmune diseases are generated by the mechanisms of hypersensitivity described previously.
- Identify the major factors that result in autoimmunity.
- Discuss the roles of genetics, infectious agents, and sex on the development of autoimmunity.
- Define cryptic antigens, epitope spreading, immunoconglutinin, rheumatoid factor, molecular mimicry, and bystander activation.

Autoimmune diseases are relatively common. They occur in about 5% of humans and probably in a similar proportion of domestic mammals. Most result from the emergence of clones of "rogue"

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lymphocytes directed against normal body components. The fact that they tend to develop late in life suggests that these diseases, like cancer, are probably the result of multiple random defects. Thus while one defect alone may be insufficient to permit an autoimmune response, multiple problems may eventually permit self-reactive lymphocytes to develop. Given the ubiquity of microbial and environmental antigens, lymphocytes are under constant pressure to proliferate. The presence of a constant supply of self-antigens is especially significant. Much of the complexity of the immune system is determined by the need to keep this lymphocyte proliferation in check.

Lymphocyte proliferation is regulated by multiple mechanisms. These include negative selection within the thymus, the requirement for multiple co-stimulatory signals, lymphocyte cooperation, and the activities of regulatory cell populations. These regulatory mechanisms often overlap so that the development of self-reactive rogue clones does not occur suddenly. It likely takes multiple accumulated defects, acting collectively in regulatory pathways, to lead to the loss of control of lymphocyte proliferation.

By evolving receptor systems that can bind to as many microbial antigens as possible, vertebrates also developed the potential for self-destruction. The random generation of antigen-binding receptors ensures that many lymphocytes are produced with receptors that can bind self-antigens. It has been estimated that as many as 50% of newly produced T cell and B cells may bind self-antigens with high affinity. These self-reactive cells are usually rigorously suppressed so that only a few animals develop autoimmune disease. However, the reasons why these individuals eventually develop autoimmune diseases while others do not are still unclear. Many factors influence susceptibility to autoimmunity. These include sex and age, genetic background, the microbiota, and virus infections. We also know that the development of autoantibodies is a relatively common event that by itself does not inevitably lead to autoimmune disease. Indeed, some autoantibodies serve a physiological function.

Because we do not know precisely what triggers autoimmunity, this chapter reviews some of the many different predisposing factors that have been identified or proposed as well as the

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mechanisms by which autoimmunity causes tissue damage and disease. As with other immune functions, both B and T cells can mediate autoimmunity. Thus in some autoimmune diseases, the disease is mediated by autoantibodies alone. In others, the damage may be mediated by T cells alone or by some combination of autoantibodies and T cells.

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Induction of Autoimmunity

Autoimmune diseases appear to develop spontaneously, and predisposing causes are rarely obvious. Nevertheless, they fall into two major categories: they can result from a normal immune response to an unusual or abnormal antigen, or they can result from an abnormal immune response to a normal antigen (Fig. 36.1). The second category is probably the most common. In these cases, the mechanisms that normally prevent the development of self-responsive T and B cells fail. Many different environmental factors and genes contribute to this failure, and the failure may not always be complete. Autoimmune diseases may result from an aberrant response to a single specific antigen; alternatively, they may be due to a general defect in the regulation of B or T cell functions.

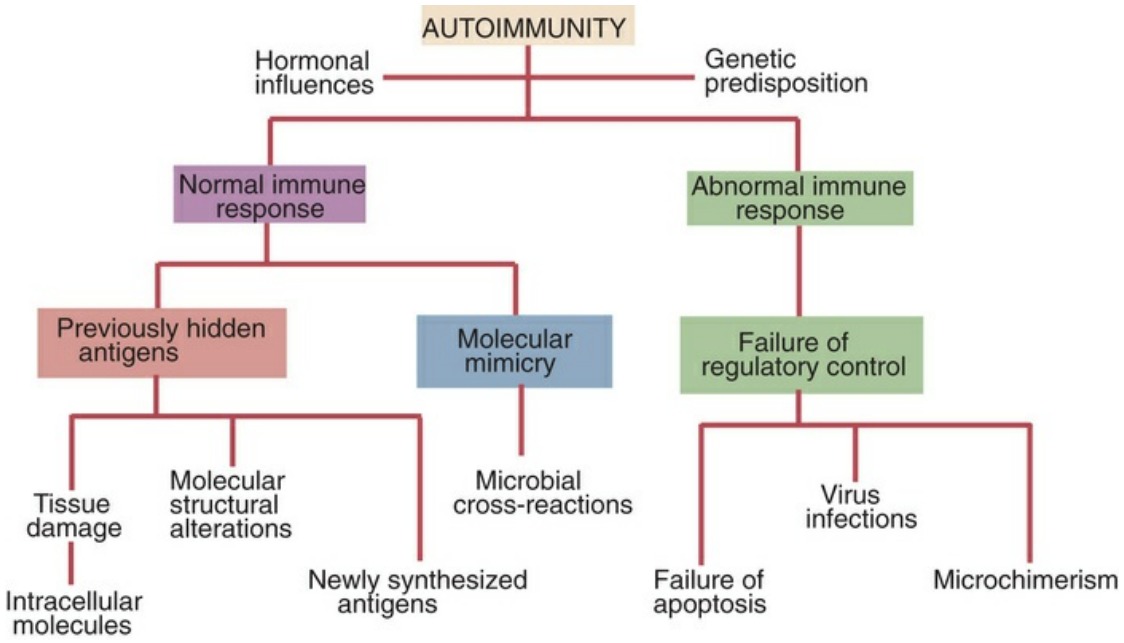


FIG. 36.1 A simplified scheme for the pathogenesis of autoimmune diseases.

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Normal Immune Responses

Many autoimmune responses simply reflect a normal immune response to an antigen that has been previously hidden or result from cross-reactivity between an infectious agent and normal body components. Many naturally occurring autoantibodies play a role in homeostasis and regulation. They are usually low-titer, low-affinity IgM or IgG antibodies directed against protein fragments, or proteins damaged by oxidation or enzymes.

Antigens Hidden in Cells or Tissues (Cryptic Antigens)

Many autoimmune responses are triggered when nontolerant T cells meet previously hidden autoantigens. After all, T cells can only be made tolerant to autoantigens if they are exposed to them. There are many autoantigens that do not induce tolerance simply because they remain hidden within cells or tissues and do not normally encounter T cells.

Although the control of the immune system requires that most self-reactive cells be eliminated, one should not assume that all autoimmune responses are bad or even cause disease. Some autoimmune responses have physiological functions. For example, red blood cells must be removed from the blood once they reach the end of their life span. This process is accomplished by autoantibodies. As red cells age, an anion transport protein called CD233 (or band 3 protein) is gradually oxidized, and a new epitope is generated. This new epitope is recognized by IgG autoantibodies. These autoantibodies bind to aged red cells and trigger their phagocytosis by splenic macrophages. CD233 is found on many cell types, and it may be that its exposure on aged cells and their subsequent removal is a major cell elimination pathway.

Many autoantigens are found in places where they never encounter circulating lymphocytes. For example, in the testes, new antigens may only appear at puberty, long after the T cell system has developed and become tolerant to autoantigens. Injury to the testes may permit proteins from damaged tissues to reach the

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bloodstream, encounter antigen-sensitive cells, and stimulate autoimmunity. Hidden antigens may also be found within cells. For example, after a heart attack, autoantibodies may be produced against the mitochondria of cardiac muscle cells. In chronic hepatitis in dogs, animals develop antibodies to liver membrane proteins. In diseases such as trypanosomiasis or tuberculosis in which widespread tissue damage occurs, autoantibodies to many different tissue antigens may be detected in serum.

Antigens Generated by Molecular Changes

The production of some autoantibodies may be triggered by the development of new epitopes on normal proteins. Two examples of autoantibodies generated in this way are the rheumatoid factors (RFs) and the immunoconglutinins (IKs, after the German spelling).

RFs are autoantibodies directed against other immunoglobulins. When an antibody binds to an antigen, the shape of the immunoglobulin molecule changes in such a way that new epitopes are exposed on its Fc region. These new epitopes may stimulate RF formation. RFs are produced in diseases in which large amounts of immune complexes are generated. These include the autoimmune disease of joints called rheumatoid arthritis and systemic lupus erythematosus (SLE), in which B cells respond to many different autoantigens.

IKs are autoantibodies directed against the complement components C2, C4, and especially C3. The epitopes that stimulate IK formation are exposed when these complement components are activated. The level of IKs in serum reflects the amount of complement activation; this, in turn, is a measure of the antigenic stimulation to which an animal is subjected. IK levels are thus nonspecific indicators of the prevalence of infectious disease within an animal population. Their physiological role is unclear, but they may enhance complement-mediated opsonization.

Receptor Editing

Both B and T cell antigen receptors are generated by random gene rearrangement. This process inevitably results in the generation of

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both nonfunctional and autoreactive antigen receptors. Once a complete antigen receptor is formed, however, rearrangement of the cell's receptor genes continues. Thus if an immature B cell produces a receptor that binds to a self-antigen, the continuing development of that B cell is blocked while its light chain receptor chains continue to undergo recombination. This replacement of one light chain by another leads to changes in receptor specificity and eventually makes the cells no longer autoreactive. Receptor editing only occurs in immature B cells. Mature B cells that bind autoantigens do not undergo receptor editing but rather are triggered to undergo apoptosis.

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Abnormal Immune Responses

Failure of Regulatory Control

Although autoimmunity may be triggered by hidden autoantigens, a sustained autoimmune response is necessary for disease to develop. This may result from a failure of the normal control mechanisms of the immune system and can be demonstrated simply by injecting mice with rat red blood cells. Following such an injection, mice not only make antibodies to the rat cells but also develop a self-limited and transient autoimmune response to their own red blood cells. This autoimmune response is rapidly controlled by regulatory cells and lasts for only a few days. If, however, regulatory cell activity in these mice is impaired, as occurs in New Zealand Black (NZB) mice, for example, these autoantibodies will persist to cause red blood cell destruction and anemia.

It is common to find autoimmune diseases associated with lymphoid tumors. For example, myasthenia gravis, an autoimmune disease involving the neuromuscular junction, is commonly associated with the presence of a thymic carcinoma. In humans, there is a four-fold increase in the incidence of rheumatoid arthritis in patients with malignant lymphoid tumors, and there is evidence for a similar association in other mammals. Since many lymphoid tumors result from a failure in immunological control mechanisms, a simultaneous failure in self-tolerance may also occur. Alternatively, some lymphoid tumors may consist of cells producing autoantibodies. It is also possible that some lymphoid tumors may develop as a result of prolonged stimulation of the immune system by autoantigens.

Potentially harmful, self-reactive lymphocytes are normally destroyed in the thymus by apoptosis triggered through CD95 (Chapter 18). Defects in CD95 or its ligand CD95L (CD178) cause autoimmunity by permitting abnormal T cells to survive. This is well demonstrated in the *lpr* strain of mice. These animals have a mutation that alters the structure of the intracellular domain of CD95 and blocks its functions. A mutation (called *gld*) in CD95L has

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a similar effect. Both *lpr* and *gld* mice develop multiple autoimmune lesions accompanied by lymphoproliferation. The autoimmune regulator *(aire)* gene regulates T cell development and permits thymic epithelial cells to express multiple self-antigens. T cells that respond to these self-antigens are eliminated. Thus humans with a defective *aire* gene develop autoimmune disease involving multiple endocrine organs and the skin.

Infection-Induced Autoimmunity

Autoimmune diseases are triggered by many environmental factors, and infectious agents are among the most important. Given, however, that infections are very common and autoimmune diseases fairly rare, they clearly cannot account for the entire autoimmune process. For example, mice infected with certain reoviruses develop an autoimmune polyendocrine disease characterized by diabetes mellitus and retarded growth. These reovirus-infected mice make autoantibodies against normal pituitary, pancreas, gastric mucosa, nuclei, glucagon, growth hormone, and insulin. Likewise, in NZB mice, persistent infection with a type C retrovirus leads to the production of autoantibodies against nucleic acids and red blood cells. Bacteria such as *Streptococcus pyogenes, Borrelia burgdorferi,* and *Leptospira interrogans* may trigger autoimmune heart disease, arthritis, and uveitis, respectively. The protozoan parasite *Trypanosoma cruzi* triggers an autoimmune cardiomyopathy.

The situation with spontaneous autoimmune disease is less clear. Many attempts have been made to isolate viruses from patients with autoimmune disease but with mixed results. For example, SLE of dogs and humans has been associated with either a type C retrovirus or paramyxovirus infection. Small quantities of the Epstein-Barr virus genome can be found in the salivary glands of humans with Sjögren's syndrome. Moreover, epidemiological evidence points to some form of a viral trigger for diseases such as multiple sclerosis, rheumatoid arthritis, and insulin-dependent diabetes mellitus in children. Just how viruses can induce autoimmunity is unclear, but three major mechanisms are recognized: molecular mimicry, epitope spreading, and bystander

activation.

Molecular Mimicry

Autoimmunity may result from molecular mimicry, a term used to describe the sharing of epitopes between an infectious agent or parasite and an autoantigen (Fig. 36.2). B cells may be triggered by a foreign epitope that cross-reacts with an autoantigen. However, they will only respond to this epitope if they receive T cell help. If nearby Th cells also recognize these microbial epitopes as foreign, they may trigger a B cell response that results in the production of autoantibodies. Once a B cell response is triggered in this way, the infectious agent may be removed while the autoimmune response continues— a "hit-and-run" process.

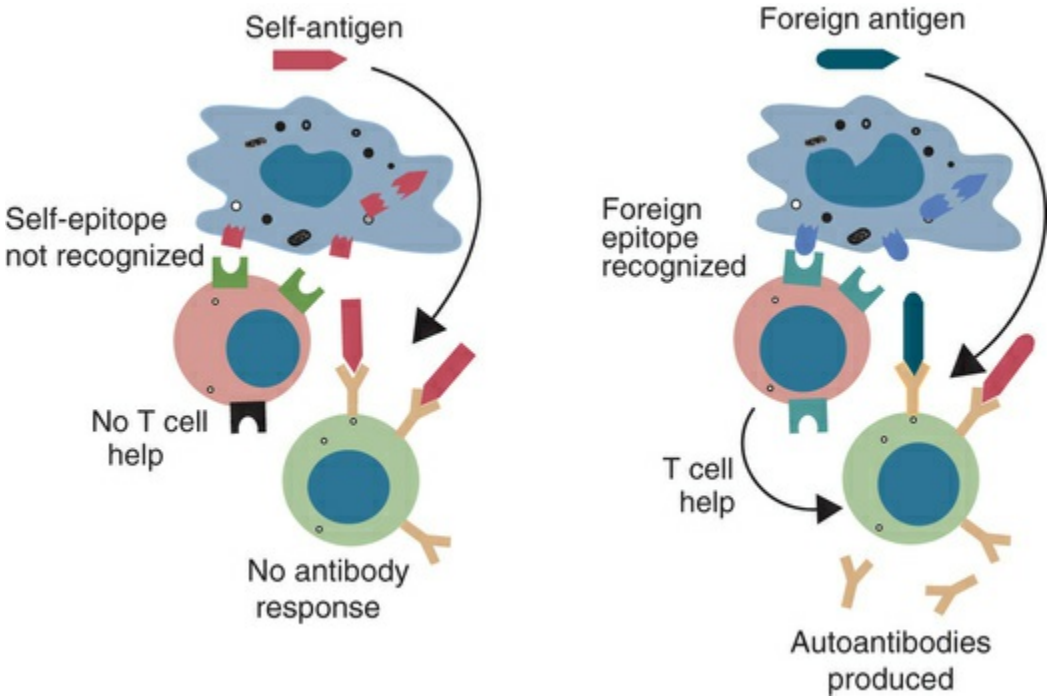


FIG. 36.2 Cross-reactions with foreign antigens may be sufficient to trigger a helper T cell population that will promote an autoimmune response by B cells. A helper effect triggered by a foreign antigen may inadvertently permit an autoimmune response to occur.

Many examples of molecular mimicry are now recognized. For example, the parasite *T. cruzi* contains antigens that cross-react with mammalian neurons and cardiac muscle. Individuals infected with *T. cruzi* make autoantibodies that can cause nervous system and

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heart disease. Molecular mimicry may also cause the heart lesions of rheumatic fever in children. Antibodies to the cell wall M-protein of group A streptococci cross-react with cardiac myosin. Children infected with certain strains of group A streptococci produce antimyocardial antibodies and develop heart disease. Some strains of streptococci may cause acute glomerulonephritis in children as a result of the production of antibodies cross-reacting with glomerular basement membranes. Other examples of molecular mimicry include the Epstein-Barr virus DNA polymerase, which cross-reacts with myelin basic protein and may be involved in the induction of multiple sclerosis, and the poliovirus capsid protein VP2, which cross-reacts with the acetylcholine receptor and may induce myasthenia gravis.

The integrin CD11a/18 (LFA-1) shares an antigenic determinant with the outer surface protein of the Lyme disease bacterium, *B. burgdorferi.* Patients infected with this organism mount an initial immune response that may then develop into autoimmunity. In about 10% of patients with Lyme arthritis, antibiotics fail to resolve the disease, suggesting that, once triggered, the autoimmune process can proceed in the absence of the bacterium.

Antibodies against microbial heat-shock proteins are found in the serum of humans and rats with rheumatoid arthritis, ankylosing spondylitis, and SLE. Injection of killed *Mycobacterium tuberculosis* in Freund's complete adjuvant can cause arthritis in rats, and T cells from these animals can transfer arthritis to normal syngeneic recipients. These T cells are responding to HSP 60, a mycobacterial heat-shock protein (Chapter 26). It has been suggested that molecular mimicry between microbial and mammalian HSP 60 may be important in rheumatoid arthritis.

Ankylosing spondylitis is an autoimmune arthritis of humans that affects the sacroiliac joints, spine, and peripheral joints. Patients also develop acute anterior uveitis (inflammation of the iris and neighboring structures in the eye). More than 95% of humans with ankylosing spondylitis possess the MHC class I allele HLA-B27, whereas in the normal population, the prevalence of this allele is less than 8%. The disease results from molecular mimicry between the hypervariable region of HLA-B27 and antigens found in *Klebsiella pneumoniae* and related bacteria. *K. pneumoniae* is found

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more frequently than normal in the intestine of patients with active ankylosing spondylitis and uveitis, and patients with active disease have elevated levels of IgA against *Klebsiella* in their sera. Cloning of B27 into mice and subsequent infection of these animals with *K. pneumoniae* causes an acute spondylitis. HLA-B27–associated ankylosing spondylitis has been described in gorillas. Up to 20% of wild gorillas may have spondylitis, and the disease has also been described in a gibbon, in baboons, and in rhesus macaques.

In porcine enzootic pneumonia caused by *Mycoplasma hyopneumoniae,* antibodies to the mycoplasma cross-react with pig lungs, and in contagious bovine pleuropneumonia, there is cross-reactivity between *Mycoplasma mycoides* antigens and normal bovine lung. It is not known to what extent these autoantibodies contribute to the pathogenesis of these diseases. There is a clearer causal relationship between *Leptospira interrogans* infection and the development of periodic ophthalmia, the leading cause of blindness in horses (Chapter 37).

Some microbial superantigens may also trigger autoimmunity. The superantigen staphylococcal enterotoxin B activates the same T cells that react with myelin and induces an autoimmune encephalitis. It has been suggested that a bacterial superantigen may trigger rheumatoid arthritis since the T cells in affected joints are enriched in cells bearing specific TCR V domains. The only known agents that can alter V region gene expression in this way are superantigens.

Epitope Spreading

In some cases, autoimmunity seems to result from a normal immune response against a foreign antigen that subsequently "spreads" to recognize self-antigens. When an immune response is initiated, the immune response is first directed against a single epitope on the inciting antigen. However, as the process continues, T and B cell receptors diversify, and responses begin to be directed against additional epitopes. At first they will react with other epitopes on the same protein. Eventually responses may spread to epitopes on autoantigens. Epitope spreading has been demonstrated in diseases such as thyrotoxicosis and diabetes mellitus and may account for some of the difficulties encountered

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in controlling these diseases.

Bystander Activation

When viruses destroy cells, previously hidden antigens may be released. These may activate nearby lymphocytes that had not been involved in the antiviral response. Additionally, T cells might, in responding to an antigen, produce a mixture of cytokines such as the tumor necrosis factors and nitric oxide, that can kill nearby cells and trigger an autoimmune response. Viruses may induce an inflammatory response that results in the release of multiple cytokines. Pathogens may trigger inappropriate lymphocyte proliferation by acting through pattern-recognition receptors to generate co-stimulatory molecules and proinflammatory mediators. These cytokines may activate previously dormant T cells. As a result, the T cells may attack autoantigens that they previously ignored. Evidence suggests that Coxsackie virus–induced diabetes mellitus is mediated in large part through bystander activation (Fig. 36.3). Prolonged infection with some viruses may induce autoimmunity as a result of chronic activation of the immune responses. Thus a prolonged polyclonal B cell activation may result in the eventual emergence of autoreactive clones.

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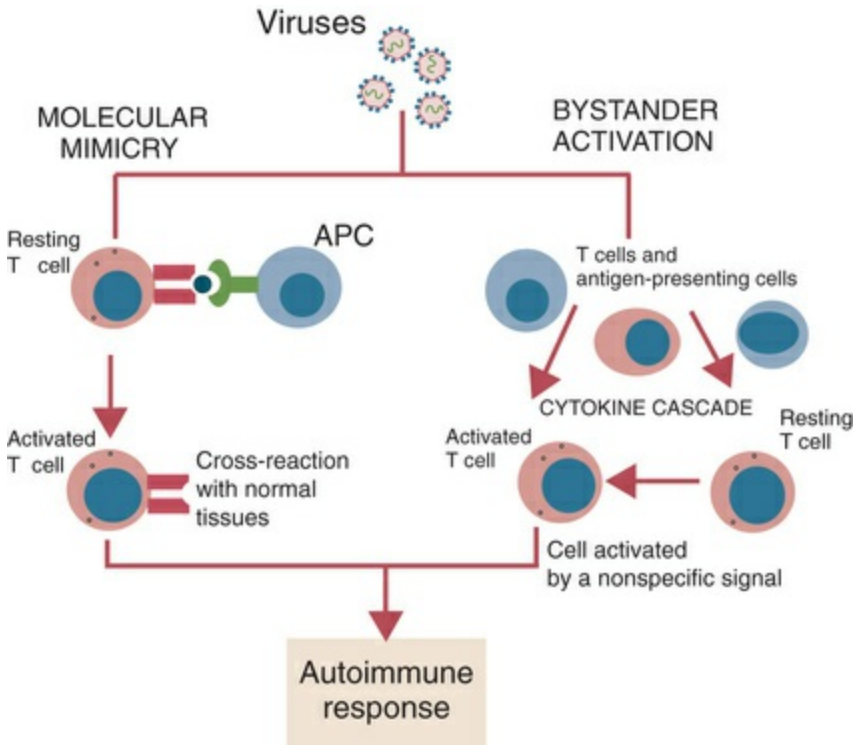


FIG. 36.3 Viruses may trigger autoimmune responses either by molecular mimicry or by bystander activation.

Microchimerism

During pregnancy, mothers and their fetuses may exchange a few cells. Some of these fetal cells may persist in a mother's body for many years after pregnancy. Conversely, a mother's cells may survive for many years in her offspring. These cells are accepted by a tolerant immune system. The process is called fetal microchimerism, and it has been suggested that these persistent cells may be the cause of some autoimmune diseases. In women with the autoimmune disease scleroderma, it is possible to find fetal T, B, and natural killer (NK) cells, as well as fetal monocytes, in their blood. It has been suggested, therefore, that scleroderma is a form of graft-versus-host disease in these patients. Transfer of cells from mother to her fetus may also cause autoimmunity. For example, small numbers of maternal cells can be detected in the blood of most boys with the autoimmune disease dermatomyositis. In all these cases, the number of persistent foreign cells is so small that they cannot be the sole cause of the autoimmune disease.

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Predisposing Factors

Genetic Predisposition

Although viruses or other infectious agents may trigger autoimmunity, it is clear that not all infected individuals develop autoimmune disease. This is because genetic factors are key determinants of disease susceptibility. Genome-wide association studies have identified hundreds of loci that harbor risk factors for autoimmune diseases. Many of these are shared by multiple diseases and perhaps identify certain common pathways (such as JAK-STAT pathways) acting in multiple diseases. It is clear from these studies, however, that the associations so far identified do not account for all genetic effects, and the effect size at any individual locus is very small.

In mice, at least 25 loci that contribute to autoimmunity if deleted or overexpressed have been identified. These include genes that code for cytokines, cytokine receptors, co-stimulators, molecules that regulate apoptosis, molecules that regulate antigen clearance, and members of cytokine or antigen-signaling cascades. Some diseases result from a defect in a single gene such as the *lpr* or *gld* mutations. Their gene products play a key role in the destruction of self-reactive T cells. In their absence, excessive T cell proliferation and autoimmunity result. Others result from inherited complement deficiencies. More commonly, the role of genes is complex. Thus genes influence the severity of disease, and no specific gene is necessary or sufficient for disease expression. Even if an animal has a complete set of susceptibility alleles at multiple loci, presence of overt disease may depend on the genetic background of the animal. This genetic complexity probably also contributes to differences in disease presentation since these may be determined by different sets of contributing genes. Genetic analysis is also complicated because susceptibility genes may or may not interact with each other. The vulnerability of a target organ to autoimmune damage may also be inherited.

The genes that most commonly associated with naturally occurring autoimmune diseases are those in the MHC. MHC

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molecules regulate the presentation of processed epitopes. In theory, therefore, they determine resistance or susceptibility to many diseases. In practice, there is strong selection against genes that predispose to susceptibility to infectious agents, and MHC genes have been selected for a strong response to most common infectious pathogens. In contrast, MHC genes causing autoimmune diseases in old, post-reproductive animals do not offer a selective disadvantage, and MHC-linked predispositions have been identified. Studies of humans have shown that almost all autoimmune diseases are linked to multiple MHC loci. Presumably an essential prerequisite for any autoimmune disease is that the autoantigen is appropriately processed and presented on an MHC molecule. Thus the structure of the MHC antigen-binding groove determines whether a specific autoantigen will trigger an immune response. Some MHC alleles appear to protect against autoimmunity, and any predisposition to autoimmunity may be the result of the net effect of both enhancing and protective genes. In addition, most autoimmune diseases are associated with multiple MHC alleles. For example, in humans the combination of HLA-A1, -B8, and -DR3 is associated with increased risk for type I diabetes, myasthenia gravis, and SLE. One possible reason for the increased predisposition of females to develop autoimmune diseases may be attributed to TLR7. This gene is located on the X-chromosome so that females have two copies while males only have one. TLR7 is closely linked to the development of systemic lupus (Chapter 38) (Box 36.1).

Box 36.1

The Effects of Spaying or Neutering on Autoimmune Diseases in Dogs

Sex hormones have significant effects on immune function, and it is unsurprising therefore that they affect the development of autoimmune diseases. In veterinary medicine, we have four distinct "genders" to assess: intact and neutered males and intact and spayed females. The relationship between spay/neutering and the development of autoimmune disease has been investigated in a population of over 90,000 dogs. Compared to intact dogs, spayed

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or neutered dogs had a significantly greater risk of hypoadrenocorticism, hypothyroidism, immune-mediated hemolytic anemia, and thrombocytopenia. Spayed females were at greater risk than neutered males for hypothyroidism and thrombocytopenia. Spayed females had a greater risk of developing systemic lupus erythematosus than intact females. There was no significant difference in risk between neutered and intact dogs for immune-mediated polyarthritis, myasthenia gravis, or the pemphigus complex.

Veterinarians should be aware of these increased risks. Neutering is associated with increased life expectancy but neutered and spayed dogs do have an increased risk of developing several autoimmune diseases.

Sundburg CR, et al: Gonadectomy effects on the risk of immune disorders in the dog: a retrospective study, *BMC Vet Res* 12:278, 2016.

Breed Predispositions

Dogs have undergone two major population bottlenecks. The first occurred when they were domesticated about 20,000 years ago. The second occurred about 200 years ago when most current dog breeds were created. Breeds have been developed as a result of aggressive phenotypic selection, in many cases resulting in inbreeding and a lack of genetic diversity. This has had two effects. First, it has permitted deleterious autosomal recessive genes to be expressed, as is seen in the increased prevalence of immunodeficiency syndromes and other immunological disorders. Second, it has resulted in a loss of MHC polymorphism. For example, DRB1*04 is found in most Boxers, DRB1*2401 may be restricted to Akitas, DRB1*01 predominates in West Highland White Terriers, DQA*0203 is restricted to Dobermans, there is a high incidence of DQA*0102 in Irish Wolfhounds and Chows, and DRB1*0101 is common in Irish Setters. These limited haplotypes ensure that the dogs in these breeds will respond to an unusually narrow range of antigens, thus reducing their resistance to infectious agents. Such dogs will also be more susceptible to immunological diseases.

The three major classes of immunologically mediated disease (autoimmunity, immunodeficiency, and atopy) tend to be

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encountered in some dog breeds more commonly than in others. Old English Sheepdogs are unusually prone to develop autoimmune blood diseases. Certain autoimmune diseases, such as polyarteritis nodosa and hypothyroidism, have familial associations. Many dogs, especially those from rare breeds with small populations, have restricted MHC polymorphism that can increase autoimmune disease susceptibility. In dogs, there are several recognized associations between autoimmunity and MHC alleles. Diabetes mellitus is associated with DLA-A3, -A7, -A10, and -B4; antinuclear antibody production is associated with DLA-12; SLE is associated with DLA-A7; and autoimmune polyarthritis is associated with certain C4 alleles. Possession of an allele of DLA-79 (DLA-79*001:02) is associated with multiple immune-mediated diseases (hemolytic anemia, thrombocytopenia, polyarthritis, and atopic dermatitis).

Inbred lines of other animals have been produced that are associated with spontaneous development of autoimmune disease. For example, inbred NZB mice spontaneously develop a syndrome that bears a striking resemblance to SLE (Chapter 38). These mice develop immune complex glomerulonephritis. They become hypergammaglobulinemic and hypocomplementemic, and they develop an autoimmune hemolytic anemia. Some also develop lymphoid tumors. NZB mice produce autoantibodies against nuclear antigens, red blood cells, and T cells, and their B cells are polyclonally activated. New Zealand White (NZW) mice are phenotypically normal, but the F1 cross between NZW and NZB mice has an even more severe SLE-like syndrome. In these animals, kidney disease is severe and is associated with high titers of antibodies to nucleic acids. Studies on the inheritance of these traits in mice suggest that they are controlled by a small number of unlinked major genes and a large number of minor genes.

Intestinal Microbiota

It is now well accepted that the intestinal microbiota both contribute to local host defenses and modulate systemic immune responses. Nutrients and microbial components are continually released into the body where they influence immune cell function.

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In critically ill animals, breakdown of the intestinal epithelium and mucosal barriers may permit excessive leakage of bacterial components into the body. Conversely, depletion of the gut microbiome, especially as a result of antibiotic treatment, may make the mucosal defenses vulnerable and perhaps reduce the priming of the systemic immune responses. Because the gut microbiota influences the development of immunological tolerance, dysbiosis may also affect the development of autoimmune disease (Chapter 21).

Non-obese diabetic (NOD) mice develop spontaneous insulin-dependent diabetes mellitus (IDDM) associated with infiltration of the pancreatic islets by lymphocytes. Their disease resembles human type 1 diabetes. The development of diabetes in these mice is influenced by their microbiota. Thus conventional NOD mice that lack MyD88 protein (MyD88 is an adaptor molecule for the toll-like receptors) do not develop diabetes, whereas totally germ-free $MyD88^-$ NOD mice do. If commensal bacteria are given to these germ-free mice, their diabetes is less severe. Somehow the interaction of the intestinal microbiota with the immune system influences the predisposition of these mice to develop diabetes.

Alterations in the intestinal microbiota also influence autoimmune diseases such as rheumatoid arthritis, ankylosing spondylitis, IDDM, and experimental autoimmune encephalitis (Chapter 21). In some mouse arthritis models, changes in the gut microbiota due to antibiotic treatment can exacerbate the disease. Antinuclear antibody production in mice is influenced by the microbiota, especially by increased colonization with segmented filamentous bacteria.

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Mechanisms of Tissue Damage in Autoimmunity

Autoimmune disease results when tissues are damaged by autoreactive T cells or antibodies. This damage is a result of hypersensitivity reactions. However, multiple mechanisms may be acting together in any such disease, and these may vary with time.

Type I Hypersensitivity

Milk allergy in cattle is an autoimmune disease in which a milk protein (α casein), normally found only in the udder, gains access to the general circulation and stimulates an immune response. This happens when milking is delayed and intramammary pressure forces the α casein into the circulation. For unknown reasons this triggers a Th2 response and IgE autoantibodies are produced. As a result, affected cows may develop acute anaphylaxis (Chapter 30). A similar condition is seen occasionally in other domestic mammals such as the mare. Although antibodies in milk proteins are commonly found in human serum after rapid weaning, type I hypersensitivity is not a usual sequel.

Type II Hypersensitivity

Autoantibodies may cause cell lysis with the assistance of complement or cytotoxic cells. Thus if autoantibodies are directed against red blood cells, autoimmune hemolytic anemia may result; if directed against platelets, thrombocytopenia will occur; and if against thyroid cells, thyroiditis will result. In one form of this process in humans, autoantibodies against thyroid-stimulating hormone (TSH) receptors on thyroid cells stimulate thyroid activity rather than its destruction. Cell surface receptors are common targets of autoimmune attack. In addition to the TSH receptor, autoantibodies attack the acetylcholine receptor in myasthenia gravis and the insulin receptor in some forms of diabetes. Autoantibodies to β-adrenoceptors (Chapter 30) have been detected in some patients with asthma. By blocking β-receptors, these

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antibodies make the airways highly irritable, and affected individuals develop severe asthma.

Type III Hypersensitivity

Autoantibodies form immune complexes with autoantigens, and these complexes may cause inflammation. This is most significant in systemic lupus erythematosus, a disease in which many different autoantibodies are produced. Immune complexes deposited in glomeruli provoke a membranoproliferative glomerulonephritis (Chapter 32). Similarly, in rheumatoid arthritis, immune complexes are deposited in joints and contribute to the local inflammatory response.

Type IV Hypersensitivity

Many autoimmune disease lesions are infiltrated with mononuclear cells, and T cells probably contribute to their pathogenesis. Cytotoxic T cells cause demyelination in experimental allergic encephalitis and multiple sclerosis. Insulin-dependent diabetes mellitus may be due to a T cell–mediated response because the diseased pancreatic islets may be infiltrated by lymphocytes. Lymphocytes from diabetics may be cytotoxic for pancreatic islet cells *in vitro.* Although cytotoxic T cells can kill cells directly, cytokines may also cause tissue damage. For example, TNF-α upregulates cell adhesion molecules, including selectins, facilitating migration of neutrophils into the lesions.

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37

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Organ-Specific Autoimmune Diseases

CHAPTER OUTLINE

Autoimmune Endocrine Disease, 409
- Lymphocytic Thyroiditis, 410
- Hyperthyroidism, 410
- Lymphocytic Parathyroiditis, 410
- Autoimmune Diabetes Mellitus, 410
- Atrophic Lymphocytic Pancreatitis, 411
- Autoimmune Adrenalitis, 411

Autoimmune Neurological Disease, 411
- Equine Polyneuritis, 411
- Canine Polyneuritis, 411
- Steroid-Responsive Meningitis-Arteritis, 411
- Necrotizing Meningoencephalitis, 412
- Degenerative Myelopathy, 412
- Cerebellar Degeneration, 412

Autoimmune Eye Disease, 412
- Equine Recurrent Uveitis, 412

VetBooks.ir

Uveodermatological Syndrome, 413
Immune-Mediated Keratoconjunctivitis, 413
Autoimmune Reproductive Diseases, 414
Autoimmune Skin Diseases, 414
Hair Follicle Diseases, 414
Alopecia Areata, 414
Blistering Diseases, 414
Skin Basement Membrane Diseases, 415
Bullous Pemphigoid, 416
Linear Immunoglobulin A Dermatosis, 416
Epidermolysis Bullosa Acquisita, 416
Relapsing Polychondritis, 417
Autoimmune Nephritis, 417
Autoimmune Blood Diseases, 417
Immune-Mediated Hemolytic Anemia, 417
Immune Suppression of Hematopoiesis, 419
Autoimmune Thrombocytopenia, 419
Autoimmune Muscle Diseases, 419
Myasthenia Gravis, 419
Polymyositis, 420
Autoimmune Masticatory Myositis, 421
Canine Cardiomyopathy, 421
Dermatomyositis, 421

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LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Understand that in domestic mammals, any organ or tissue is a potential victim of autoimmune attack.
- Recognize that the most common autoimmune diseases in domestic mammals involve the endocrine system, the skin, and blood cells.
- Understand why treatment of autoimmune diseases usually involves suppression of the destructive inflammatory lesion by corticosteroids.
- Understand the principles of the antiglobulin test and the indirect immunofluorescence assay.
- Understand that in diseases such as diabetes mellitus, some cases may be due to autoimmunity while others are not.
- Explain that in many animal diseases, the development of autoantibodies is a consequence not a cause of the disease.

Autoimmune diseases that mainly affect a single organ or tissue presumably result from an abnormal response to a small number of self-antigens and do not necessarily reflect loss of control of the immune system as a whole. All tissues of the body are potentially susceptible to this form of immunological attack. Nevertheless, autoimmune diseases directed against endocrine organs, skin, blood, and the nervous system, tend to be most common. Their prevalence depends to a great extent on the species and breed of an animal as well as its age.

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Autoimmune Endocrine Disease

Although domestic animals develop autoimmune endocrine diseases, they differ from humans insofar as these tend to be targeted at single organs rather than involving multiple endocrine glands. Occasionally a dog may experience two or more autoimmune endocrine disorders simultaneously (autoimmune polyglandular syndrome), usually affecting the thyroid and adrenals, but this is uncommon.

Lymphocytic Thyroiditis

Dogs, humans, and chickens suffer from autoimmune thyroiditis. In humans, this results from the production of autoantibodies against thyroglobulin or thyroid peroxidase. These antibodies may also react with triiodothyronine (T_3) or thyroxin (T_4). In dogs, many develop thyroiditis in the absence of these autoantibodies and cell-mediated type 1 responses are responsible for thyroid destruction. Several dog breeds are predisposed to thyroiditis, and relatives of affected animals may have anti-thyroid antibodies although clinically normal. A familial form of hypothyroidism has been demonstrated in Beagles and Great Danes. Dogs from high-risk breeds such as Dobermans tend to develop the disease when young, whereas dogs from low-risk breeds tend to develop it when older. Unfortunately, by the time the disease is diagnosed, the dog may already have bred. Affected thyroids are infiltrated with plasma cells and lymphocytes, and germinal center formation may occur (Fig. 37.1). The invading lymphocytes probably cause epithelial cell destruction through antibody-dependent cell-mediated cytotoxicity (ADCC) and T cell cytotoxicity.

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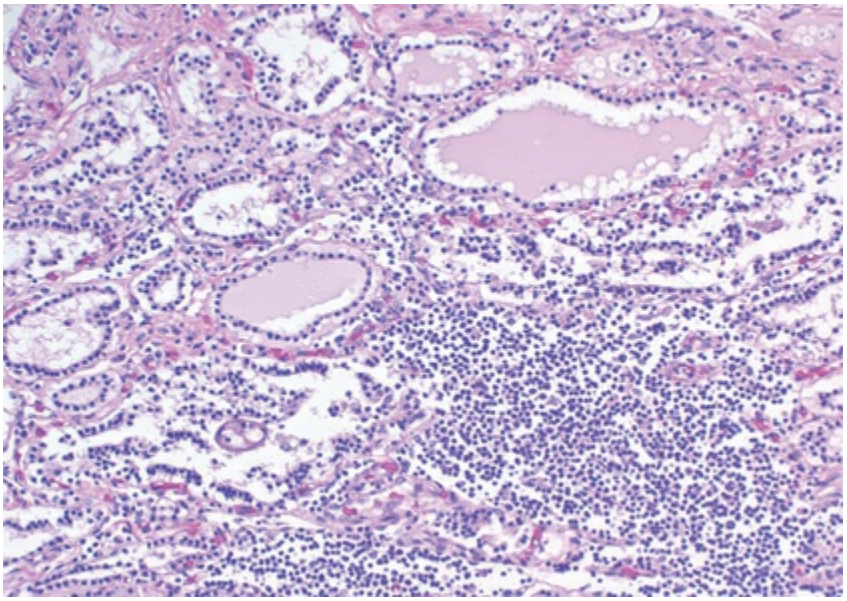


FIG. 37.1 A lymphocytic nodule in the thyroid of a dog suffering from autoimmune thyroiditis. Original magnification ×100. (Courtesy Dr. G. Stoica.)

In dogs, clinical signs appear after about 75% of the thyroid is destroyed. These signs are those of hypothyroidism; that is, the animals are fat and inactive and show patchy hair loss. The most common problems are a dry, dull, coarse coat; scaling; hypotrichosis; slow hair regrowth; hyperpigmentation; myxedema; and pyoderma. Other signs include myopathy, hyperlipidemia, hypothermia, anestrus, galactorrhea, diarrhea or constipation, and polyneuropathy. Tests of thyroid function such as a radioimmunoassay for plasma T_4 or T_3 only confirm the existence of hypothyroidism. A thyroid-stimulating hormone (TSH) response test is more useful because it can confirm the inability of the affected thyroid to respond. (Plasma T_4 levels are measured before and after injection of TSH.) In order to confirm autoimmune thyroiditis, a biopsy must show the characteristic lymphocytic infiltration. Antithyroid antibodies must be detected in serum using an enzyme-linked immunosorbent assay (ELISA), immunoblot, or an indirect fluorescent antibody test (Chapter 42). As pointed out previously, there is poor correlation between antithyroid antibody titers and disease severity, reflecting the importance of cell-mediated processes in this species. Management of affected animals involves replacement therapy with sodium levothyroxine (synthetic T_4). Improvement should be seen within 4 to 6 weeks. There is no

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cure for this disease, and the success of treatment depends on effective replacement therapy.

Hyperthyroidism

Hyperthyroidism is a disease of old cats. Autoantibodies to thyroid peroxidase have been demonstrated in almost one-third of cases of feline hyperthyroidism, and about 10% of these animals also have antinuclear antibodies. Lymphocytic infiltration is also observed in about one-third of cases. It is probable that these are secondary to the disease process and not its cause.

Lymphocytic Parathyroiditis

Dogs and cats can develop autoimmune hypoparathyroidism. Affected animals usually have a history of neurological or neuromuscular disease, especially seizures. On investigation, animals are profoundly hypocalcemic, and serum parathormone levels are severely reduced. Normal parathyroid tissue is replaced by lymphocytes and plasma cells. Once hypocalcemic tetany is controlled, these animals may be treated by oral vitamin D and calcium administration. It would be logical to administer immunosuppressive therapy.

Autoimmune Diabetes Mellitus

Canine diabetes mellitus is a heterogeneous disease and most cases have an unknown etiology. Some are probably immunologically mediated. The canine disease is associated with pancreatic islet atrophy and a loss of β cells. In some cases, the islets may be infiltrated by lymphocytes and the dogs may make autoantibodies to canine proinsulin. Experimentally, it has been shown that circulating mononuclear cells from some diabetic dogs can suppress insulin production by cultured mouse islet cells. Additionally, serum from some diabetic dogs may lyse these islet cells in the presence of complement. When diabetic dog serum was tested for antibodies against cultured β cells by immunofluorescence, 9 of 23 dogs showed strongly positive reactions, and an additional three showed a weak reaction. Only 1 of 15 normal dogs gave a positive

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response.

Humans with juvenile onset (type 1) diabetes have circulating antibodies against the 67-kDa isoform of glutamic acid decarboxylase (GAD65) and/or insulinoma antigen-2 (IA-2). Some diabetic dogs also possess these autoantibodies. Thus 4 of 30 diabetic dogs had autoantibodies to GAD65, and three had autoantibodies to IA-2. Two had autoantibodies to both antigens.

Certain DLA haplotypes are more prevalent in breeds such as the Samoyed, Tibetan Terrier, and Cairn Terrier that have a higher risk of developing diabetes. However, these haplotypes are not uncommon in other breeds. A predisposition to neonatal diabetes mellitus has also been observed in Keeshonds, where it appears to be controlled by a single autosomal recessive gene. Boxers, in contrast, rarely get this disease. Genes that may influence the development of canine diabetes include MHC class II genes, genes coding for the CTLA-4 promoter, and those for interferon-γ (IFN-γ), interleukin-12 (IL-12), IL-4, and IL-10.

Diabetes mellitus is rare in cattle. Affected animals have atrophied and reduced numbers of pancreatic islets with partial or complete loss of β cells. Lymphocytes commonly infiltrate the remaining islets.

Atrophic Lymphocytic Pancreatitis

The most common cause of an exocrine pancreatic deficiency in dogs is atrophy associated with lymphocyte infiltration. The disease has a high heritability as shown by its prevalence in German Shepherd dogs and Rough-coated Collies. The infiltrating lymphocytes are primarily $CD4^+$ and $CD8^+$ T cells. The $CD8^+$ cells are associated with areas of pancreatic necrosis. Some of these dogs have low levels of antibodies against pancreatic acinar cells, so this may be, in part, an autoimmune disease. Genetic studies have pointed to genes located within the MHC as associated with the disease. One haplotype containing a novel allele of DLA-88 is highly associated with the disease, while other haplotypes may confer some protection.

Autoimmune Adrenalitis

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Dogs may suffer from lymphocyte-mediated destruction of the adrenal cortex. Affected animals present with depression, weak pulse, bradycardia, abdominal pain, vomiting, diarrhea, dehydration, and hypothermia. As a result of excessive sodium and chloride loss, animals develop hypovolemia and acidosis, leading to circulatory shock, hyperkalemia, and cardiac arrhythmias. Blood corticosteroid levels are low in these animals. This disease has been observed in association with hypothyroidism.

A syndrome has been described in Italian Greyhounds that resembles autoimmune polyendocrine syndrome type 2 in humans. This is not a random collection of unlinked diseases but a syndrome with a common etiology but diverse clinical manifestations. It involves mainly endocrine tissues, adrenals, thyroid, pancreas, gonads, and skin. It tends to occur in females at mid-life.

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Autoimmune Neurological Disease

An autoimmune brain disease known as experimental allergic encephalomyelitis (EAE) can be produced by immunizing mice with brain tissue emulsified in Freund's complete adjuvant. The mice develop focal encephalitis and myelitis, possibly with paralysis. Their brain lesions consist of vasculitis, mononuclear cell infiltration, perivascular demyelination, and axon damage. Antibodies to brain tissue can be detected in the serum of these animals, although the lesion itself is a result of a cell-mediated response.

A similar encephalitis used to occur following administration of rabies vaccines containing brain tissue to humans. For this reason, the use of adult brain tissue was stopped, and suckling mouse brain tissue obtained prior to myelination substituted. Post-distemper demyelinating leukoencephalopathy may also be of autoimmune origin, although the production of antimyelin antibodies appears to be common response to central nervous tissue damage, regardless of its cause.

Equine Polyneuritis

Equine polyneuritis (neuritis of the cauda equina) is an uncommon disease affecting the sacral and coccygeal nerves. Affected horses show hyperesthesia followed by progressive paralysis of the tail, rectum, and bladder and localized anesthesia in the same region. The disease may also be associated with facial and trigeminal paralysis. Although sacral and lumbar involvement is usually bilateral, the cranial nerve involvement is often unilateral. A chronic granulomatous inflammation develops in the region of the extradural nerve roots. Affected nerves are thickened and discolored. There is a loss of myelinated axons; infiltration by macrophages, lymphocytes, giant cells, and plasma cells; and deposition of fibrous material in the perineurium. In severe cases, the nerve trunks may be almost totally destroyed. Affected horses have circulating antibodies to a peripheral myelin protein called P2. Although equine polyneuritis may be an autoimmune disease,

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equine adenovirus-1 has been isolated from its lesions, so the cause is complex. Because of the severe nerve damage, immunosuppressive or antiinflammatory therapy is rarely successful. Neuritis of the cauda equina has also been reported in a dog.

Canine Polyneuritis

Canine polyneuritis or coonhound paralysis affects dogs following a bite or scratch from a raccoon. It presents as an ascending symmetrical flaccid paralysis with mild sensory impairment. The bitten limb is usually affected first, but the disease is progressive and will worsen for 10 to 12 days following the bite. In severe cases, the dog may develop flaccid quadriplegia and lose the ability to swallow, bark, or breathe. The disease is, however, self-limiting, and if respiration is not impaired, the prognosis is good. Dogs usually recover completely. Affected nerves show demyelination and axonal degeneration with macrophage infiltration. An acute polyneuritis similar to coonhound paralysis has also been described following vaccination of dogs with rabies or other vaccines.

Coonhound paralysis and post-vaccinal polyneuritis both closely resemble Guillain-Barré syndrome in humans. This syndrome may follow upper respiratory tract infection, gastrointestinal disease, or even vaccination. It is mediated by autoantibodies against peripheral nerve glycolipids. Management of Guillain-Barré syndrome requires plasmapheresis and administration of intravenous immunoglobulins (IVIG). Veterinarians treating canine polyneuritis have traditionally administered corticosteroids, but their effectiveness is unclear.

Steroid-Responsive Meningitis-Arteritis

Steroid-responsive meningitis-arteritis (SRMA) is characterized by inflammation of the meningeal arteries and cervical meningitis. Two different forms of the disease are recognized. In the acute form, affected dogs show anorexia, fever, lameness, and listlessness followed by progressive cervical rigidity; hyperesthesia along the vertebral column; generalized, cervical, or spinal pain; ataxia;

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seizures; and behavioral changes. The typical disease course consists of severe episodes with symptom-free remissions. The less common chronic form may develop following relapses of acute disease or inadequate treatment. Additional neurological signs consistent with lesions in the brain and spinal cord such as paresis or ataxia may develop. These dogs may have concurrent immune-mediated polyarthritis. The prognosis in young dogs is fair to good since aggressive antiinflammatory and immunosuppressive therapy using prednisolone or prednisone leads to rapid clinical improvement. Once the disease is in remission, the steroid dose should be gradually reduced to the minimum necessary to prevent relapses. Treatment may be stopped 6 months after clinical status, cerebrospinal fluid (CSF), and blood return to normal. It may not be possible to discontinue treatment completely in chronic cases, although azathioprine is effective in such cases. Large dogs, such as Boxers, Weimaraners, and Bernese Mountain Dogs, are commonly affected, although the disease has also been reported in Beagles. Dogs under 2 years of age are most commonly affected.

This disease is characterized by increased IgA production, an acute-phase response, and the development of a necrotizing vasculitis. Several cytokines play a role in this process. IL-6 and vascular endothelial growth factor (VEGF) are significantly increased in cerebrospinal fluid. The changes in IL-6 levels may promote Th17 cell differentiation. The CSF in acute cases of SRMA contains high IgA and CXCL8 levels and mature neutrophils. Serum IgA, C-reactive protein and α_2-macroglobulin are also elevated. T cell production of IL-2 and IFN-γ is depressed, whereas Th2 production of IL-4 is enhanced and probably accounts for the increased IgA production. About 30% of these dogs have a positive lupus erythematosus (LE) cell test but no detectable antinuclear antibodies (Chapter 38). In chronic cases, the CSF contains predominantly mononuclear cells. On necropsy, the spinal meningeal arteries show fibrinoid degeneration, intimal or medial necrosis, and hyalinization, and are infiltrated with lymphocytes, plasma cells, macrophages, and a few neutrophils. Complete obliteration of the blood vessel lumina may occur, whereas rupture and thrombosis of inflamed vessels may lead to hemorrhage, compression, and infarction.

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Necrotizing Meningoencephalitis

Three somewhat similar inflammatory diseases of the canine central nervous system are recognized. These are necrotizing meningoencephalitis (NME), necrotizing leukoencephalitis (NLE), and granulomatous meningoencephalomyelitis (GME). They each have distinct lesions, clinical manifestations, and breed predispositions. They may be immunological in origin. Canine NME is an inflammatory disease of unknown etiology that has been described in several toy breeds such as Pugs (young females), Papillons, Shih Tzu, Maltese Terriers, Pekinese, and Chihuahuas. The necrotic lesions are multifocal and asymmetrical, restricted to the gray and white matter in the cerebra, and are accompanied by a severe meningitis. Macrophages predominate in the lesions, but both scattered T cells and dendritic cells are present in the lesions, whereas B cells are restricted to the meninges. Dogs with NME have autoantibodies to glial fibrillary acidic protein, but the significance of these is unclear. In at least one case, an affected dog had a concomitant glomerulonephritis with smooth linear basement membrane staining for IgG, suggesting the presence of anti–basement membrane antibodies.

NLE has been reported in Yorkshire Terriers and French Bulldogs. It is similar to NME but the necrotic foci are found predominantly in the white matter of the forebrain and brainstem. These foci are characterized by cavitation, necrosis, demyelination, and perivascular cuffing. The primary infiltrating cells are T cells. Some investigators consider NLE to be a variant of NME.

The third form of canine nonsuppurative encephalitis, GME, may account for a quarter of canine central nervous system diseases. GME is characterized by the formation of multifocal granulomas around blood vessels in the cerebellum and brainstem. T cells and macrophages predominate in the lesions. GME may be disseminated, focal, or ocular. The prognosis is poor, although aggressive treatment with corticosteroids may be beneficial. IFN-γ and IL-17 are elevated in NME and GME. The IL-17 in GME appears to be produced by macrophages rather than T cells.

Degenerative Myelopathy

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Affected dogs show progressive ataxia affecting the hind limbs until they can no longer walk. Forelimb problems eventually develop, and dogs die 6 to 12 months after disease onset. On necropsy, these dogs have a degenerative myelopathy with widespread demyelination and loss of axons in the thoracolumbar region. The cause of the disease is unknown, but some investigators believe it to be immune mediated. Affected dogs have circulating immune complexes, depressed lymphocyte mitotic responses, and deposits of IgG and C3 in the lesions and nearby normal tissues. Boxers and Newfoundland dogs affected with inflammatory myopathies have circulating autoantibodies to sarcolemmal autoantigens. It is not clear whether these autoantibodies are a cause or effect of the myopathy. However, their detection may be a useful diagnostic test.

Cerebellar Degeneration

Cerebellar degeneration has been observed in Coton de Tulear puppies. It is associated with a depleted granular cell layer and microglial cell activation caused by T cell destruction of granular cells.

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Autoimmune Eye Disease

Equine Recurrent Uveitis

The most common cause of blindness in horses is recurrent uveitis (or periodic ophthalmia). Horses suffer repeated episodes of intraocular inflammation mainly involving the anterior uveal tract. In acute cases, they develop ocular pain, blepharospasm, lacrimation, corneal changes such as edema and vascularization, and photophobia. Some horses develop a posterior uveitis, including vitritis and retinitis, resulting in photoreceptor destruction. Each attack gets progressively more severe and gradually spreads to involve other eye tissues until complete blindness results. The eye lesions are infiltrated with $CD4^{+}$Th1 cells and neutrophils with extensive fibrin and C3 deposition, and the production of IL-2 and IFN-γ. Th17 cells may be responsible for chronic disease. The major autoantigen implicated is the interphotoreceptor retinoid-binding protein with subsequent epitope spreading to the retinal S-protein and other autoantigens, including recoverin and cellular retinaldehyde-binding protein. Affected horses may also have circulating antibodies to a protein found on the inner leaflet of the outer membrane of *Leptospira interrogans* called LruC. The titer of these antibodies tends to rise during a flare-up of the lesion and drop while in remission. If horses are immunized with either equine cornea or certain serovars of killed *L. interrogans,* they develop corneal opacity 10 days later at the time when antibodies appear in the bloodstream. Partial antigenic identity exists between equine corneas and these *L. interrogans* serovars, and some cases may be due to molecular mimicry of *L. interrogans* LruC. Other cases may be due to persistent retinal infection with leptospires. Some cases may be associated with *Borrelia burgdorferi* infection or with the nematode *Onchocerca cervicalis.* Systemic and topical corticosteroid therapy is required to bring the inflammation under control, although the disease usually recurs. Encouraging results have been obtained with slow-release cyclosporine implants.

Appaloosa horses are predisposed to developing equine

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recurrent uveitis. Three single nucleotide polymorphisms (SNPs) are significantly correlated with the development of this disease. One is on chromosome 1, and the other two are associated with the equine MHC region (ELA). In German Warmbloods, the ELA-A9 haplotype is associated with increased susceptibility.

Uveodermatological Syndrome

Uveodermatological syndrome is a sporadic disease of dogs. A similar disease, Vogt-Koyanagi-Harada syndrome, occurs in humans. Affected dogs develop uveitis and skin depigmentation with whitening of the hair (poliosis) and skin (vitiligo). The eye lesions develop first and most animals present with sudden blindness or chronic uveitis. The early lesions vary from a severe panuveitis to a bilateral anterior uveitis. Some dogs may have detached retinas, and there may be progressive depigmentation of the retina and iris. Depigmentation of the hair and skin gradually follows the onset of eye lesions. Some cases may be generalized, involving the eyelids, nasal planum, lips, scrotum, and foot pads (Fig. 37.2). These depigmented areas may become ulcerated and crusted.



FIG. 37.2 A case of uveodermatological syndrome. Note ocular clouding, alopecia, and depigmentation of the nasal planum.
(Courtesy Drs. R. Kennis, J. Dziezc, and L. Wadsworth.)

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There is a diffuse infiltration of the uveal tract with lymphocytes, plasma cells, and macrophages. Many of the macrophages contain ingested melanin. The skin lesions consist of a mononuclear (macrophages, giant cells, lymphocytes, plasma cells) infiltration of the dermal-epidermal junction (Fig. 37.3). The amount of melanin in the epidermis and hair follicles is greatly reduced. In humans, Vogt-Koyanagi-Harada syndrome is believed to be a result of an autoimmune response against melanocytes. In dogs no consistent immunological abnormalities have been observed.

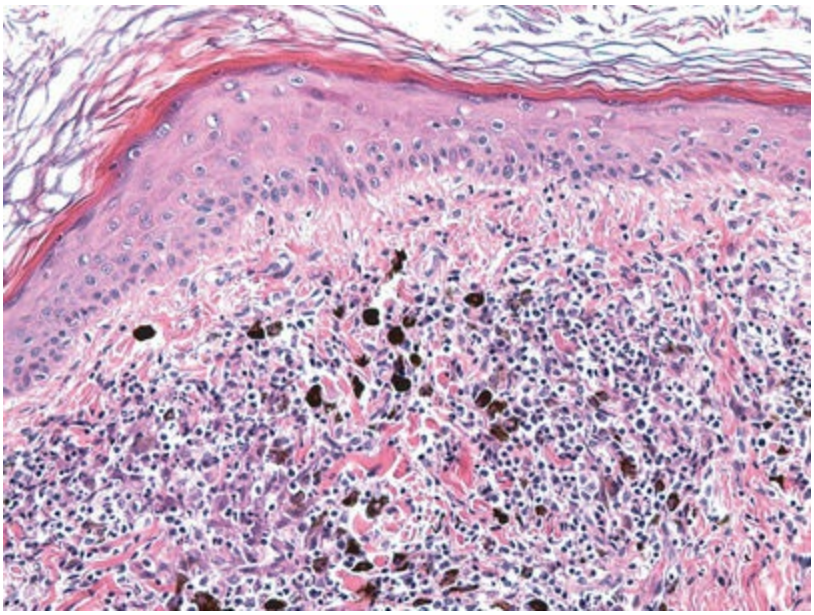


FIG. 37.3 A histological section of skin from a case of uveodermatological syndrome. Note the major lymphocyte infiltration associated with the skin melanocytes. It is the destruction of these melanocytes that leads to depigmentation. (Courtesy Dr. J. Mansell.)

Management of the eye lesions with ocular corticosteroids and of the skin lesions with systemic corticosteroids has been beneficial, although the disease may recur when therapy is terminated. Azathioprine may also be given if corticosteroids are insufficient to stop disease progression.

Immune-Mediated Keratoconjunctivitis

This is a common eye condition in horses. It presents as a chronic,

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yellowish-white diffuse corneal opacity without ulceration or significant uveitis. The depth of the corneal lesion determines the severity of the disease. The lesion can be located anywhere from the epithelium to the endothelium. It usually affects only one eye. It appears to result from an immune reaction to infectious agents or to an autoimmune response. Treatment with topical dexamethasone plus antibiotics is appropriate. The deeper the lesion, the slower will be recovery and the poorer the prognosis. The condition often recurs.

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Autoimmune Reproductive Diseases

If the testes are damaged so that hidden antigens are released, an autoimmune response may cause orchitis. Experimentally, autoimmune orchitis may be produced in male animals by injection of testicular extracts emulsified in Freund's complete adjuvant. Autoantibodies to sperm may also be detected in some animals following injury to the testes or long-standing obstruction of the seminiferous ducts. For example, dogs infected with *Brucella canis* have chronic epididymitis and become sensitized by sperm antigens carried to the circulation after phagocytosis by macrophages. These sperm antigens stimulate the production of IgG or IgA autoantibodies. The autoantibodies can agglutinate and immobilize sperm, causing infertility.

In stallions and cows, antisperm autoantibodies may be associated with reduced fertility or infertility. In certain lines of black mink, 20% to 30% of older males are infertile as a result of high levels of antisperm antibodies. The animals have a monocytic orchitis, and immune complexes are deposited along the basal lamina of the seminiferous tubules.

Dermatologists recognize an autoimmune dermatitis in which intact female dogs develop a hypersensitivity to endogenous progesterone or estrogen. The disease presents as a bilaterally symmetrical intense pruritus, erythema, and papular eruption. Its development usually coincides with estrus or pseudopregnancy. Corticosteroid treatment may have little effect, but testosterone may help.

Many vaccine companies are interested in production-enhancing vaccines. These vaccines commonly interfere with normal hormone production or reproductive behavior by inducing an autoimmune response. Thus a vaccine designed to neutralize production of gonadotropin-releasing hormone effectively lowers testosterone levels. This results in improved meat quality, faster growth, and reduced aggression by bulls. This vaccine is also used to reduce aggressive behavior in male pigs and block the production of androstenone, the lipophilic steroid that contributes to boar taint, the offensive odor associated with cooked boar meat. In horses, a

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similar vaccine is used to control estrus and estrus-related misbehavior. Similar vaccines may be used as contraceptives or to treat benign prostatic hyperplasia in dogs. If dogs are immunized with bovine or ovine luteinizing hormone (LH), the autoantibodies produced may neutralize their own LH. Similarly, it is possible to produce autoantibodies that neutralize LH-releasing hormone. As a result, the reproductive cycle is abolished in females, and testicular, epididymal, and prostatic atrophy occurs in males. Other experimental immunocontraceptive vaccines have been directed against prostaglandin $F_{2\alpha}$, reproductive steroids, the LH receptor, and zona pellucida protein.

Sheep immunized with polyandroalbumin (androstenedione-7-carboxyethyl thioester linked to human serum albumin) have about 23% more lambs than untreated sheep. The ewes are given two doses of this vaccine before mating. It is believed that the vaccine induces autoantibodies that reduce serum androstenedione levels.

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Autoimmune Skin Diseases

Many autoimmune skin diseases are recognized. These diseases may affect hair follicles, basal keratinocytes, or the skin basement membrane. Hair follicle disease can lead to alopecia. Diseases involving basal keratinocytes or basement membranes result in cell separation within the skin and the consequent development of bullae (blisters or vesicles) (Fig. 37.4). As a result, dermatologists use the terms *pemphigus* or *pemphigoid* to describe them, after the Greek word *pemphix* meaning "blister."

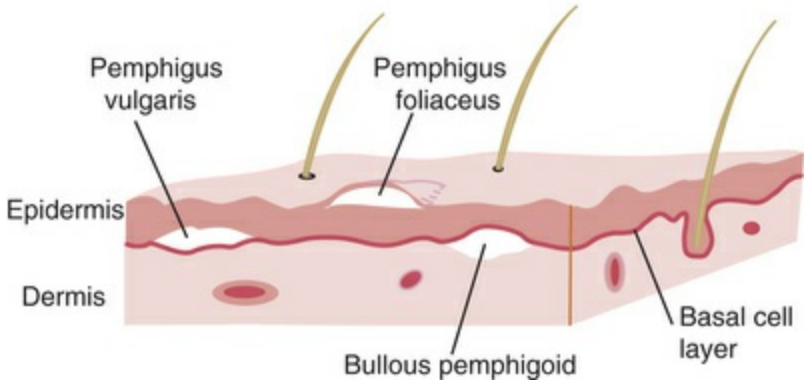


FIG. 37.4 The differential histology of the autoimmune skin diseases. Note the location of the vesicle in relation to the epidermis.

Hair Follicle Diseases

Alopecia Areata

Alopecia areata is characterized by inflammatory hair loss. It has been reported in humans, other primates, dogs, cats, horses, and cattle. In dogs, it is a rare disease. The alopecia starts locally, often on the head, but may spread to involve the entire body. It is often symmetrical. The hair follicles are infiltrated with $CD4^+$ and $CD8^+$ T cells and Langerhans cells. IgG antibodies are directed against the lower hair follicles. C3 or IgM may also be present. This immune attack may be directed against a protein called trichohyalin located in the inner root sheath of hair follicles. Alopecia areata responds to

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corticosteroid treatment, but spontaneous hair regrowth also occurs. Other autoimmune diseases that lead to hair loss include pseudopelade. It differs from alopecia areata in the precise location of the inflammatory infiltrate within the hair follicles. Likewise, some cases of pemphigus vulgaris (see later) may also be restricted to hair follicles.

Blistering Diseases

Blistering skin diseases have been described in humans, dogs, horses, and cats. Known as the pemphigus complex, they are classified according to the location of the lesions within the epidermis. Some lesions develop deep within the epidermis. For example, the most severe form (although very rare) is called pemphigus vulgaris. In this disease, bullae (blisters) develop around the mucocutaneous junctions, especially the nose, lips, eyes, prepuce, and anus, on the tongue, and the inner surface of the ear. These bullae rupture easily, leaving weeping, denuded areas that may become secondarily infected. Histological examination of intact bullae shows separation of the skin cells (acantholysis) in the suprabasal region of the lower epidermis (Fig. 37.5). The acantholysis results when autoantibodies destroy the desmosomes that bind skin cells together. In pemphigus vulgaris, the autoantigen is a desmosome protein called desmoglein-3. Binding of antibodies to desmoglein-3 activates the proto-oncogene *c-myc* and leads to keratinocyte proliferation. As a result, the keratinocytes above the lesion proliferate and fail to express adhesion proteins so that they separate from each other. Eventually this leads to acantholysis and bulla formation.

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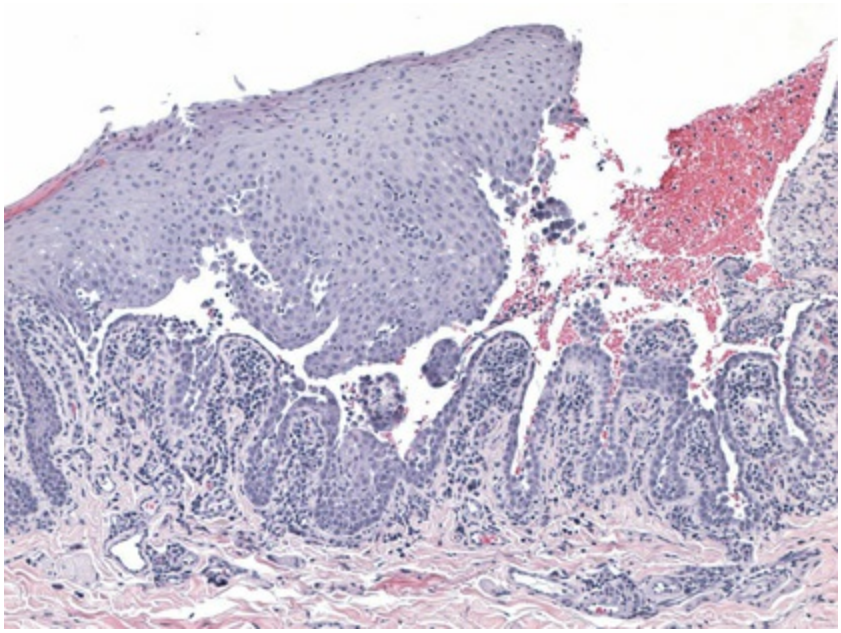


FIG. 37.5 A section of an oral lesion of pemphigus vulgaris in a dog. Note the cleft formation at the base of the epidermis accompanied by extensive cellular infiltration. (Courtesy Dr. J. Mansell.)

Pemphigus foliaceus (PF) is the commonest autoimmune disease of dogs. It is a blistering disease in which the lesions develop superficially in the epidermis (Fig. 37.6). As a result, it is a milder disease than pemphigus vulgaris. It has been described in humans, dogs, cats, goats, and horses. The bullae are not confined to mucocutaneous junctions or the muzzle. Histology reveals that the bullae develop in the subcorneal region. These bullae are very fragile, rupture easily, and therefore rarely persist. IgG is the dominant serum autoantibody. The major autoantigen in human PF is desmoglein-1, a cell adhesion protein found in squamous cell desmosomes. In dogs, the major autoantigen is a different desmosome protein called desmocollin-1. Some cases of canine PF develop in response to the use of antibiotics such as trimethoprim-sulfadiazine, oxacillin, cephalexin, and ampicillin, as well as some topical flea control products. They appear to result from the binding of drug thiol groups to cell membranes.

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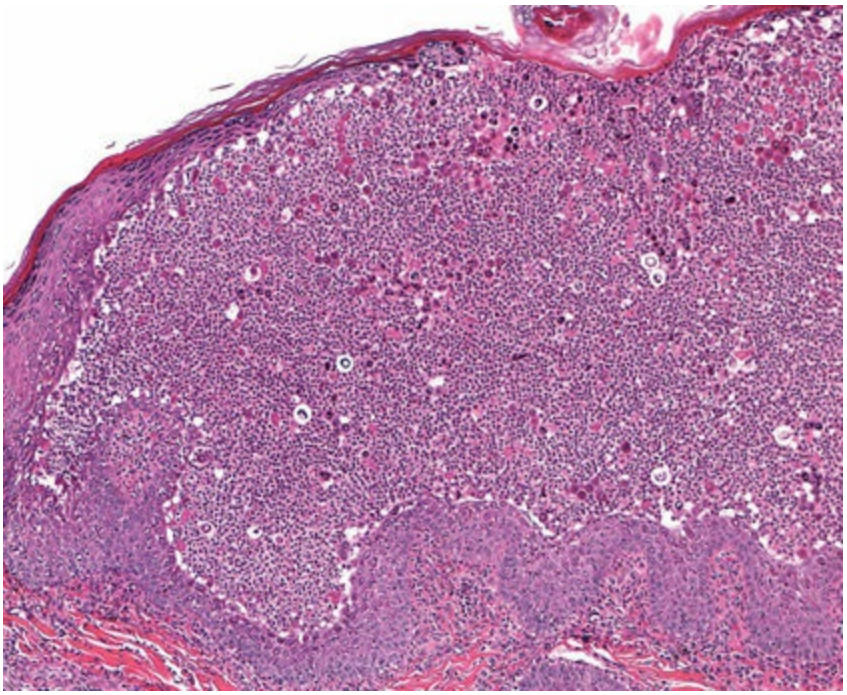


FIG. 37.6 A section from a case of canine pemphigus foliaceus. Note the subcorneal location of the cell-filled vesicle. (Courtesy Dr. J. Mansell.)

A mild variant of PF is pemphigus erythematosus. The lesions in this disease tend to be confined to the face and ears and are very similar to those of systemic lupus erythematosus (SLE). Indeed, some dogs with pemphigus erythematosus may have antinuclear antibodies in their serum. Panepidermal pustular pemphigus (pemphigus vegetans) is another rare and mild variant of PF in which papillomatous proliferation of the base of the bullae occurs on healing.

A fifth form of pemphigus, called paraneoplastic pemphigus, occurs in humans and has been recorded in a dog. It develops in association with lymphoid or solid tumors. It resembles pemphigus vulgaris, but multiple autoantibodies against skin antigens are present.

A topical ectoparasiticide containing fipronil, amitraz and S-methoprene has been associated with the development of an acantholytic pustular dermatitis resembling PF. One or two applications were sufficient to induce disease. Anti-keratinocyte IgG was detected in the epidermis of 8 of 19 cases, while serum anti-keratinocyte IgG was detected in 10 of 14 cases. Eleven of fourteen dogs had detectable antibodies to desmocollin-1. Thus the lesions closely resembled naturally occurring PF.

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Direct immunofluorescent examination of pemphigus lesions reveals immunoglobulins deposited on the intercellular cement in a typical "chicken-wire" pattern (Fig. 37.7).

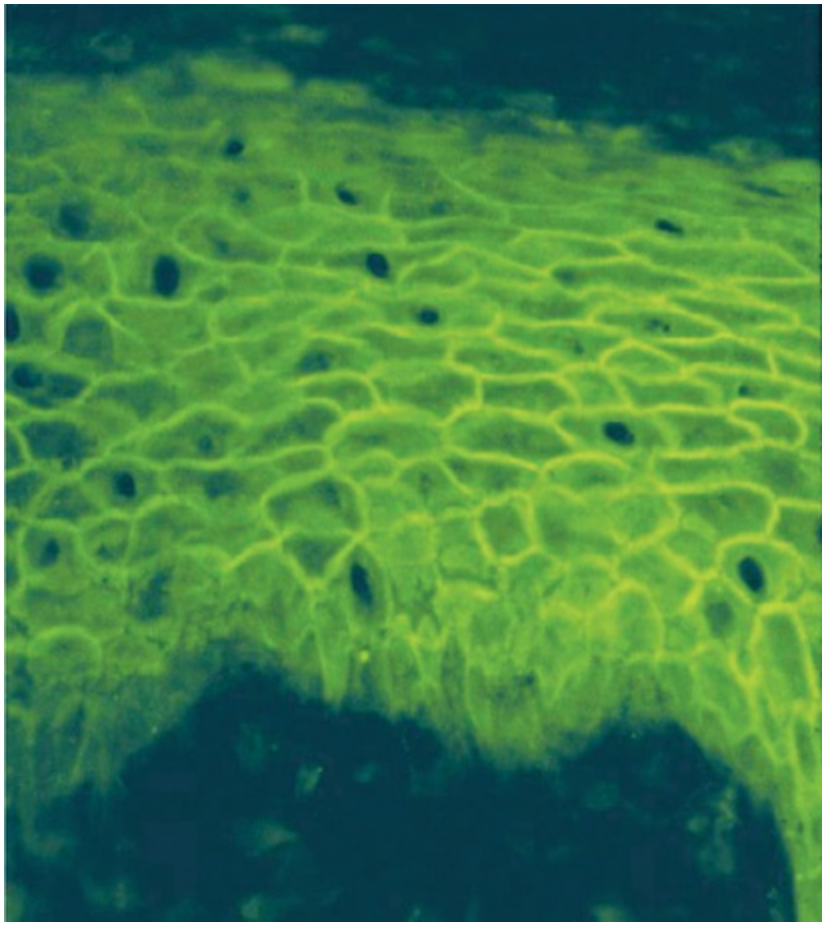


FIG. 37.7 Direct immunofluorescence of a section of normal dog skin that has been incubated in serum from a dog with pemphigus vulgaris. The intercellular cement is stained. (Courtesy Dr. K. Credille.)

It is important to differentiate among the forms of pemphigus for prognostic reasons. Pemphigus vulgaris has a poor prognosis; treatment tends to be unsatisfactory, and the lesions are persistent. In contrast, PF is milder, and the results of treatment may be more satisfactory. Treatment primarily involves the use of corticosteroids. In refractory cases, azathioprine, cyclophosphamide, chlorambucil, cyclosporine, or gold salts such as aurothioglucose may be of assistance. As with other autoimmune diseases, these diseases often recur when treatment is stopped.

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Skin Basement Membrane Diseases

A second set of blistering diseases is associated with the development of autoantibodies against the skin basement membrane. As a result, affected dogs develop subepidermal bullae. Several such diseases have been identified in dogs and other domestic animals. They include bullous pemphigoid, linear IgA dermatosis, and epidermolysis bullosa acquisita.

Bullous Pemphigoid

Bullous pemphigoid is a rare skin disease that resembles pemphigus vulgaris. Collies, Shetland Sheepdogs, and Dobermans appear to be predisposed to it. It has also been described in humans, pigs, horses, and cats. Multiple bullae develop around mucocutaneous junctions and in the groin and axillae. However, the disease differs from pemphigus vulgaris in that the bullae develop in the subepidermis (and are therefore less likely to rupture). They tend to be filled with fibrin as well as mononuclear cells or eosinophils, and they heal spontaneously. Bullous pemphigoid results from the development of autoantibodies against type XVII collagen. This molecule is a component of hemidesmosomes, the structures that attach basal keratinocytes to the basement membrane (Fig. 37.8). The presence of IgG on the basement membrane may be demonstrated by immunofluorescence that reveals intense linear staining. The prognosis of bullous pemphigoid is usually poor, but mild cases may recover after treatment with corticosteroids. More commonly, aggressive treatment such as high doses of prednisolone, supplemented if necessary with cyclophosphamide, azathioprine, and chlorambucil, may be needed. Some dogs may develop a bullous pemphigoid-like disease in response to autoantibodies against the basement membrane protein laminin-5.

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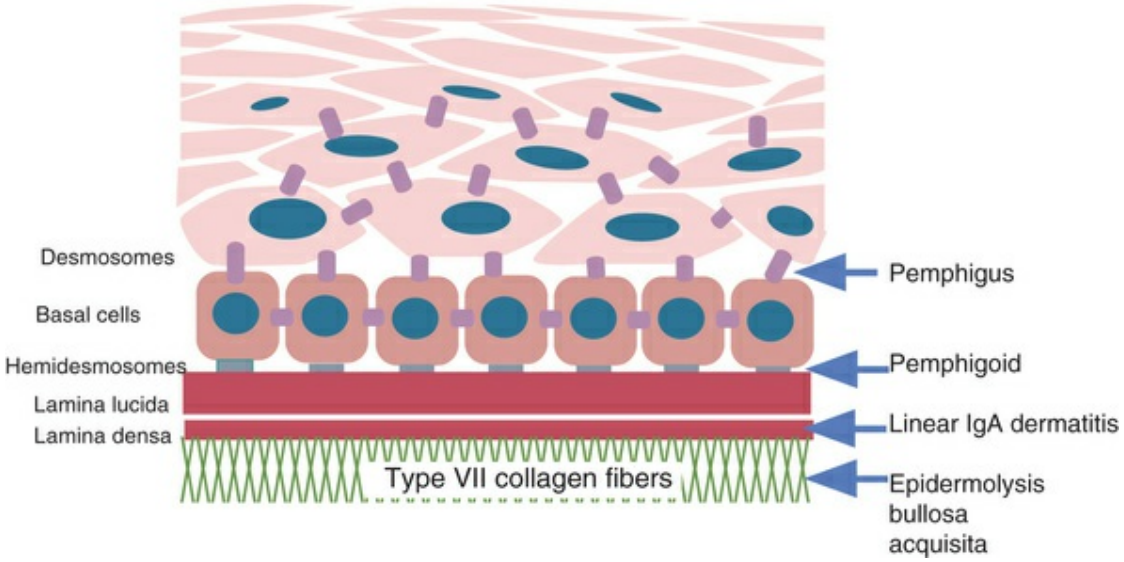


FIG. 37.8 The structures of the skin showing the major features that can act as autoantigens.

Linear Immunoglobulin A Dermatosis

Another group of skin diseases is characterized by the deposition of IgA in the lamina lucida of the skin basement membrane. One such disease, called dermatitis herpetiformis, has been recorded in a Beagle, whereas a linear IgA dermatosis has been recorded in Dachshunds. Both diseases present with pruritic pustular and papular lesions, resembling pyoderma, with eosinophil-filled subepidermal bullae. The target autoantigen has been identified as a processed extracellular form of collagen XVII. The drug dapsone has been recommended as the specific treatment for these diseases.

Epidermolysis Bullosa Acquisita

A generalized skin disease characterized by severe subepidermal blistering and ulcerative lesions has been identified in dogs, especially young Great Danes. The bullae originate from erythematous areas on the skin and rapidly progress to ulcers. Dogs develop generalized urticaria, oral ulceration, and eventually cutaneous sloughing. A localized variant of the disease has been observed in German Shorthaired Pointers. The dermis and epidermis separate, and neutrophils accumulate within the superficial dermis. The neutrophil infiltration may eventually result in microabscess formation. Secondary changes include deep

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ulceration, necrosis, and bacterial infection. Affected animals develop IgA and IgG autoantibodies against the anchoring fibrils of the lower basement membrane (lamina densa). These autoantibodies are specific for type VII collagen and distinctly different from those responsible for bullous pemphigoid. Glucocorticoid therapy may be of benefit, although secondary bacterial infection can cause complications. Another subset of canine subepidermal blistering diseases results from the production of IgG autoantibodies to the basement membrane component, laminin-332. The skin blistering and ulceration in these cases is associated with microscopic subepidermal vesiculation.

Relapsing Polychondritis

Autoimmunity against type II cartilage has been described in humans and in cats. The animals present with bilateral curling of the ears and ocular changes. The cartilage is infiltrated with plasma cells and lymphocytes. A similar proliferative and necrotizing otitis in kittens is associated with $CD3^+$ T cells found in close approximation to apoptotic keratinocytes, suggesting that some form of T cell–mediated cytotoxicity is occurring. Application of topical tacrolimus cream led to resolution of the lesions within a few weeks.

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Autoimmune Nephritis

Horses may develop autoantibodies to glomerular basement membranes resulting in glomerulonephritis and renal failure. Immunofluorescence studies of affected kidneys show that the basement membrane is evenly coated with a smooth, linear deposit of immunoglobulin. The autoantibodies may provoke proliferation of the glomerular epithelial cells and epithelial crescent formation.

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Autoimmune Blood Diseases

Immune-Mediated Hemolytic Anemia

Autoantibodies to red blood cell antigens cause their destruction and result in immune-mediated hemolytic anemia (IMHA). These hemolytic anemias are well recognized in humans and dogs and have been recorded in cattle, horses, cats, mice, rabbits, and raccoons, as well as birds.

Affected dogs are anemic. Thus pallor, weakness, and lethargy are accompanied by fever, icterus, and hepatosplenomegaly. The anemia may be associated with tachycardia, anorexia, vomiting, or diarrhea. Clinical signs are contingent on the speed of development of the disease, its severity, and the mechanism of red cell destruction. This destruction may result from intravascular hemolysis (destruction within the bloodstream) mediated by complement or, much more commonly, by removal of antibody-coated red cells by the macrophages of the spleen and liver (extravascular hemolysis) (Fig. 37.9). In dogs, the disease occurs more often in females. The average age of onset is about 4 to 5 years. There is a genetic predisposition to IMHA in Cocker Spaniels and Miniature Schnauzers. The "causes" of IMHA are unknown, although some cases may be attributable to alterations in red cell surface antigens induced by drugs or viruses. In dogs, the autoantibodies are primarily directed against red cell glycophorins, the cytoskeletal protein spectrin, and the membrane anion exchange protein CD233 (band 3). About one-third of IMHA cases are associated with other immunological abnormalities such as SLE (Chapter 38) or autoimmune thrombocytopenia, or with the presence of lymphoid and other tumors. Its onset may be associated with obvious stress such as vaccination (Chapter 25), anaplasmosis, viral disease, or hormonal imbalances as in pregnancy or pyometra.

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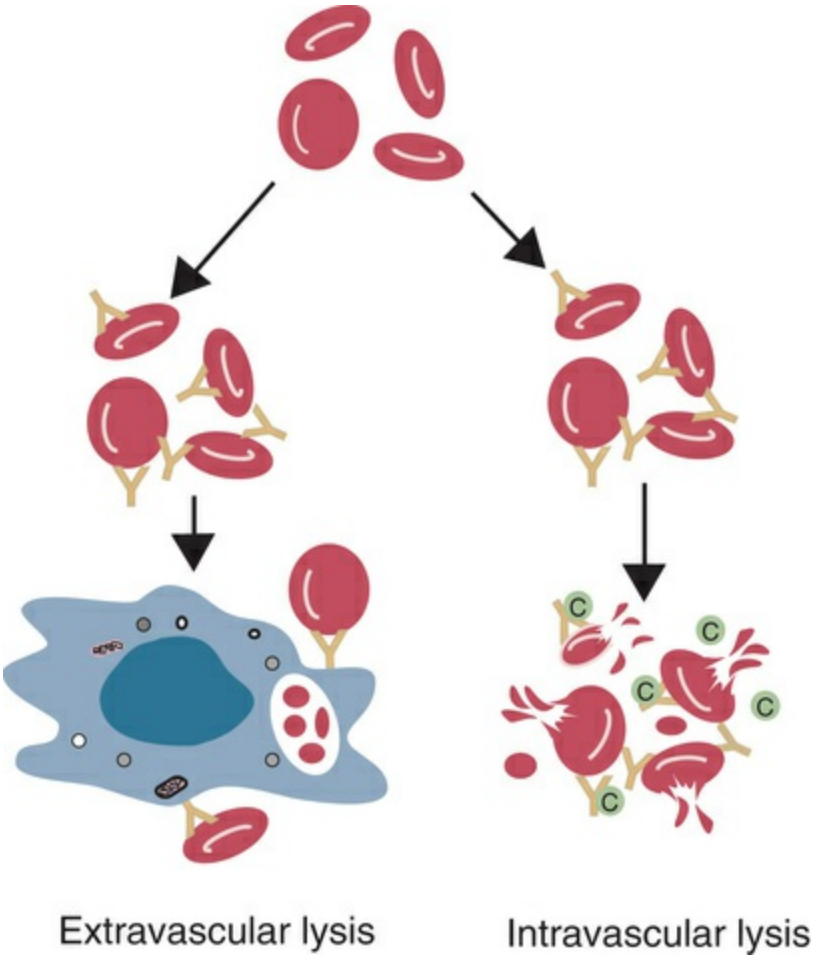


FIG. 37.9 The basic differences between intravascular and extravascular hemolysis.

IMHAs in dogs are classified according to the antibody class involved, the optimal temperature at which the autoantibodies react, and the nature of the hemolytic process (Table 37.1).

TABLE 37.1
Classification of Immune-Mediated Hemolytic Anemias

Class	Predominant Antibody	Activity	Optimal Temperatire (° C)	Site of Red Cell Removal	Clinical Effect
I	G ≫ M	Agglutinin	37	Spleen	Intravascular agglutination
II	M	Hemolysin	37	Liver	Intravascular hemolysis
III	G	Incomplete	37	Spleen	Anemia
IV	M	Agglutinin	4	Liver	Cyanosis and infarction of extremities
V	M	Incomplete	4	Liver	Anemia

Class I: Caused by autoantibodies that agglutinate red cells at body temperature. The agglutination may be seen when a drop of blood is placed on a glass slide. Both IgG and IgM antibodies are

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involved. Since IgG does not activate complement efficiently, the red cells are mainly destroyed by phagocytosis in the spleen. In very severe cases, a blood smear may show erythrophagocytosis by neutrophils and monocytes.

Class II: IgM antibodies activate complement and destroy red cells by intravascular hemolysis. This results in hemoglobinemia, hemoglobinuria, icterus, and very severe anemia. Affected dogs are anemic, weak, and possibly jaundiced. Kupffer cells in the liver or macrophages in lymph nodes preferentially remove red cells with complement on their surface, so these animals develop hepatomegaly and lymphadenopathy.

Class III: Most cases of IMHA in dogs and cats are mediated by IgG1 and IgG4 antibodies, which bind to red cells at 37° C but do not activate complement or agglutinate the red cells. IgG antibodies can only form short bridges (15-25 nm) between cells. As a result, they cannot counteract the zeta potential of the red cells and will not cause direct agglutination. (In contrast, IgM antibodies form long bridges [30-50 nm] and can agglutinate cells despite their zeta potential.) Affected red cells are opsonized and removed by splenic macrophages. Splenomegaly is a consistent feature of class III disease.

Class IV: Some IgM antibodies cannot agglutinate red cells at body temperature but can do so when the blood is chilled. These antibodies are called cold agglutinins. They can be detected by cooling blood to between 10° and 4° C, at which point clumping occurs. The agglutination is reversed on rewarming. As blood circulates through the extremities (tail, toes, ears, and so forth) of affected animals, it may be cooled sufficiently to permit red cell agglutination within capillaries. This can lead to vascular stasis, blockage, tissue ischemia, and, eventually, necrosis. Affected animals may therefore present with necrotic lesions at the extremities, and anemia may not be a significant feature. As might be anticipated, this form of IMHA is most severe in the winter.

Class V: This is mediated by IgM antibodies that bind red cells when

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chilled to 4° C but do not agglutinate them. These antibodies can only be identified by an antiglobulin test conducted in the cold. They do not induce necrosis of extremities but can activate complement leading to intravascular hemolysis.

Hematology reflects the severe anemia and a regenerative response by the bone marrow. Blood smears commonly show spherocytes, which are small round red cells that lack a central pale area. They result from the partial phagocytosis of antibody-coated red cells. The number of spherocytes in blood is a measure of the intensity of red cell destruction.

To diagnose IMHA associated with the presence of non-agglutinating or incomplete antibodies (classes II, III, V), it is necessary to use a direct antiglobulin test (Chapter 42). The red cells of the affected animal are collected in anticoagulant, washed free of serum, and incubated in an antiglobulin serum. The best antiglobulin for this purpose is a polyclonal one with activity against IgM, IgG, and complement. Red cells coated with autoantibody or complement will be cross-linked and agglutinated by the antiglobulin. Occasionally IgM may have a low affinity for the red cells, so it elutes, leaving only complement on their surface.

It is important to emphasize that blood samples for immunological testing should be collected before immunosuppressive therapy begins. It is also important to note that in the cat, most cases of antiglobulin-positive hemolytic anemia are secondary to feline leukemia virus or *Mycoplasma haemofelis (Haemobartonella felis)* infections. The disease in cats has a more favorable prognosis than in dogs. Dogs with IMHA have increased concentrations of C-reactive protein and α-1 acid glycoprotein, and decreased serum albumin. This acute-phase response is not predictive of survival, duration of hospitalization, or number of transfusions required, but it normalizes rapidly with disease stabilization.

Treatment of IMHA involves prevention of further hemolysis, treatment of hypoxia, prevention of thromboembolism, and aggressive supportive care. The major cause of death is thromboembolic disease. Administration of high doses of corticosteroids, specifically prednisolone, reduces

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erythrophagocytosis by mononuclear cells and is the most effective treatment for IgG-mediated disease. Treated animals may respond within 24 to 48 hours. Corticosteroids are of much less benefit in the management of intravascular hemolysis mediated by IgM and complement and do not induce significant immunosuppression in these animals. In such cases, corticosteroid treatment may be supplemented with other agents such as cyclosporine or mycophenolate mofetil. Low-dose aspirin or heparin may reduce the risk of thromboembolism. Splenectomy should only be considered when more conservative therapy has failed and may help cases of refractory class III disease.

Acute immunologically mediated anemias occur in horses following infection with *Streptococcus fecalis,* in sheep following leptospirosis, in cats with mycoplasmosis (hemobartonellosis), in dogs with babesiosis, and in pigs with eperythrozoonosis. In these cases, IgM cold agglutinins clump red cells from normal animals of the same species when chilled. Antibodies to hemoglobin are found in the serum of cattle severely infected with *Arcanobacterium pyogenes,* perhaps as a result of bacterial hemolysis.

IMHA occurs in horses with lymphosarcomas and melanomas. The animals are depressed and pyrexic and exhibit splenomegaly, jaundice, and hemoglobinuria. Some show red cell autoagglutination. These horses have IgG-coated red cells. Dexamethasone treatment can induce remission.

Immune Suppression of Hematopoiesis

In humans, dogs, and cats, autoantibodies to erythroid stem cells may cause red cell aplasia, and autoantibodies to myeloid stem cells may provoke an immune neutropenia. In dogs, red cell aplasia has been associated with an IgG that inhibits erythroid stem cell differentiation. Severe, persistent, immune-mediated neutropenia does occur in dogs. Diagnosis is based largely on excluding other causes of the neutropenia together with a favorable response to steroid and immunosuppressive therapy. These diseases can only be diagnosed by careful hematological analysis and by demonstration of autoantibodies by immunofluorescence on bone marrow smears. These tests are not easy and have not been

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validated in domestic species. Affected animals may benefit from high doses of corticosteroids or immunosuppressive therapy. Immune-mediated bone marrow aplasia is rare in cats and usually only affects erythrocyte progenitors. It has been recorded in a ferret. An immune-mediated, vaccine-induced neonatal pancytopenia does occur in cattle (Chapter 31).

Autoimmune Thrombocytopenia

Autoimmune thrombocytopenia (AITP) due to an immune attack on platelets has been reported in horses, dogs, and, rarely, cats. Affected animals usually present with multiple petechiae in the skin, gingiva, other mucous membranes, and conjunctiva. Epistaxis, melena, and hematuria may occur. The predominant cause of death in these dogs is severe gastrointestinal hemorrhage. Antibodies against platelet antigens cause extravascular destruction of opsonized platelets in the spleen. As a result, affected animals have unusually low platelet counts and a prolonged bleeding time. The disease is commonly observed in association with IMHA and SLE. The thrombocytopenia occurring in animals with multiple myeloma or other lymphoid tumors, with ehrlichiosis and leishmaniasis, or following certain drug treatments may be due to the nonspecific binding of IgG to platelets. (Drug-induced immune-mediated thrombocytopenia in humans is associated with quinine and vancomycin use.) In dogs, the average age of onset is 6 years. Predisposed breeds include Airedales, Dobermans, Old English Sheepdogs, Cocker Spaniels, and Poodles. Antibodies to platelets may be measured by direct immunofluorescence on bone marrow aspirates looking for positive staining on megakaryocytes. However, an alternative test is one that measures the release of factor III from platelets after exposure to autoantibodies. This may be performed by incubating platelet-rich plasma with a globulin fraction of the serum under test and estimating the amount of procoagulant activity released. In about 75% of cases the antibodies are of the IgG class. Most cases of AITP in cats are probably secondary to feline leukemia virus infection.

Immunosuppressive doses of a corticosteroid such as prednisolone are used to treat AITP. Vincristine may also produce a

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good clinical response since it binds to platelets and kills macrophages when they phagocytose the antibody-coated platelets. There is limited experience with other drugs such as cyclosporine, cyclophosphamide, azathioprine, mycophenolate mofetil, or leflunomide, although some positive clinical results have been reported. Splenectomy may help when other forms of therapy have failed. IVIG therapy may also be beneficial.

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Autoimmune Muscle Diseases

Dogs suffer from several different immune-mediated inflammatory muscle diseases.

Myasthenia Gravis

Myasthenia gravis is a disease of skeletal muscle characterized by abnormal fatigue and weakness after mild exercise. It occurs in humans, ferrets, dogs, and cats. Myasthenia gravis results from a failure of transmission of nerve impulses across the motor endplate of striated muscle as a result of a deficiency of acetylcholine receptors (Fig. 37.10). In Jack Russell Terriers, Springer Spaniels, and Fox Terriers, an inherited deficiency of these receptors occurs. This congenital form is therefore a disease of very young dogs.

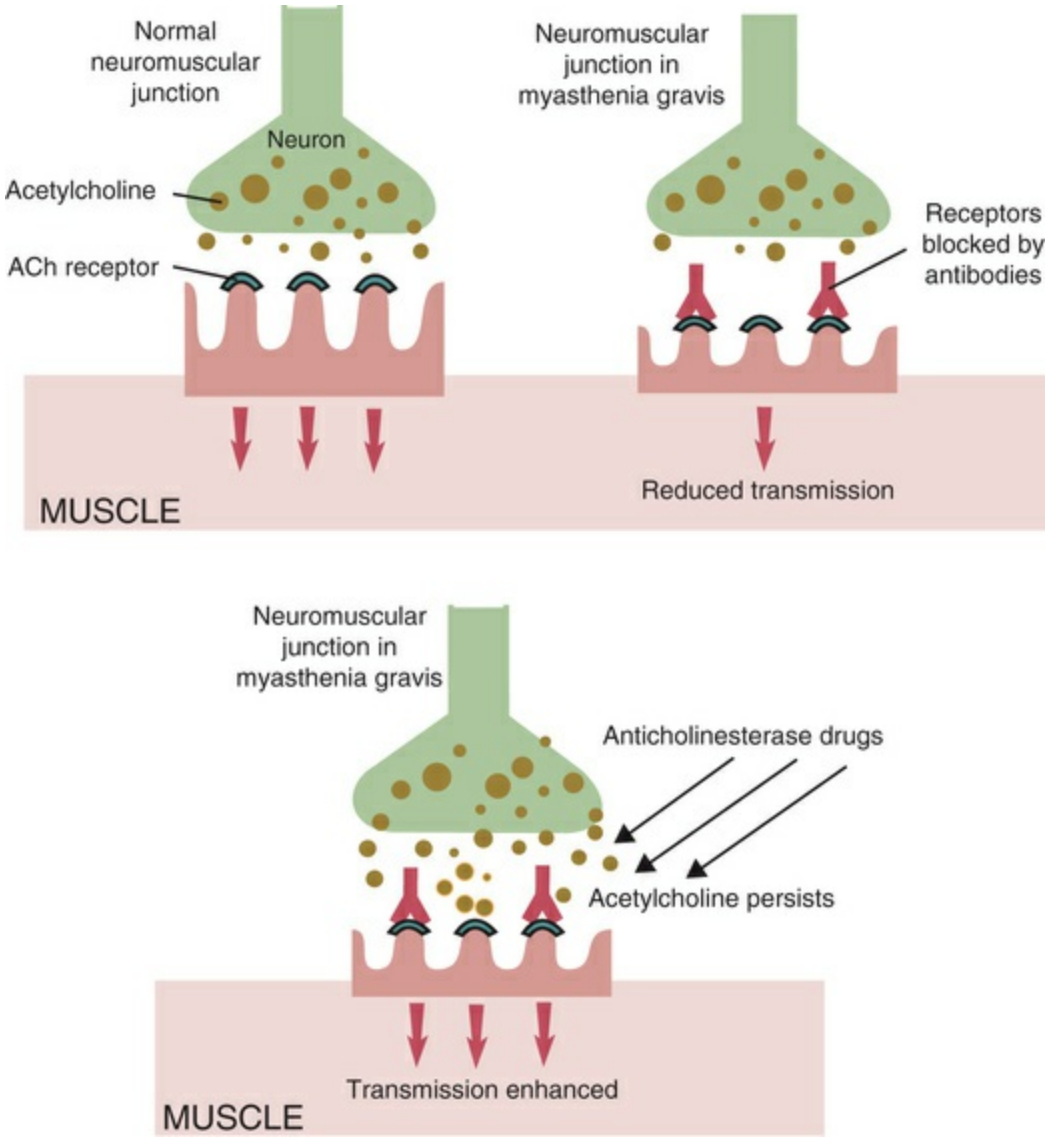


FIG. 37.10 The pathogenesis of myasthenia gravis. Destruction of acetylcholine receptors prevents effective neuromuscular transmission. Blockage of cholinesterase activity by anticholinesterase drugs permits acetylcholine to accumulate and so enhances neuromuscular transmission.

In adult dogs, however, the acetylcholine receptor deficiency is due to autoantibodies. These IgG antibodies accelerate degradation of the receptors, block their acetylcholine-binding sites, and trigger complement-mediated damage. As a result, the number of functional acetylcholine receptors is significantly reduced. Dogs may also make autoantibodies against titin, an intracellular muscle protein, and the ryanodine receptor, a Ca^{2+} release channel in striated muscle.

In normal muscles, the binding of acetylcholine to its receptor opens a sodium channel to produce a localized endplate potential.

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If the amplitude of the endplate potential is sufficient, this will generate an action potential and trigger muscle contraction. The endplate potential from a normal neuromuscular junction is more than sufficient to generate a muscle action potential. In myasthenic junctions, however, the endplate potentials fail to trigger action potentials in many muscle fibers. This is manifested as muscle weakness. Repeating the stimulus leads to a progressive increase in weakness as transmission failure occurs at more and more neuromuscular junctions, since the amount of acetylcholine released from a nerve terminal usually declines after the first few impulses.

The disease may develop in any dog, but certain breeds are predisposed to it. German Shepherds, Golden Retrievers, Labradors, and Dachshunds appear to develop more severe disease. Rottweilers appear to be at low risk. In cats, there is a predisposition in Abyssinians and related Somalis.

In some animals, the thymus may show medullary hyperplasia, germinal center formation, or even a thymic carcinoma, and surgical thymectomy may lead to clinical improvement. About 3% of dog cases and up to 52% of cat cases are associated with the presence of a mediastinal mass, most frequently a thymoma.

Animals may present with a history of difficulty swallowing, regurgitation, labored breathing, and generalized muscle weakness. Megaesophagus is common. Clinically different diseases may be recognized. Thus focal myasthenia gravis is characterized by megaesophagus and facial paralysis without limb muscle weakness; generalized myasthenia gravis cases develop limb muscle weakness associated with facial paralysis and megaesophagus; and acute fulminating myasthenia gravis develops when the disease rapidly leads to quadriplegia and respiratory difficulty. Almost 60% of cases are generalized or fulminating. Without treatment, about half of affected animals die, whereas the others may show spontaneous remissions. Aspiration pneumonia is the main cause of death in myasthenic dogs. Administration of a short-acting anticholinesterase drug such as edrophonium chloride (Tensilon) leads to a rapid gain in muscle strength. The anticholinesterase, by permitting the acetylcholine to accumulate at the neuromuscular junction, enables the remaining receptors to be stimulated more

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effectively. Dogs with transient myasthenia gravis may be supported temporarily with long-acting anticholinesterase drugs such as pyridostigmine bromide or neostigmine methyl sulfate. Dogs with progressive disease that show no signs of remission may benefit from immunosuppression. Positive clinical responses have been reported in dogs treated with prednisone or azathioprine or both. However, corticosteroid treatment may result in transient exacerbation of symptoms. Plasmapheresis has been used for short-term therapy to stabilize patients before thymectomy.

Polymyositis

A generalized autoimmune myositis occurs in large dogs such as German Shepherds, Boxers, and Retrievers. Hungarian Vizslas suffer from a breed-specific inflammatory myopathy. In all these cases, the animals show progressive muscle weakness not associated with exercise intolerance. Changes in laryngeal muscle function lead to a change in the voice. Megaesophagus may lead to dysphagia and if severe can result in aspiration pneumonia. Affected animals may develop a shifting lameness. Animals may be febrile and develop leukocytosis and eosinophilia. Biopsies show muscle fiber degeneration, necrosis, and vacuolation, and affected muscles may be infiltrated by $CD8^+$ lymphocytes and plasma cells. About 50% of affected dogs have antinuclear antibodies or antibodies to sarcolemma, or both. Corticosteroids are the treatment of choice.

A similar immune-mediated myositis has been recorded in Quarter Horses and related breeds. It causes rapid atrophy of the gluteal and epaxial muscles. The affected muscles are infiltrated with macrophages and $CD4^+$ T cells with lesser numbers of $CD8^+$ T cells and B cells. It too may be treatable with corticosteroids. MHC class I and II are expressed on the sarcolemma of some myofibers of affected horses but not in normal horses.

Autoimmune Masticatory Myositis

Dogs may develop a focal myositis confined to the muscles of mastication. The major autoantigen is masticatory myosin-binding

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protein-C found only in masticatory muscle fibers. Animals present with pain and atrophy or swelling of the masticatory muscles resulting in difficulty in opening (trismus) or closing the jaw. Affected animals may also develop conjunctivitis or exophthalmos. Histology of affected muscles shows inflammatory or degenerative lesions affecting the M2 myofibrils. Myositis with lymphocytes and plasma cells predominates, and some lesions may contain many eosinophils. Myofiber atrophy, perimysial or endomysial fibrosis, and muscle fiber necrosis are consistent features. Immunoglobulins may be detected in biopsy specimens of affected muscles, and circulating antibodies to the M2 myofibrils have been demonstrated by an immunoperoxidase assay. Corticosteroids such as prednisone are used for treatment, but the prognosis is guarded. Cavalier King Charles Spaniels may be predisposed to this disease.

Canine Cardiomyopathy

English Cocker Spaniels may develop a cardiomyopathy with antinuclear and antimitochondrial autoantibodies and reduced serum IgA levels. It is associated with a specific C4 allotype (C4-4). The autoantigen has not been identified, but in humans similar cardiomyopathies are due to autoantibodies directed against the adenine nucleotide translocator of mitochondria.

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Dermatomyositis

A familial disease of dogs that resembles dermatomyositis in humans has been described in Collies and Shetland Sheepdogs. It is a complement-mediated microangiopathy in which complement-mediated vascular damage leads to muscle ischemia. The disease is inherited as an autosomal dominant involving a locus on chromosome 35, although expression is highly variable. A similar dermatomyositis-like disease has been described in other breeds such as Pembroke Welsh Corgi, Lakeland Terrier, Chow, Jack Russell Terrier, German Shepherd, and Rottweilers. Dogs develop dermatitis with a less obvious myositis. Puppies appear normal at birth, but skin lesions develop between 7 and 11 weeks of age, and myositis develops between 12 and 23 weeks. In other studies, the dermatitis developed at 3 to 6 months of age, and myositis was detected after the dermatitis was investigated. The dermatitis first develops on the face; subsequently, lesions may spread to the limbs and trunk, especially over bony prominences. These early lesions are erythematous and eventually lead to vesicle and pustule formation. There is diffuse hair follicle atrophy and keratinocyte degeneration that can lead to ulceration (Fig. 37.11). Once the vesicles rupture, they ulcerate and crust. Lesions may be found on the bridge of the nose and around the eyes and show hair loss and changes in pigmentation. There may be local lymph node enlargement. The clinical course and severity are variable, but skin lesions usually resolve by 1 year of age.

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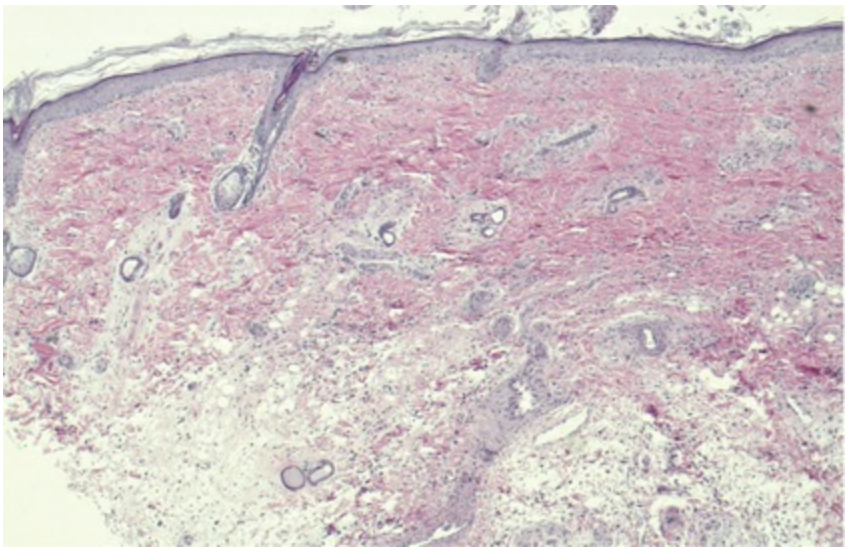


FIG. 37.11 Skin lesion in a case of dermatomyositis. Note the diffuse ischemic hair follicle atrophy and multifocal basal keratinocyte degradation in the epidermis. (Courtesy Dr. J. Mansell.)

Muscle disease follows the onset of skin disease, but there is poor correlation between the severity of the two lesions. The most common sign of myositis is masseter and temporal muscle atrophy. Some severely affected puppies may have difficulty eating as a result of the myositis and thus grow poorly. If the muscles of the esophagus are affected, megaesophagus may develop, and secondary aspiration pneumonia results. Generalized lymphoid hyperplasia may also occur in these dogs. Many dogs outgrow the disease and are left with moderate hyperpigmentation, some hypopigmentation and alopecia, and atrophy of the muscles of mastication. Other dogs develop a progressive disease with severe dermatitis and myositis. Dogs with progressive disease may also develop signs of immunodeficiency, especially pyoderma and septicemia, as well as demodicosis. On necropsy, myositis may be seen in the esophagus and arteritis in the skin, muscle, and bladder.

The key autoimmune target in dermatomyositis is capillary endothelium. The skin and muscle lesions are a result of ischemic vasculopathy. Thus there are swollen endothelial cells, vacuolization, capillary necrosis, inflammation, and ischemia. The onset and progression of the disease correlate with a rise in circulating immune complexes and serum IgG, but the reason for these increases is unclear. Immune complexes and IgG levels return to normal as the disease resolves, suggesting a causal association. Muscle biopsy, especially of the temporal muscle, shows multifocal

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accumulations of lymphocytes, plasma cells, and macrophages, as well as a few neutrophils and eosinophils. The myofibers are atrophied and may show fragmentation and vacuolation. Symptomatic and corticosteroid treatment may be of benefit in severely affected cases.

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Chronic Active Hepatitis

Doberman Pinschers may develop an autoimmune hepatitis. The symptoms are typical of liver disease with anorexia, depression, weight loss, diarrhea, polydipsia, polyuria, icterus, and eventually ascites. The disease commonly presents between 3 and 6 years of age but may have been present subclinically for many years. On necropsy, there is intense inflammation and scar tissue formation around small hepatic veins in the liver. The lesions contain lymphocytes, plasma cells, and macrophages. The disease eventually causes progressive fibrosis and destruction of hepatocytes. About half of affected dogs develop antibodies to hepatocyte cell membranes. These antibody-positive dogs have more severe disease than dogs without antibodies. In addition, lymphocytes from about 75% of affected dogs respond to liver membrane proteins in vitro. Hepatocytes from affected dogs, but not from normal dogs, express MHC class II antigens. This MHC expression correlates with the severity of the disease, whereas corticosteroid treatment reduced both MHC expression and disease severity. It has been suggested therefore that the disease results from a cell-mediated attack on abnormally expressed MHC molecules or an antigen associated with them.

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38

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Immune-Mediated Inflammatory Diseases

CHAPTER OUTLINE

Systemic Lupus Erythematosus, 423
Pathogenesis, 424
Equine Lupus, 426
Canine Lupus, 426
Feline Lupus, 427
Diagnosis, 427
Treatment, 427
Discoid Lupus Erythematosus, 428
Sjögren's Syndrome, 428
Keratoconjunctivitis Sicca, 428
Chronic Superficial Keratitis, 429
Autoimmune Polyarthritis, 429
Erosive Polyarthritis, 429
Rheumatoid Arthritis, 429
Nonerosive Polyarthritis, 432
Equine Polyarthritis/Polysynovitis, 432
Canine Polyarthritis, 432

Lupus Polyarthritis, 432
Polyarthritis With Polymyositis, 432
Idiopathic Polyarthritis, 432
Feline Polyarthritis, 433
Cruciate Ligament Rupture, 433
Immune Vasculitis, 433

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Explain how some inflammatory diseases may result from immune attack on many different organs at the same time.
- Describe the probable pathogenesis of systemic lupus erythematosus (SLE).
- Explain how many different forms of arthritis are immunologically mediated.
- Describe the pathogenesis of rheumatoid arthritis.
- Define rheumatoid factor (RF) and antinuclear antibodies.
- List the diagnostic criteria for systemic lupus erythematosus.
- List the diagnostic criteria for rheumatoid arthritis.
- Describe the classification of the immune-mediated arthritides.
- List the appropriate treatments for systemic lupus and rheumatoid arthritis.

Animals suffer from inflammatory diseases involving multiple organ systems. In human medicine, these have been called "rheumatic" diseases, "connective tissue" diseases, or "collagen" diseases based on outdated views on their pathogenesis. These diseases or syndromes are interrelated and have many overlapping clinical features (Fig. 38.1). One common feature is extensive and uncontrolled inflammation, and it may be useful to consider them to be caused by innate autoimmunity or "autoinflammatory diseases." Because of their similarities, it is sometimes difficult to

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come to a specific clinical diagnosis.

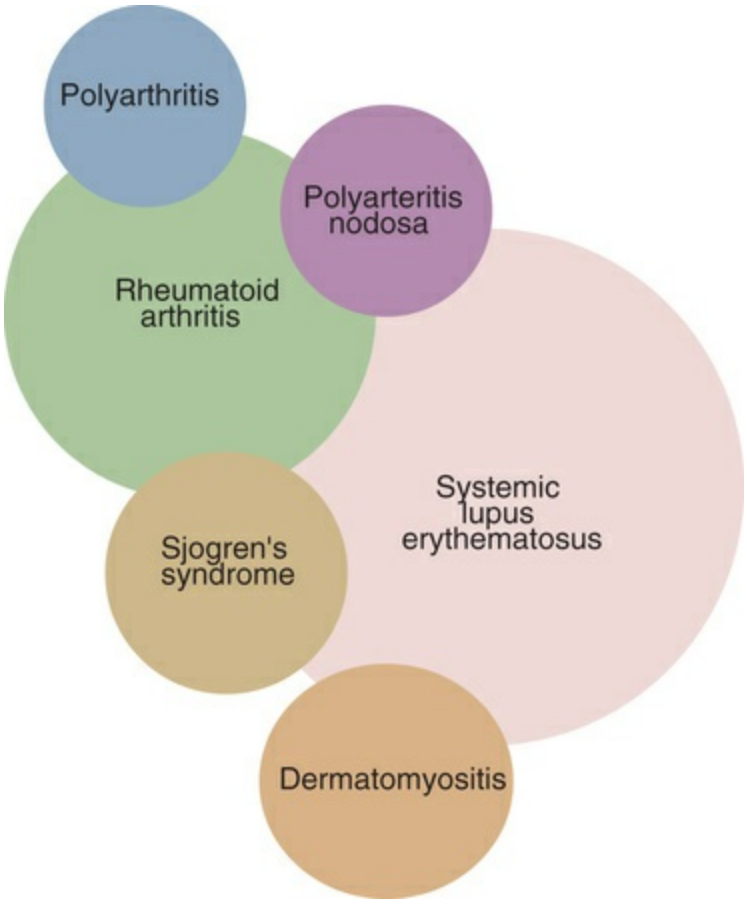


FIG. 38.1 The interrelationships among the diseases discussed in this chapter. The diagram is somewhat simplified since polyarthritis may be associated with polymyositis.

These inflammatory diseases include systemic lupus erythematosus (SLE), rheumatoid arthritis, nonerosive forms of arthritis, vasculitis, and Sjögren's syndrome. Although all these diseases have some form of autoimmune component, they do not simply result from autoantibodies causing tissue destruction. Most are associated with the presence of immune complexes and complement in tissues resulting in chronic inflammation. Many result from uncontrolled inflammatory cytokine production or abnormalities in the complement system. Their initiating factors are unknown, but some may be triggered by infectious agents acting through toll-like receptors (TLRs). All exhibit a significant genetic predisposition, commonly with linkage to the MHC.

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Systemic Lupus Erythematosus

SLE is a complex disease syndrome (or perhaps even multiple diseases) that has been described in humans, other primates, mice, horses, dogs, and cats. It is characterized by a broad and bewildering diversity of different symptoms and a wide variety of disease courses as symptoms flare and recede over time. The factors that cause lupus are complex and poorly defined (Fig. 38.2). Its development is affected by environmental factors, including infectious agents, hormones, drugs, food, and the intestinal microbiota in association with the effects of many different genes. Patients develop multiple autoantibodies, changes in T cell function, defective phagocytosis, impaired apoptosis, multiorgan inflammation, and oncogene expression.

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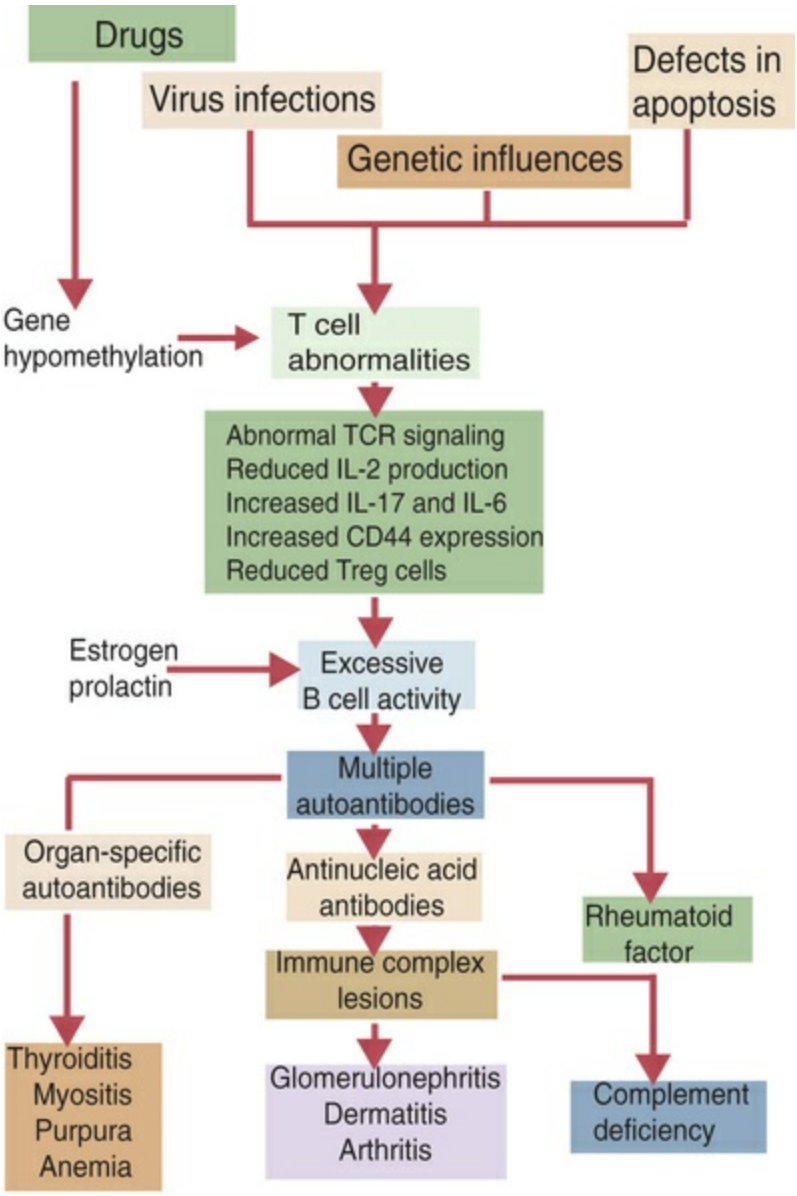


FIG. 38.2 A diagram showing a possible pathogenesis of systemic lupus erythematosus.

Pathogenesis

Genetic factors.

Lupus development in humans can be caused by deficiencies of the complement components C1q or C4. Genes associated with dendritic cell functions, immune complex processing, T cell functions and signaling, apoptosis, and B cell functions have all been implicated in influencing lupus risk. Lupus in humans is predominantly a disease of women.

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T cell abnormalities.

T cell antigen receptor functions are altered in lupus. The TCR-CD3 complex, instead of signaling through CD3ζ as in normal cells, signals through the common γ chain of the FcR. As a result, interleukin-2 (IL-2) and effector T cell function are reduced, leading to immunosuppression. There is also evidence for increased IL-17 production in some lupus patients.

B cell abnormalities.

B cells are central to the pathogenesis of lupus since they are the source of the autoantibodies responsible for its lesions. The hallmark of all forms of lupus is the development of autoantibodies against nuclear components, including nucleic acids, histones, ribonucleoproteins, and chromatin. These antinuclear antibodies (ANAs) are found in 97% to 100% of dogs with lupus compared with 16% to 20% of normal control animals. About 16 different nuclear antigens have been described in humans. Dogs mainly develop autoantibodies against histones and ribonucleoproteins. The nucleic acids that provoke ANA production probably come from three major sources: invading bacteria, neutrophil NETs, and apoptotic cells.

Since mammalian and bacterial DNA have a conserved backbone, it is possible that animals with lupus may respond to bacterial infection by producing antibodies that cross-react with their own DNA. For example, the NZB/NZW mouse strain develops a lupus-like syndrome when immunized with bacterial DNA. This induces anti-DNA antibodies that form immune complexes and cause arthritis, skin rashes, and vascular disease.

When neutrophils expel DNA nets to capture bacteria, they release their nuclear contents. The components of these nets are potential autoantigens and may trigger autoantibody formation. Free DNA released by bacteria or mitochondria or as a result of NETosis can bind to TLR7 and 9 or IL-26, and so trigger innate responses. Antibody-DNA immune complexes may also bind to TLR9 and activate B cells by triggering both their TLRs and Fc receptors. FcRγ and TLR-mediated uptake of immune complexes and nucleic acids activates plasmacytoid dendritic cells and triggers IFN-α production. It is no coincidence that some TLRs such as TLR7

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and TLR8 are more active in females than males. The immune responses in lupus are closely associated with IFN-α production, and the level of this cytokine correlates with disease activity. IFN-α also promotes inflammation by activating macrophages and autoreactive T cells, and many of the immunologic or pathogenic features of lupus are driven by IFN-α. The production of ANAs in lupus may also result if TLR7 and TLR9 lose the ability to discriminate between microbial and self-DNA. If, at the same time, some of their B cells undergo somatic mutation that enables their BCRs to bind self-DNA, the ingredients necessary for a profound antibody response to mammalian DNA come together.

Antinuclear antibodies can bind soluble nuclear antigens to form immune complexes that are deposited in glomeruli, resulting in development of a membranoproliferative glomerulonephritis (MPGN) (Chapter 32). These immune complexes can also activate neutrophils, causing them to release even more DNA and nucleoproteins through NETosis. The immune complexes may also be deposited in arteriolar walls, where they cause fibrinoid necrosis and fibrosis, or in synovia, where they provoke arthritis.

ANAs bind to the nuclei of degenerating cells to produce round or oval structures called hematoxylin bodies in the skin, kidney, lung, lymph nodes, spleen, and heart. Within the bone marrow, opsonized nuclei may be phagocytosed, giving rise to lupus erythematosus (LE) cells (Fig. 38.3).

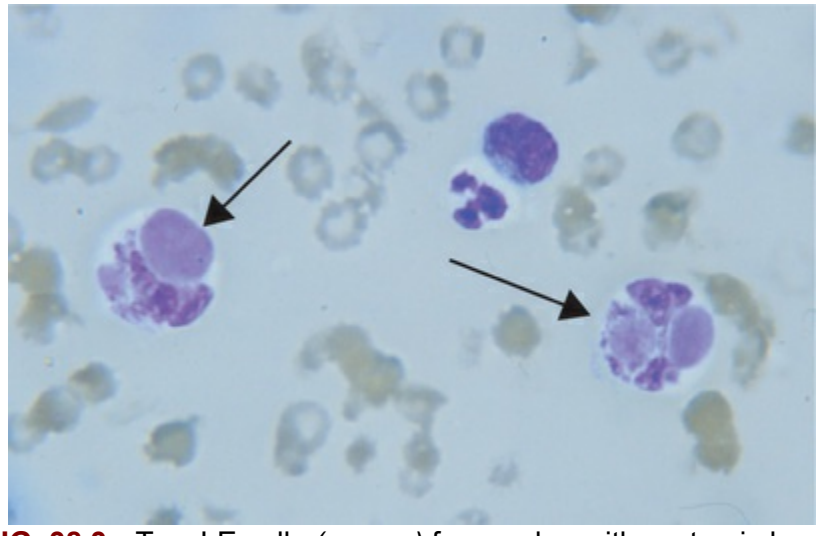


FIG. 38.3 Two LE cells *(arrows)* from a dog with systemic lupus

erythematosus. Original magnification ×1300.

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Impaired apoptosis.

Although failure of apoptosis leading to activation of autoimmune B cells and multiple autoimmune disorders is a feature of lupus, its initiating cause remains obscure. Normally, apoptotic cells are removed by macrophages without causing inflammation (Chapter 18). Macrophages from lupus patients, however, show defective phagocytosis of apoptotic cells, which thus accumulate in tissues. Apoptotic blebs or NETs from these cells may be trapped and processed by dendritic cells, thereby triggering autoantibody formation. The defect in apoptosis is most obvious in the skin of affected animals, where ultraviolet radiation damages cells and so triggers apoptotic cell death. In humans, lupus skin lesions are commonly restricted to the bridge of the nose and the area around the eyes since apoptosis is triggered by UV radiation in sunlight. Nucleic acids from these cells may activate dendritic cells, then act as autoantigens and trigger immune responses to chromatin. Autoantibodies generate immune complexes and thus tissue damage. Complement components mediate the efficient clearance of apoptotic cells, so complement deficiencies, especially C1q or C4 deficiency, are also associated with the development of lupus-like syndromes. As described in Chapter 4, some lupus patients have a deficiency of the complement receptor CD35. As a result, immune complexes are not bound to red cells or platelets and therefore are not removed from the circulation. These immune complexes may then be deposited in the glomeruli or in joints resulting in MPGN and arthritis.

Multiple autoantibodies.

Although ANAs are characteristic of lupus, many other autoantibodies are produced in these animals, suggesting grossly abnormal B cell function. Autoantibodies to red cells induce a hemolytic anemia. Antibodies to platelets induce a thrombocytopenia. Antilymphocyte antibodies may interfere with immune regulation. About 20% of dogs with lupus produce antibodies to IgG (rheumatoid factors). Antimuscle antibodies may

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cause myositis, and anti-myocardial antibodies may provoke myocarditis or endocarditis. Antibodies to skin basement membrane cause a dermatitis characterized by changes in epidermal thickness, focal mononuclear cell infiltration, collagen degeneration, and immunoglobulin deposits at the dermoepidermal junction. These deposits form a "lupus band," seen in many other autoimmune skin diseases in addition to lupus (Fig. 38.4). The results of this excessive immune reactivity are also reflected in a polyclonal gammopathy, enlargement of lymph nodes and spleen, and thymic enlargement with germinal center formation.

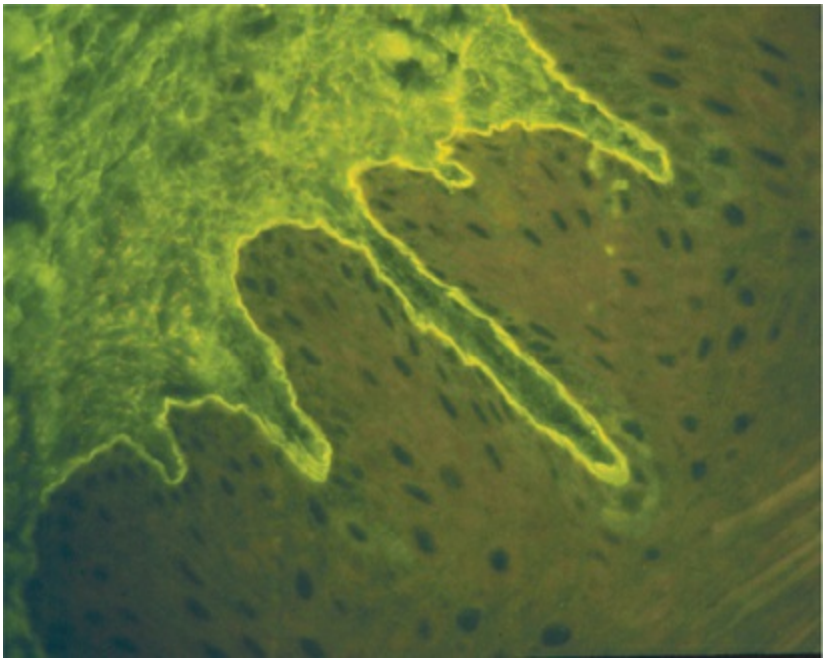


FIG. 38.4 A lupus band in a section of monkey esophagus. The indirect immunofluorescence assay shows IgG deposition on the skin basement membrane. (Courtesy Dr. F.C. Heck.)

Affected animals show abnormalities in B cell signaling and migration, overexpression of CD154 (CD40L), and enhanced production of IL-6 and IL-10. Some experimental mouse models show overexpression of B cell stimulatory molecules by T cells and dendritic cells. It is therefore possible that the production of multiple autoantibodies in lupus is a combined result of defective apoptosis, overstimulation of B cells, and a failure to eliminate self-reactive B cells. The diversity of autoantibodies in lupus can cause

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an equally great variety of clinical symptoms. Polyarthritis, fever, proteinuria, anemia, and skin diseases are the most common abnormalities, but pericarditis, myocarditis, myositis, lymphadenopathy, and pneumonia have also been reported.

Equine Lupus

Lupus presents as a generalized skin disease in the horse (alopecia, dermal ulceration, and crusting), usually accompanied by an antiglobulin-positive anemia. The disease is remarkable insofar as affected horses may be almost totally hairless (Fig. 38.5). Affected horses are ANA positive, although LE cell tests are equivocal in this species. Skin biopsies show basement membrane degeneration and immunoglobulin deposition typical of lupus. Affected horses may also have glomerulonephritis, synovitis, and lymphadenopathy. Treatment of reported cases has been unsuccessful.



FIG. 38.5 A filly with systemic lupus erythematosus. Note the generalized alopecia and crusting. (From Geor RJ, Clark EG, Haines DM, Napier PG: Systemic lupus erythematosus in a filly, *J Am Vet Med Assoc* 197:1489, 1990.)

Canine Lupus

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Lupus affects middle-aged dogs (between 2 and 12 years of age) and affects males more than females. The disease is commonly seen in Collies, German Shepherds, Nova Scotia Duck Tolling Retrievers, and Shetland Sheepdogs, but Beagles, Irish Setters, Poodles, and Afghan Hounds are also affected. Lupus (or positive lupus serology) may occur in related animals, supporting the importance of genetic factors. For example, dogs possessing the MHC class I antigen DLA-A7 are at increased risk and those possessing DLA-A1 and -B5 are at decreased risk for developing disease (Fig. 38.6). When dogs with lupus are bred, the number of affected offspring is higher than can be accounted for genetically, suggesting that the disease may be vertically transmitted. A type C retrovirus has been suggested as a potential trigger of lupus.

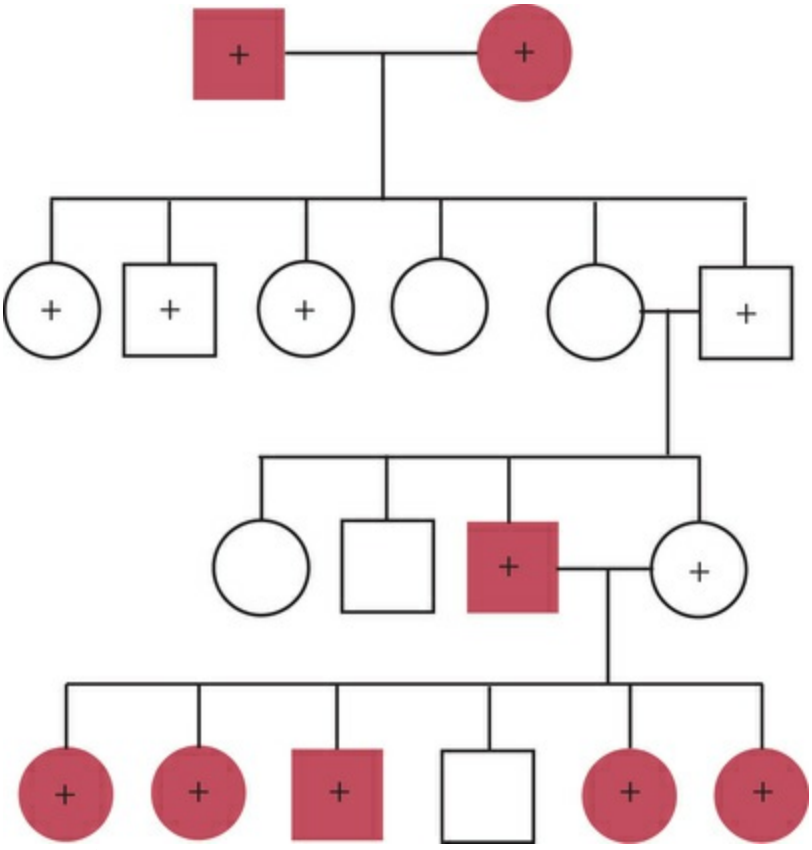


FIG. 38.6 The inheritance of canine systemic lupus erythematosus. This diagram shows four generations of a single family of dogs. Colored squares (male) or circles (female) denote those animals exhibiting clinical signs of systemic lupus; "+" denotes animals positive for antinuclear antibodies. (From Teichner M, Krumbacher K, Doxiadis I, et al: Systemic lupus erythematosus in dogs: association to the major histocompatibility complex class I antigen DLA-A7, *Clin Immunol*

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Dogs may present with one or more signs of disease. However, the disease is progressive, so the severity of the lesions and the number of organ systems involved gradually increases in untreated cases. The most characteristic presentation is a fever accompanied by a symmetrical, nonerosive polyarthritis. Indeed, as many as 90% of dogs with lupus may develop arthritis at some stage. Other common presenting signs include renal failure (65%), skin disease (60%), lymphadenopathy or splenomegaly (50%), leukopenia (20%), hemolytic anemia (13%), and thrombocytopenia (4%). Dogs may also show myositis (8%) or pericarditis (8%) and neurological abnormalities (1.6%). The leukopenia involves a major loss of $CD8^+$ T cells with a somewhat smaller loss of $CD4^+$ T cells so that the CD4/CD8 ratio may climb as high as six, compared with a normal value of about 1.7. The skin lesions are highly variable but are commonly restricted to areas exposed to sunlight. With this great variety of clinical presentations to choose from, it is not surprising that lupus is difficult to diagnose.

Several unique variants of lupus have been described in dogs. All are very rare, and many are associated with specific breeds, strongly suggesting a genetic predisposition. For example, vesicular systemic lupus is seen in Shetland Sheepdogs and Rough Collies. It is characterized by vesicular erosive and ulcerative skin lesions, subepidermal vesicles, and immunoglobulin deposition at the dermal-epidermal junction. Affected animals have antibodies against type VII collagen as well as ANAs.

Exfoliative lupus dermatitis has been described in German Shorthaired Pointers. Young adult dogs develop scaling and alopecia on the muzzle, pinnae, and dorsum. Some dogs may exhibit signs of pain and arthritis. Others may develop anemia and thrombocytopenia. Skin histology shows hyperkeratosis with a lymphocytic interface dermatitis similar to that seen in human lupus. IgG is deposited in the epidermal and follicular basement membranes. These dogs have circulating autoantibodies to epidermal basement membranes. Affected animals respond poorly to immunosuppressive therapy. This disease is inherited in an autosomal recessive manner.

Another lupus-related disease has been described in Gordon

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Setters. These dogs developed a symmetrical onychodystrophy, malformations, and loss of the claws. As a result, affected animals show lameness, severe discomfort, and acute pain. Some develop ANAs. A possibly related disease of Gordon Setters is black hair follicular dysplasia. In this disease, dogs begin to shed their black hair without normal regrowth. The remaining black hair is either short and stiff or thin and easily removed. Many affected dogs have positive ANA titers. These two diseases, occurring in the same breed and often in the same individual, may be closely related.

Mucocutaneous lupus is a form associated with skin lesion development at mucocutaneous junctions such as the perigenital and perianal areas, with less involvement at perioral and periocular sites. A chronic cutaneous form of lupus with bilaterally symmetrical alopecia and hyperpigmentation has been described in a Doberman.

Feline Lupus

Lupus is uncommon in cats, in which it usually presents as an antiglobulin-positive anemia. Other clinical manifestations include fever, skin disease, thrombocytopenia, polyarthritis, and renal failure. The ANA test must be interpreted with care in cats since many normal cats are ANA positive.

Diagnosis

A simple diagnostic rule for lupus could be stated as follows: Suspect lupus in an animal with multiple disorders such as those described previously and either a positive test for ANA or a positive test for LE cells (Box 38.1).

Box 38.1

Diagnostic Criteria for Systemic Lupus Erythematosus

Any two of the following must be present:

Characteristic skin lesions

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Polyarthritis

Antiglobulin-positive hemolytic anemia

Thrombocytopenia

Proteinuria

And either:

A positive ANA test

Or:

A positive LE cell test

ANAs are normally demonstrated by immunofluorescence. Cultured cells or frozen sections of mouse or rat liver on a microscope slide are used as a source of antigen. Dilutions of a patient's serum are applied to this, and the slide is incubated and then washed off. Binding of ANA to the cell nuclei is revealed by incubating the tissue in a fluorescein-labeled antiglobulin to canine or feline immunoglobulins and then rewashing. Several different nuclear staining patterns have been described for humans and their clinical correlations identified. In animals, staining patterns have been less thoroughly investigated, and their significance is less clear. A homogeneous staining pattern or staining of the nuclear rim is of greatest diagnostic significance but nucleolar fluorescence is not (Fig. 38.7). Dogs with a speckled fluorescence pattern tend to have autoimmune diseases other than lupus. Some normal dogs, dogs undergoing treatment with certain drugs (griseofulvin, penicillin, sulfonamides, tetracyclines, phenytoin, procainamide), and some dogs with liver disease or lymphosarcoma may have detectable ANAs. ANAs are also found in dogs infected with *Bartonella vinsonii* subsp. *berkhoffii, Ehrlichia canis,* and *Leishmania infantum.* Dogs infected with multiple vector-borne organisms are especially likely to be ANA positive. Thus non-specific ANAs may be a result of many different neoplastic, inflammatory, and autoimmune diseases. ANA test results must therefore be used

with caution. Administration of propylthiouracil to cats with hyperthyroidism may result in the development of a syndrome resembling lupus. This may include the development of an antiglobulin-positive anemia as well as positive ANA reactions.

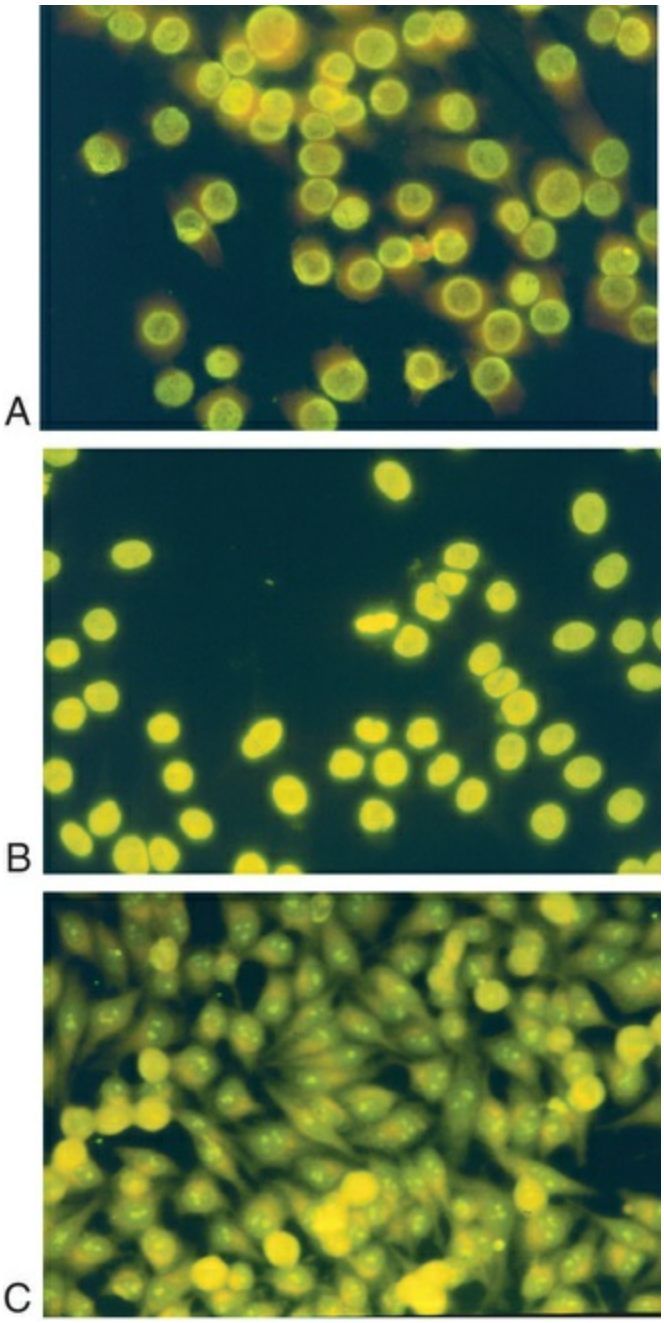


FIG. 38.7 Three positive ANA reactions. These are indirect fluorescent antibody reactions in which dog serum under test is layered onto a cell culture. After washing, the bound antibody is detected using a fluorescent antiglobulin. Although "rim" fluorescence **(A)** has traditionally been considered a positive reaction, the staining pattern obtained appears to depend in large

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part on the way the cells are fixed. These can therefore show diffuse staining **(B)** or nucleolar fluorescence **(C).** (Courtesy Dr. F.C. Heck.)

LE cells, as previously mentioned, are neutrophils that have phagocytosed nuclear material from apoptotic cells (see Fig. 38.3). Their presence may be detected in the bone marrow and occasionally in buffy coat preparations from animals with lupus. It is usually necessary, however, to produce them *in vitro.* This can be accomplished by allowing the blood of an affected animal to clot and then incubating it at 37° C for 2 hours. During this time, normal neutrophils will phagocytose the nuclei of any apoptotic cells. Pressing it through a fine mesh then disrupts the clot, the resulting cell suspension is centrifuged, and the buffy coat is smeared, stained, and examined. LE cells are not a reliable diagnostic feature of systemic lupus in domestic animals since there is a high prevalence of both false-positive and false-negative results.

Treatment

Lupus in animals usually responds well to high doses of corticosteroids (prednisolone or prednisone), accompanied, if necessary, by cyclophosphamide, azathioprine, or chlorambucil. Levamisole (Chapter 41) has also been used with success. However, more drastic measures, such as plasmapheresis, may be needed in refractory cases.

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Discoid Lupus Erythematosus

Discoid lupus erythematosus is a variant of SLE that occurs in two forms. The commonest form is characterized by the occurrence of facial skin lesions alone. A rarer form is generalized discoid lupus erythematosus. There are no other pathological lesions, and ANA and LE tests are negative or weakly positive. It occurs in dogs, cats, horses, and humans. Facial discoid lupus has been described in Collies and Collie crosses, German Shepherds, Siberian Huskies, and Shetland Sheepdogs. They commonly present with nasal dermatitis with depigmentation, erythema, erosion, ulceration, scaling, and crusting. A vesicular form of the disease has been reported in Shetland Sheepdogs. Occasionally the feet may be affected, and some dogs may have oral ulcers. C3, IgA, IgG, or IgM may be detected in the skin basement membrane in a typical lupus band. The skin lesions may be infiltrated with mononuclear and plasma cells. It is treated with corticosteroids, and the prognosis is good. Since the lesions are exacerbated by sunlight, it is appropriate to use sunscreens and encourage the owner to keep the animal out of intense sunlight.

Discoid lupus in cats is characterized by a nonpruritic scaling and crusting dermatitis almost totally confined to the pinnae of the ear. There may be some ulceration and papule or pustule formation. Skin biopsy shows mononuclear infiltration of the basal cell layer with degeneration of basal cells. Direct immunofluorescence of skin sections shows a lupus band. Affected cats have negative or low ANA titers and negative LE cell tests. Treatment with corticosteroids is effective.

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Sjögren's Syndrome

In this syndrome, autoimmune attack on salivary and lacrimal glands leads to conjunctival dryness (keratoconjunctivitis sicca) and mouth dryness (xerostomia). Affected animals subsequently develop gingivitis, dental caries, and excessive thirst. Sjögren's syndrome is often associated with rheumatoid arthritis, systemic lupus, polymyositis, and autoimmune thyroiditis. Affected dogs develop antibodies to nictitating membrane epithelial cells and, less consistently, to lacrimal and salivary glands or to the pancreas, and these organs may be infiltrated with lymphocytes and other mononuclear cells. Most affected animals (90%) are hypergammaglobulinemic and have ANAs (40%) and RFs (34%). Many have other autoimmune lesions such as polyarthritis, hypothyroidism, and glomerulonephritis.

Keratoconjunctivitis Sicca

In keratoconjunctivitis sicca, one of the most common ophthalmic diseases of dogs, tear film production is greatly reduced, and animals therefore develop corneal dryness. The resulting painful abrasion leads to blepharospasm. A mucoid or mucopurulent ocular discharge, blepharitis, conjunctivitis, and secondary bacterial infections develop. Corneal ulceration may occur and progress to perforation if untreated.

The disease is diagnosed by use of the Schirmer tear test. A 5 × 30 mm strip of filter paper is placed in the medioventral cul-de-sac for 1 minute. Normal dog tears wet between 14 and 24 mm of paper/minute, but in keratoconjunctivitis sicca cases, the tears usually wet less than 10 mm, and many wet less than 5 mm. The breeds at highest relative risk include English Bulldogs, West Highland White Terriers, Lhasa Apsos, Pugs, Cocker Spaniels, and Pekinese. The disease may be treated by use of artificial tears. It is also logical to use immunosuppressive agents in refractory cases. For example, cyclosporine ophthalmic drops appear to be effective, although it may take 2 to 3 weeks before improved lacrimation is seen.

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Keratoconjunctivitis sicca has been reported in a horse. The 3-year-old animal presented with bilateral ulcerative keratoconjunctivitis and improved clinically with ophthalmic cyclosporine therapy. The lacrimal glands showed an eosinophil infiltration with lesser numbers of lymphocytes, plasma cells, and macrophages. Although this suggests an immunological origin for the disease, it must be pointed out that non-immunological mechanisms such as facial nerve damage can also result in corneal dryness.

Chronic Superficial Keratitis

Chronic superficial keratitis is a common inflammatory ocular disease of dogs in which blood vessels, lymphocytes, plasma cells, and melanocytes invade the superficial corneal stroma. Immunoglobulin deposits may be present. Eventually the pigmented granulation tissue causes corneal opacity. The disease is believed to be immune mediated, although its cause is unknown. It is more prevalent in dogs living at high altitudes, where UV exposure is great. It is also most prevalent in German Shepherds and is associated with specific MHC class II haplotypes.

Horses can also develop an immune-mediated keratitis. It causes chronic corneal opacity without ulceration or severe uveitis. It is usually unilateral and its severity depends largely on the depth of the lesion. Thus it may be epithelial, superficial stromal, deep stromal, or endothelial. In superficial stromal immune-mediated keratitis, there are increased numbers of T cells (but not B cells). Both $CD4^+$ and $CD8^+$ T cells are present as are IgG, IgM, and IgA. However, anti-corneal antibodies are not detected in either serum or aqueous humor. The most damaging form is endothelitiis, in which the cornea becomes edematous, and it may not respond well to immunosuppressive therapy.

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Autoimmune Polyarthritis

Animals develop immunologically mediated joint diseases, most of which are associated with the deposition of immunoglobulins or immune complexes within joints. Their classification is based on the presence or absence of joint erosion.

Erosive Polyarthritis

Rheumatoid Arthritis

The most important immune-mediated erosive polyarthritis in humans is rheumatoid arthritis. Rheumatoid arthritis is a common, crippling disease affecting about 1% of humans. A very similar disease is seen in domestic animals, especially dogs, in which there is no obvious breed or sex predilection. Dogs with rheumatoid arthritis may present with chronic depression, anorexia, and pyrexia, in addition to lameness, which tends to be most severe after rest (e.g., immediately after waking in the morning). The disease mainly affects peripheral joints, especially the carpal joints, which show symmetrical swelling and stiffness. Rheumatoid arthritis tends to be progressive and eventually leads to severe joint erosion and deformities. In advanced cases, affected joints may fuse as a result of the formation of bony ankyloses. Radiological findings are variable, but the swelling usually involves soft tissues only, and there may be subchondral rarefaction, cartilage erosion, and narrowing of the joint space.

Pathogenesis.

Rheumatoid arthritis is a chronic inflammatory disease. It commences as a lymphocytic synovitis with neutrophils in the joint fluid. As the inflammation continues, the synovia swell and proliferate. Outgrowths of the proliferating synovia eventually extend into the joint cavities, where they are called pannus. Pannus consists of fibrous vascular tissue that, as it invades the joint cavity, releases proteases that erode the articular cartilage and, ultimately, the neighboring bony structures. As the arthritis progresses, the

infiltrating lymphocytes form lymphoid nodules and germinal centers within the synovia. Amyloidosis, arteritis, glomerulonephritis, and lymphatic hyperplasia are occasional complications of rheumatoid arthritis (Fig. 38.8).

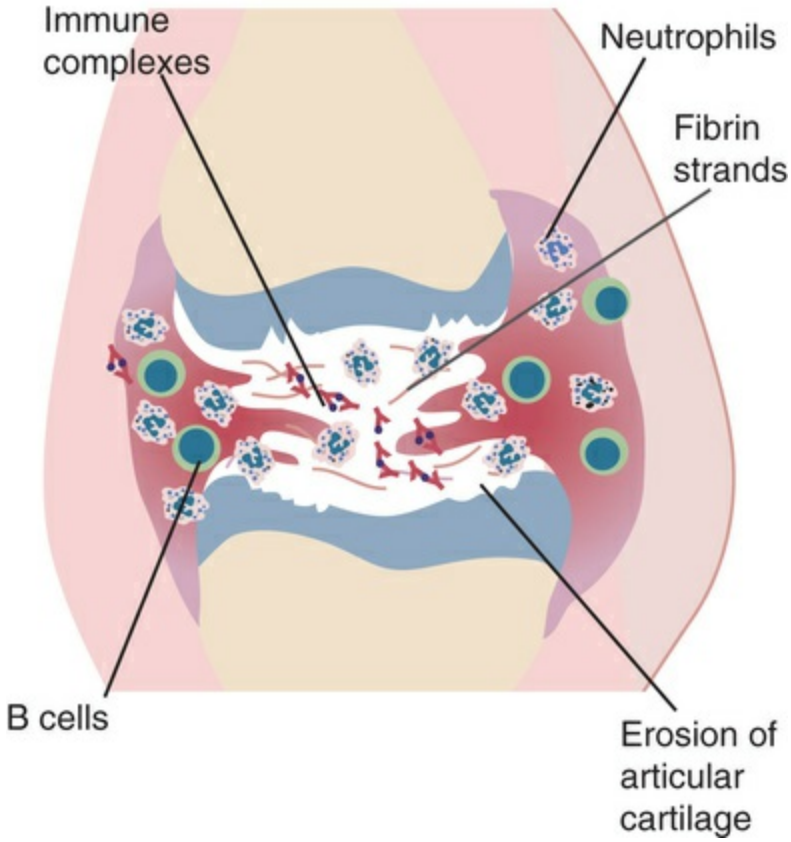


FIG. 38.8 A schematic diagram showing how joints are damaged in rheumatoid arthritis.

It is probable that many different stimuli, especially infectious agents, trigger rheumatoid arthritis in susceptible animals. Infectious agents implicated in the human disease include Epstein-Barr virus (a herpesvirus), parvoviruses, and mycobacteria.. The composition of the intestinal microbiota also influences the development of human disease. In domestic mammals, *Mycoplasma hyorhinis, Erysipelothrix rhusiopathiae,* and *Borrelia burgdorferi* each produce a chronic arthritis that resembles rheumatoid arthritis. Dogs with rheumatoid arthritis have antibodies to canine distemper in their synovial fluids, antibodies that are not present in dogs with osteoarthritis. Immune complexes can be precipitated out of the

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synovial fluid of dogs with rheumatoid arthritis, and analysis of these complexes by Western blotting shows the presence of canine distemper virus antigens. Thus distemper virus may be present in canine rheumatoid joints and may play a role in the pathogenesis of the disease.

Susceptibility to and severity of rheumatoid arthritis in humans are linked to the expression of certain MHC class II molecules (HLA-DR). This susceptibility is associated with the presence of a conserved 5-amino acid sequence located in the HLA-DRB1 antigen-binding groove and known as the "RA shared epitope." Presumably these MHC molecules can bind and present self-peptides. They may also serve as auto-antigens as well. It is interesting to note that this same conserved RA shared epitope is found on canine DLA-DRB1 and is associated with susceptibility to RA in some dog breeds. Some MHC class III genes also affect susceptibility to canine RA. For example, possession of the C4 allotype C4-4 is associated with the development of autoimmune polyarthritis. Despite this, it has been estimated that non-MHC genes contribute as much as 75% of the genetic susceptibility to rheumatoid arthritis.

Although rheumatoid arthritis is generally considered an autoimmune disease, the identity of the autoantigens involved is unclear. Four that have been implicated are IgG, collagen, citrullinated proteins, and glycosaminoglycans (Fig. 38.9). The development of rheumatoid factors to IgG is characteristic of rheumatoid arthritis. These RFs are directed against epitopes on the C_H2 domains of antigen-bound IgG. They can belong to any immunoglobulin class, including IgE, although IgG RFs are by far the most common. The IgG in rheumatoid arthritis patients is less glycosylated than normal IgG, and it may be that this abnormal IgG can act as an immunogen in a susceptible animal. RFs are found not only in rheumatoid arthritis but also in SLE and other diseases in which extensive immune complex formation occurs. RFs are also present in the serum and synovial fluid of some dogs with osteoarthritis (including cruciate disease) or infective arthritis.

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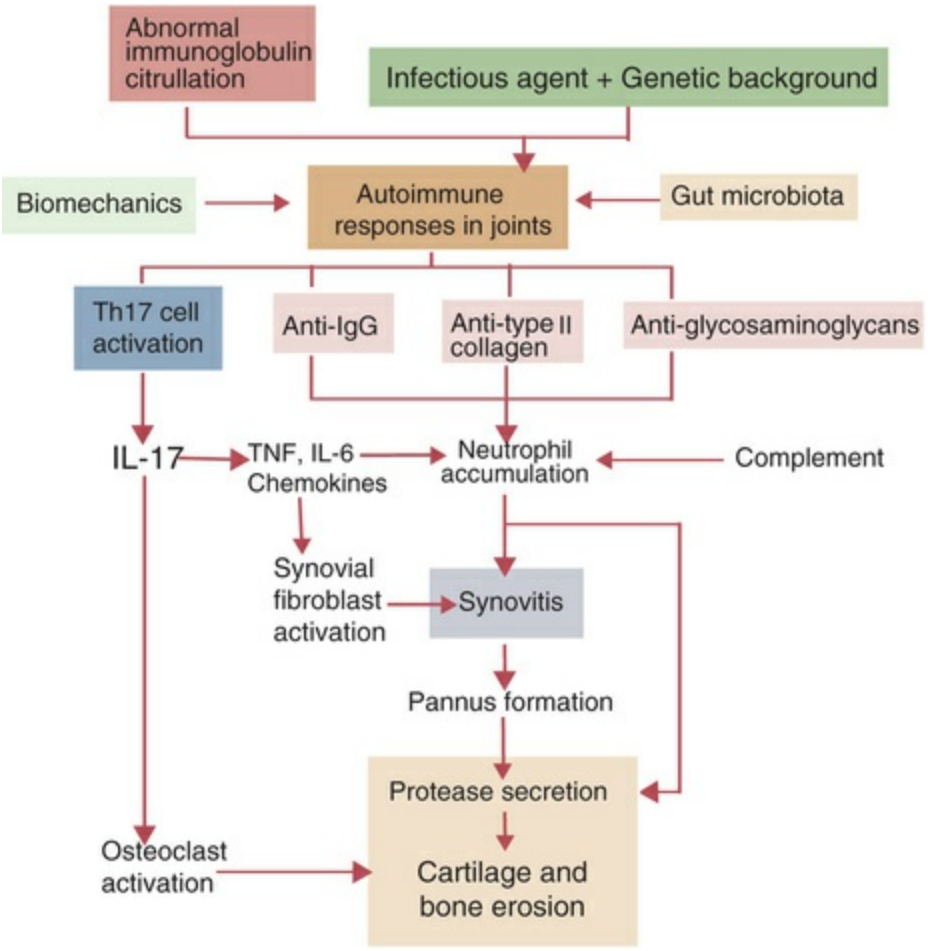


FIG. 38.9 A schematic diagram showing the possible pathogenesis of rheumatoid arthritis.

RFs will agglutinate antibody-coated particles. In humans, latex beads coated with IgG are used for this purpose. In dogs, it is easier to make a canine antisheep erythrocyte serum and coat sheep erythrocytes with this in a subagglutinating dose. After washing, these coated erythrocytes will agglutinate when mixed with RF–positive dog serum.

Although RFs are of diagnostic importance, their clinical significance is less clear. RFs are found in joint fluid, where their titer tends to correlate with the severity of the lesions, and the lesions themselves may be exacerbated by intraarticular inoculation of autologous immunoglobulins. Nevertheless, some individuals with rheumatoid arthritis may not have detectable RFs, and it is not uncommon to find others who have no arthritis despite the presence of RF in their serum. Thus the measurement of RF in dogs is of doubtful specificity.

Other evidence suggests that autoantibodies to collagen may be

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important. Type II collagen predominates in articular cartilage and may act as an autoantigen. Autoantibodies to type II collagen can be detected in the serum and synovial fluid of dogs with rheumatoid arthritis, infective arthritis, and osteoarthritis. Affected humans develop a cell-mediated response to denatured collagen II and III, and horses with chronic, nonsuppurative arthritis, osteoarthritis, or traumatic arthritis develop antibodies to horse collagens I and II. These antibodies, as well as immune complexes, can therefore be found in the synovial fluid of horses with many different joint diseases. An autoimmune disease that closely resembles rheumatoid arthritis develops in rats or sheep immunized with type II collagen. Evidence from experimental mice and some humans suggests that T cells directed against hyaluronic acid, heparin, and chondroitin sulfates may induce an arthritis resembling rheumatoid arthritis.

It has also been suggested that rheumatoid arthritis may result from immune responses against citrullinated proteins. Citrulline is derived from arginine by conversion of a ketamine side chain to a ketone during inflammation. This change fundamentally alters the behavior of proteins and probably plays a role in the preparation of intracellular proteins for apoptosis. Citrullinated proteins are expressed in inflamed joints. Patients develop high levels of autoantibodies to these neoantigens before rheumatoid arthritis lesions develop, and these autoantibodies appear to be specific for this disease. They are rarely found in healthy people or in other diseases. It is possible therefore that the key initial lesion in this disease involves autoimmunity to these modified proteins.

Whatever the precise initiating factors, the first stage in the development of rheumatoid arthritis probably involves unregulated activation of Th17 cells within the synovial membrane. The presence of IL-17 activates synovial fibroblasts. Cytokines such as IL-1, IL-6, IL-22, granulocyte-macrophage colony-stimulating factor, and tumor necrosis factor-α (TNF-α) are produced by stromal and endothelial cells. IL-33 is released by synovial fibroblasts. Levels of IFN-γ and IL-2 are very low in synovial fluid, suggesting that Th1 cells are not important in this disease. Inflammatory chemokines such as CXCL8 (IL-8) also accumulate. The production of IL-17 and the escape of IL-33, together with

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chemokines, C5a, leukotriene B_4, and platelet-activating factor, results in the accumulation of neutrophils in the synovial fluid. Phagocytosis of immune complexes and tissue debris leads to protease escape and the release of oxidants. IL-1, IL-17, and TNF-α stimulate cartilage degradation by activating fibroblast-like cells that line the synovia and by stimulating the release of metalloproteases. Metalloprotease-2 and metalloprotease-9 from chondrocytes and macrophages are increased in canine RA joint fluid and can degrade the articular cartilage and ligaments. Activated platelets may enter the joint space and aggravate the process by producing more IL-1. More importantly, IL-17, TNF-α, and high mobility group box protein-1 (HMGB1) from activated T cells activate bone-destroying osteoclasts. Collectively these reactions lead to bone and cartilage erosion and the characteristic joint pathology.

Macrophage cytokines also trigger synovial angiogenesis. Circulating lymphocytes, home to newly formed capillaries, emigrate into the tissues and aggregate around the blood vessels. These infiltrating lymphocytes are primarily activated $CD4^+$ T cells. B cell emigration into the tissues eventually leads to local RF production. The RFs form immune complexes and activate complement. Some immune complexes may precipitate out within the superficial layers of the articular cartilage.

The progressive development of inflammation within synovia leads first to morning stiffness. The joints become warm as the blood flow increases, but because the inflammation is restricted to the synovia, the skin rarely becomes red. The animal may show depression and fatigue as a result of the systemic effects of IL-1 and TNF-α. If the joints develop effusions, they will be obviously swollen. As the disease progresses, the inflamed synovia invades the cartilage, ligaments, and bone, and destroys the articular cartilage. Synovial lining cells, small blood vessels, and fibroblasts proliferate. Large numbers of macrophages are found in the pannus, as well as MHC class II^+ B cells and dendritic cells.

Diagnosis.

Diagnosis of rheumatoid arthritis in animals is based on the criteria established for human rheumatoid arthritis. They are listed in Box

38.2. Most signs should have been present for at least 6 weeks. In addition, steps should be taken to exclude SLE (by testing for ANA) and to exclude an infectious cause for the arthritis. In practice, cytologic evaluation of synovial fluid with a protein concentration of >3.0 g/dL and a nucleated cell count of >3,000 cells/mL consisting of >12% neutrophils identifies an inflammatory arthritis, especially when supported by radiographic evidence of erosion.

Box 38.2

Diagnostic Criteria for Canine Rheumatoid Arthritis

- Stiffness or joint pain, especially after periods of inactivity
- Symmetrical joint swelling, especially if multiple joints are involved
- Sterile synovial fluid containing inflammatory cells, especially neutrophils
- Positive rheumatoid factor test
- Erosive polyarthritis with characteristic histology

Treatment.

Treatment of canine rheumatoid arthritis tends to be unsatisfactory, and the long-term prognosis of the disease is poor. Nonsteroidal antiinflammatory drugs, such as aspirin, carprofen, or etodolac, have been the first choice in treating early, uncomplicated cases of rheumatoid arthritis, although their efficacy is unclear. Corticosteroids such as prednisolone should be reserved for late, severe cases in which salicylates have proved inadequate. Local steroid injections into affected joints will produce rapid relief and clinical remission. However, the joints are still subjected to stress, disease progression is not slowed, and the corticosteroids delay healing and promote articular degeneration. Their use may therefore permit articular damage to proceed unabated. Recently,

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encouraging results have been obtained in humans by the aggressive use of the immunosuppressive agent methotrexate. Monoclonal antibodies to TNF-α (infliximab), to CD4, to thymocytes, or to IL-2R have also helped prevent bone erosion in humans, as has administration of recombinant TNF-α receptors bound to IgG (etanercept). The immunosuppressive drug leflunomide appears to be as effective as methotrexate. Slow-acting immunosuppressive agents, such as the gold salts, sodium aurothiomalate, and aurothioglucose, and antimalarials such as chloroquine, are also used in humans, but they are expensive, results have been erratic, and experience with these in animals is limited. (Gold salts inhibit the release of HMGB1 from activated macrophages.) Appropriate surgery may improve joint stability and reduce pain.

Nonerosive Polyarthritis

The second major group of immune-mediated arthritides consists of those in which the joint cartilage is not eroded and the inflammatory lesion is largely confined to the joint capsule and synovia. Many of these clinically resemble rheumatoid arthritis but may be differentiated by their nonerosive character.

Equine Polyarthritis/Polysynovitis

Polyarthritis has been reported in foals in association with a lupus-like syndrome. Affected foals (up to 3 months of age) present with multiple swollen joints involving all four limbs and a persistent fever. In some cases, other synovial sheaths, including tendon sheaths and bursae, are affected. The synovial effusions are sterile, but synovial biopsies show lymphocyte and plasma cell infiltration with some immunoglobulin deposits. The cells in the joint fluid are mainly neutrophils. These animals are negative for RF, ANA, and LE cells. Many of these animals have a lesion within the thorax, especially *Rhodococcus equi* pneumonia. This is classified as a type II disease. It is possible that immune complexes originating in the lungs may lodge in the synovia and trigger the synovitis. The polyarthritis usually resolves as the primary lesion resolves.

A type I immune-mediated polyarthritis has also been recorded

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in horses. In these cases, animals lose weight, develop an intermittent fever, and have effusions in multiple joints leading to stiffness. They have systemic signs of inflammation, including anemia, leukocytosis, hyperfibrinogenemia, and hyperglobulinemia. The synovial effusion is sterile, and immunoglobulins are present in the synovial membrane. The condition usually resolves with steroid and immunosuppressive therapy.

Canine Polyarthritis

Dogs may develop several distinct nonerosive polyarthritides, which can be divided into three major categories: arthritis associated with SLE, arthritis associated with a myositis, and idiopathic polyarthritis. Breeds predisposed to polyarthritis include German Shepherds, Irish Setters, Shetland Sheepdogs, Cocker Spaniels, and Springer Spaniels. The main clinical features are stiffness, pyrexia, anorexia, and lethargy. Leflunomide appears to be an effective alternative to oral corticosteroids for the treatment of this disease.

Lupus Polyarthritis

Polyarthritis is a common feature of SLE. Diagnosis is contingent on making a firm diagnosis of lupus. Thus it is necessary to show multiple-system involvement, a significant titer of serum ANAs, and immunopathological features consistent with lupus.

Polyarthritis With Polymyositis

A disease characterized by both nonerosive polyarthritis and polymyositis is recognized in young dogs. Most recorded cases have been seen in spaniels. The animals are stiff and have painful joints, fever, lethargy, weakness, muscle atrophy, and muscle pain. They are negative for both ANA and RF. The arthritis is symmetrical, involving multiple joints. The animals also have a symmetrical inflammatory myopathy with myalgia, atrophy, and muscle contracture. The synovial fluid shows high white cell counts, especially neutrophils. Muscle biopsies show a neutrophil or mononuclear cell infiltrate, or both, with muscle fiber atrophy

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and degeneration. Synovial biopsies show a neutrophil and mononuclear cell infiltration with a fibrinous exudate. IgG, IgM, and complement are deposited in the walls of the synovial vessels. Animals may be treated with corticosteroids and immunosuppressive agents such as cyclophosphamide.

Idiopathic Polyarthritis

Most cases of canine polyarthritis fit none of the categories described previously. Although these cases are nonerosive and possess the characteristics of type III hypersensitivity, their precise etiology is unknown. They can be classified into four types (Table 38.1). Type I disease is polyarthritis alone. Type II disease is a reactive arthritis associated with infections in the respiratory or urinary tract, tooth infections, or cellulitis. Type III disease is associated with the presence of gastroenteritis, diarrhea, or ulcerative colitis. It is not clear whether this type of disease is truly distinguishable from type II disease. Type IV disease is associated with the presence of tumors, including seminomas and carcinomas.

TABLE 38.1

Classification of Nonerosive Polyarthritis in Dogs

Type	Disease Associations
I	Uncomplicated polyarthritis without other disease associations
II	Polyarthritis associated with infectious lesions remote from the joints (e.g., respiratory or urinary infections)
III	Polyarthritis associated with gastrointestinal disease
IV	Polyarthritis associated with neoplastic disease remote from the joints

From Bennett DJ: Canine idiopathic polyarthritis, *Small Anim Pract* 28:909-928, 1987.

An example of type I polyarthritis is the juvenile polyarthritis syndrome seen in Akitas between the ages of 9 weeks and 8 months. These dogs have a cyclical high fever lasting 24 to 48 hours before resolving and evidence of severe, incapacitating joint pain with soft tissue swelling. Radiology shows hepatosplenomegaly and lymphadenopathy. Some animals may have meningitis or meningoencephalitis. Their erythrocytes may be antiglobulin positive. Synovial fluid shows no evidence of infection, although large numbers of neutrophils are present. The dogs are usually negative for RF and ANA. Pedigree analysis suggests that the disease is inherited. Some dogs respond positively to corticosteroid

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treatment. In refractory cases, azathioprine may also be required.

Idiopathic polyarthritis tends to be most common in male dogs, and about half of the cases are seen in young dogs between 1 and 3.5 years of age. Most of the animals show fever, anorexia, and lethargy. The animals are lame and have a history of stiffness after rest. The most commonly affected joints are the stifle, elbow, and carpus. The onset of lameness is sudden in most cases and is associated with obvious muscle atrophy. There is no significant joint erosion, although periarticular soft tissue swelling and synovial effusions are common. Some cases may have proliferative periosteal changes. All cases are negative for RF and ANA. The joint fluid is sterile. Synovial biopsies show hypertrophy with a neutrophil or a mononuclear cell infiltration, or both. Fibrin deposits are seen in most cases, as is fibrosis. Most lesions contain IgM, IgG, and complement deposits, and some contain IgA-producing plasma cells. Some affected dogs may have a glomerulonephritis. Animals respond well to corticosteroids.

Feline Polyarthritis

Chronic progressive polyarthritis of male cats is characterized by polyarthritis with either osteopenia or periosteal new bone formation. Periarticular erosions and eventual collapse or subchondral erosions, joint instabilities, and deformities closely resembling those of rheumatoid arthritis are also seen. Affected cats are commonly infected with feline syncytia-forming virus (FSV) or feline leukemia virus (FeLV), or both. (The incidence of FSV in these cats is two to four times higher, and the incidence of FeLV is 6 to 10 times higher than in normal cats.) It is described here because of suggestions that it is of immunological origin. These suggestions are based on the massive lymphocyte and plasma cell infiltration of affected joints and the presence of an immune complex glomerulonephritis. However, affected cats are RF and ANA negative, and their serum immunoglobulin levels tend to be close to normal. Corticosteroids lessen the severity of clinical signs. Combination therapy with corticosteroids and azathioprine or cyclophosphamide can induce temporary remissions.

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Cruciate Ligament Rupture

While genetic/structural causes predominate, immunological abnormalities are also associated with spontaneous rupture of the anterior cruciate ligament in dogs. For example, the synovia of affected dogs contains B cells, IgG-positive plasma cells, and numerous MHC class II^+, $CD1c^+$ dendritic cells. Thus it resembles the lesions seen in rheumatoid arthritis. Cruciate ligaments are mainly composed of type I collagen. Autoantibodies to both type I and type II collagen are found in synovial fluid following cruciate ligament rupture (secondary to osteoarthritis), usually bound in immune complexes. They are probably secondary to tissue damage. CXCL8 (IL-8) levels rise in joints before cruciate ligament rupture, implying that inflammation precedes rupture.

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Immune Vasculitis

Several forms of immune-mediated vasculitis have been described in domestic animals. Their precise relationships are unclear, and, as a result, they have been given several different names including canine juvenile polyarteritis, polyarteritis nodosa, and leukocytoclastic vasculitis.

Canine juvenile polyarteritis primarily affects Beagles less than 2 years of age. The animals show episodes of anorexia, persistent fever of greater than 40° C, and a hunched stance with lowered head and a stiff gait, indicating severe neck pain. They may show cyclical remissions and relapses. The animals have a neutrophilia and elevated acute-phase proteins. Dogs have elevated serum IgM and IgA but normal IgG. Blood B cells are increased, but their T cells are decreased, as is their response to mitogens. On necropsy, there are few gross lesions. There may be some hemorrhage in lymph nodes. Histologically, they have a systemic vasculitis and perivasculitis. In the acute disease, there is necrotizing vasculitis with fibrinoid necrosis and a massive inflammatory cell infiltration involving the small- and medium-sized arteries of the heart, mediastinum, and cervical spinal cord (Fig. 38.10). Immunoglobulins are deposited in the walls of these arteries. During remissions, the vascular lesions consist of intimal and medial fibrosis and a mild perivasculitis, the residue of previous acute vasculitis. Chronically affected dogs may develop generalized amyloidosis.

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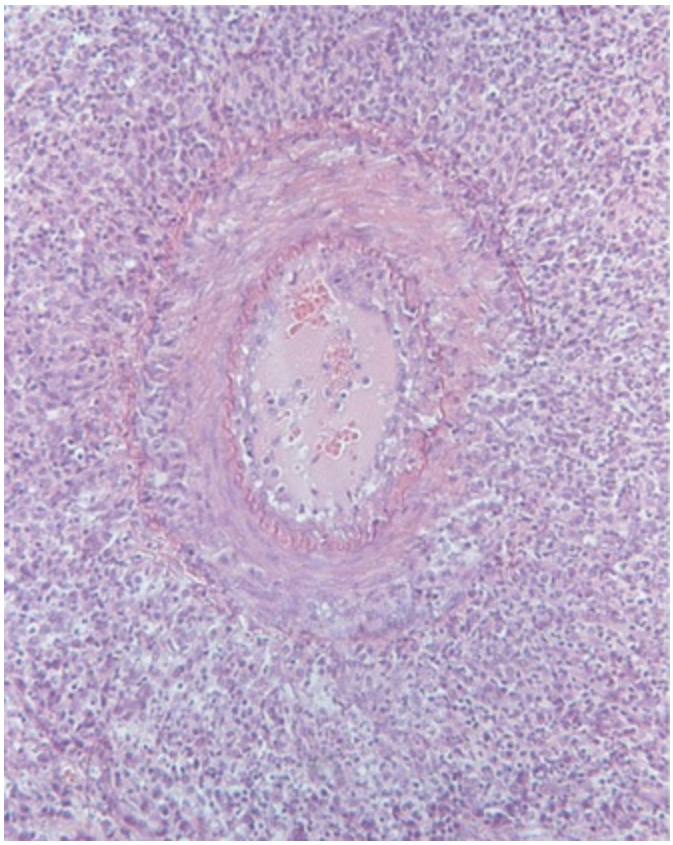


FIG. 38.10 An extramural coronary artery from a Beagle suffering from juvenile polyarteritis. This medium-sized muscular artery is characterized by medial necrosis, ruptured elastic laminae, and severe perivascular accumulations of neutrophils, lymphocytes, and macrophages. (H&E stain.) (From Snyder PW, Kazacos EA, Scott-Moncrieff JC, et al: Pathologic features of naturally occurring juvenile polyarteritis in beagle dogs, *Vet Pathol* 32:337-345, 1995.)

Polyarteritis nodosa occurs in humans, pigs, dogs, and cats. It is characterized by a widespread, focal necrosis of the media of small- and medium-sized muscular arteries. The lesions are found in many organs, especially in the kidney. Vessels in the skin are rarely involved.

On occasion, focal vascular lesions characterized by neutrophil infiltration may develop in small blood vessels throughout the body, but especially in skin. Affected dogs have mucocutaneous ulcers, bullae, edema, polyarthropathy, myopathy, anorexia, intermittent fever, and lethargy. Although called hypersensitivity vasculitis, a foreign antigen can be found in only a small proportion of cases. For this reason, a better name for this condition may be leukocytoclastic vasculitis. The cause or causes of polyarteritis nodosa and hypersensitivity vasculitis are unknown. Their histopathology suggests that they are a form of type III

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hypersensitivity reaction, perhaps triggered by an infectious agent. Immunosuppression with corticosteroids, together with cyclophosphamide, has given encouraging results in treating canine hypersensitivity vasculitis. Polyarteritis nodosa is usually detected as an incidental finding on necropsy, although ocular defects may present clinically if the arteries of the eye are involved.

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39

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Primary Immunodeficiencies

CHAPTER OUTLINE

Inherited Defects in Innate Immunity, 435

Chédiak-Higashi Syndrome, 436

Pelger-Huët Anomaly, 436

Canine Leukocyte Adhesion Deficiency (LAD), 436

Bovine Leukocyte Adhesion Deficiency, 437

Canine Cyclical Neutropenia, 438

Other Examples of Defective Neutrophil Function, 438

Inherited Defects in the Adaptive Immune System, 439

Immunodeficiencies of Horses, 439

Severe Combined Immunodeficiency, 439

Pathogenesis, 440

Immunoglobulin Deficiencies, 441

Common Variable Immunodeficiency, 441

Foal Immunodeficiency Syndrome, 441

Prevalence of Equine Immunodeficiencies, 442

Immunodeficiencies of Cattle, 442
Severe Combined Immunodeficiency, 442
Selective Immunoglobulin G2 Deficiency, 442
Hereditary Parakeratosis, 442
Other Immunodeficiencies, 442
Immunodeficiencies of Pigs, 442
Porcine SCID, 442
Immunodeficiencies of Dogs, 443
Combined Immunodeficiencies, 443
Immunoglobulin Deficiencies, 444
T Cell Deficiencies, 445
Uncharacterized Immunodeficiencies, 446
Immunodeficiencies of Cats, 446
Hypotrichosis With Thymic Aplasia, 446
Immunodeficiencies of Mice, 446
Nude Mice, 446
Severe Combined Immunodeficiency Mice, 447
Moth-Eaten Mice, 447
X-Linked Immunodeficiency, 447
Immunodeficiencies of Humans, 447
T Cell Deficiencies, 447
B Cell Deficiencies, 447

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LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Understand and explain the differences between primary and secondary immunodeficiency diseases.
- Explain the characteristic features that would suggest a primary immunodeficiency syndrome.
- Explain how, as a result of genetic mutations, defects may develop in the developing immune system.
- Understand that many different inherited defects have now been identified in domestic animals.
- Describe how defects in innate immunity include deficiencies in phagocytosis, leukocyte adherence, and intracellular killing, leading to increased susceptibility to bacterial diseases.
- Explain why defects in T cell function generally predispose an animal to overwhelming virus infections.
- Explain how defects in B cell function and immunoglobulin production predispose animals to overwhelming bacterial disease.
- Understand why combined immunodeficiencies are most severe since affected animals lack resistance to all infectious agents.
- Define and explain the pathogenesis of leukocyte adherence deficiency and severe combined immunodeficiency.
- Understand the significance of “nude” mice as research subjects.

A defect in either the innate or adaptive immune systems usually becomes apparent when affected animals show unusual susceptibility to infectious or parasitic diseases. Deficiencies in the immune systems may result from inherited or genetic defects (primary immunodeficiencies); or a direct result of some other cause (secondary or acquired immunodeficiencies). This chapter describes some of the primary immunodeficiencies recorded in domestic mammals.

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Inherited Defects in Innate Immunity

Inherited deficiencies in innate immunity include defects at various stages of phagocytosis as well as the complement deficiencies described previously (Chapter 4). Phagocytic defects are well recognized in domestic animals.

Chédiak-Higashi Syndrome

Chédiak-Higashi syndrome is an inherited disease of Hereford, Japanese Black, and Brangus cattle; Aleutian mink; blue smoke Persian cats; white tigers; beige *(bg/bg)* mice; orcas; and humans. It is an autosomal recessive disease resulting from a mutation in the *lysosomal trafficking regulator (LYST)* gene encoding, a protein that controls lysosomal membrane fusion. In Chédiak-Higashi cattle, there is a missense A:T → G:C mutation that results in replacement of a histidine with an arginine residue. In Aleutian mink, the mutation is a base deletion in *LYST* that causes a frame shift, leading to premature termination. The defect produces abnormally large secretory granules in neutrophils, monocytes, eosinophils, and pigment cells (Fig. 39.1). The enlarged neutrophil granules result from the fusion of primary and secondary granules. These granules are more fragile than normal, rupturing spontaneously and causing tissue damage. These leukocytes have defective chemotactic responsiveness, reduced motility, and reduced intracellular killing. Cytotoxic T cells and NK cells cannot excrete their granzyme-rich granules.

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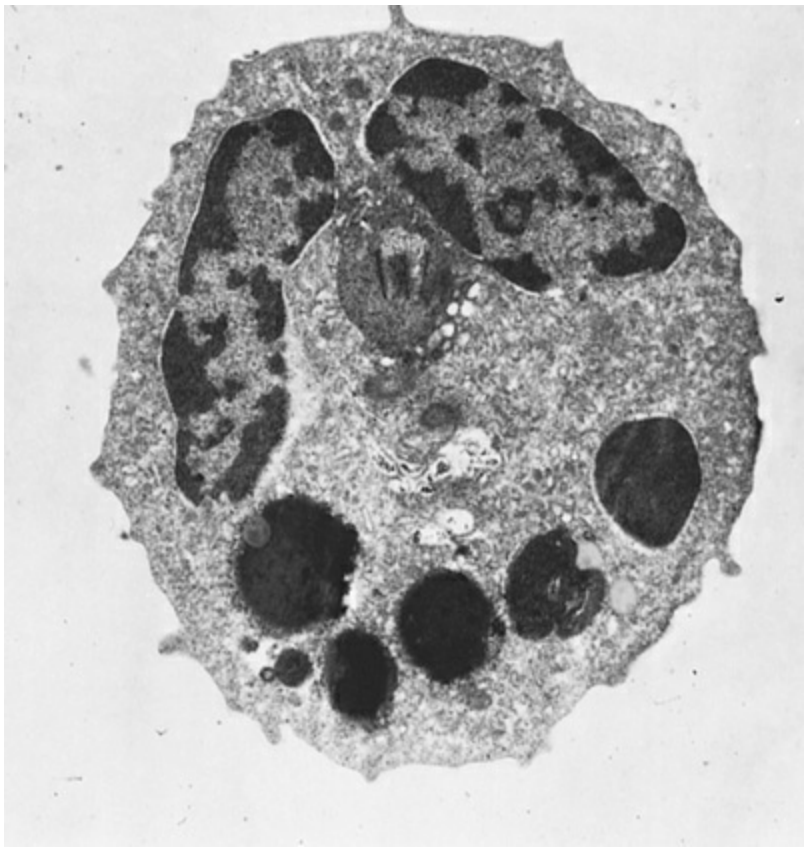


FIG. 39.1 A neutrophil from a Chédiak-Higashi syndrome calf with enlarged cytoplasmic granules. (Courtesy Dr. H.W. Leipold.)

Clinically, the syndrome is associated with multiple abnormalities. In hair, the melanosomes fuse together, causing the dilution of coat color (sometimes only obvious in the newborn) and light-colored irises (pseudoalbinism). Other eye abnormalities include photophobia, and animals may develop cataracts. Their eyes have a red fundic light reflection rather than the normal yellow-green. Because of the neutrophil defects, affected animals may be more susceptible to respiratory infections and neonatal septicemia. As a result, affected animals may show increased susceptibility to tumors and to infections such as the Aleutian disease parvovirus in mink. Platelets from affected animals also contain enlarged lysosomes, and, as a result, affected animals may bleed excessively after surgery and develop hematomas at injection sites. Death due to acute hemorrhage is common. Chédiak-Higashi syndrome may be diagnosed by examining a stained blood smear for the presence of grossly enlarged granules within leukocytes or by examining hair shafts for enlarged melanosomes.

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Pelger-Huët Anomaly

Pelger-Huët anomaly is an inherited disorder characterized by a failure of granulocyte nuclei to segment into lobes. The neutrophils therefore appear on first sight to be very immature (a left shift). The anomaly is usually detected when an animal is observed to have a persistent left shift that cannot be reconciled with its good health. Although Pelger-Huët neutrophils closely resemble band forms, their nuclear chromatin is condensed, reflecting their maturity. In humans, the anomaly is due to a mutation in the gene coding for lamin B, a nuclear membrane receptor that interacts with chromatin to determine the shape of the nucleus. Pelger-Huët anomaly has been observed in humans, Arabian horses, domestic shorthair cats, and various dog breeds such as Cocker Spaniels, Basenjis, Boston Terriers, Foxhounds, and Coonhounds. In Foxhounds and Australian Shepherds, the anomaly is inherited as an autosomal dominant trait. It has a minimal effect on the health of animals. Nevertheless, fewer pups are weaned from affected dogs than from unaffected ones. In addition, Pelger-Huët neutrophils are less able to emigrate from blood vessels in vivo. Their B cell responses may also be impaired.

Canine Leukocyte Adhesion Deficiency (LAD)

In order for neutrophils to leave inflamed blood vessels, they must first bind to vascular endothelium. This adhesion is mediated by neutrophil integrins. In the absence of these integrins, neutrophils cannot bind to endothelial cells and so cannot emigrate into tissues (Fig. 39.2). As a result, bacteria in tissues can grow freely without fear of attack by neutrophils.

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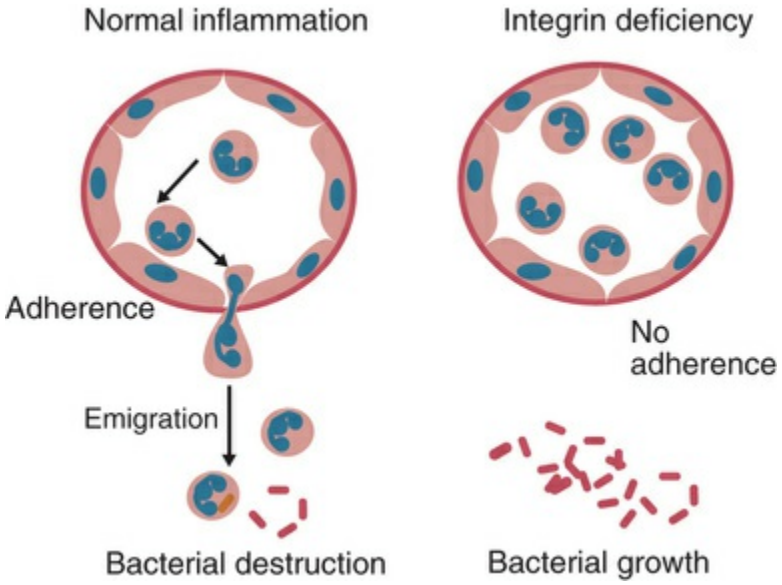


FIG. 39.2 Integrins are required to bind neutrophils firmly to blood vessel walls. This permits the neutrophils to emigrate to sites of bacterial invasion. In the absence of integrins, neutrophil emigration fails to occur. As a result, invading bacteria can grow unmolested in the tissues.

Three different forms of LAD are recognized. The commonest form, LAD-I, results from a loss-of-function mutation on the gene encoding the β2-integrin (CD18) and has been reported in Irish Setters and related breeds. LAD-II results from a defect in fucose metabolism that leads to a deficiency of the carbohydrate structure sialyl-Lewis-X and impairs neutrophil rolling. It has not been reported in dogs. LAD-III results from defects in the activation of β-integrins as a result of mutations in the Kindlin-3 gene. Kindlin-3 is a protein that is essential for β-integrin activation.

Canine leukocyte adhesion deficiency (CLAD) is a LAD-I that results from a defect in the integrin Mac-1 (CD11b/CD18). In Mac-1–deficient dogs, neutrophils cannot respond to chemoattractants, trap complement-coated bacteria (Mac-1 is a complement receptor), or bind to endothelial cells. Affected dogs suffer recurrent infections, despite the fact that their blood neutrophil numbers are high.

CLAD has been described in Irish Red Setters (as well as in the related Red and White Setter breed), in which it is an autosomal recessive disease. Affected animals die early in life as a result of recurrent severe bacterial infections (osteomyelitis, omphalophlebitis, gingivitis), lymphadenopathy, impaired pus

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formation, delayed wound healing, weight loss, and fever. Animals have a leukocytosis (>200,000/μL), primarily a neutrophilia and eosinophilia. Although their granulocytes look normal, functional tests reveal defects in adhesion-dependent activities, including impaired adhesion to glass or plastic surfaces. They cannot ingest C3b-opsonized particles. Migration in response to chemotactic stimuli is poor. Neither CD11b nor CD18 can be detected by immunofluorescence.

The CLAD lesion results from a single missense mutation in the β chain of CD18 that results in the replacement of a cysteine by a serine. As a result, the mutation disrupts a disulfide bond in CD18 and alters its structure and function. CD11b (the α chain) is not expressed because it must be associated with the β chain before the dimer can be expressed on the cell surface. A diagnostic PCR test for CLAD has been developed. Matched related bone marrow allografts from normal animals have been given to CLAD dogs and effectively "cured" the disease.

Another form of CLAD results from excessive downregulation of β_2-integrin. This has been reported in mixed-breed dogs that present with recurrent pyogenic infections. Their neutrophils produce significantly reduced amounts of CD18 and hence β_2-integrin. As a result of this reduced expression, defects occur in adhesion-dependent neutrophil functions, as well as superoxide production.

Cases of LAD-III have been reported in a German Shepherd dog and in a German Shepherd x Rottweiler cross. The dogs developed pyrexia, persistent leukocytosis, severe periodontal disease, lameness, mucosal hemorrhages, and poor wound healing. Because of defects in thrombocyte function, life-threatening hemorrhage was the most significant cause of death in these animals. The second case was shown to be homozygous for a 12-nucleotide insertion mutation in the gene encoding Kindlin-3.

Bovine Leukocyte Adhesion Deficiency

A LAD-1 has been described in Holstein calves. Bovine leukocyte adhesion deficiency (BLAD) is an autosomal recessive trait characterized by recurrent bacterial infections, anorexia, oral

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ulceration, gingivitis, periodontitis, chronic pneumonia, stunted growth, delayed wound healing, peripheral lymphadenopathy, and a persistent extreme neutrophilia. Affected calves usually die between 2 and 7 months of age. The survivors grow slowly and may develop amyloidosis. These calves have large numbers of intravascular neutrophils but very few extravascular neutrophils, even in the presence of invading bacteria.

Because T cells also express CD18, BLAD calves show poor delayed hypersensitivity responses. Their neutrophils show reduced responsiveness to chemotactic stimuli and diminished superoxide production and myeloperoxidase activity. They have increased expression of Fc receptors but decreased binding of C3b and IgM on neutrophils, implying a defect in receptor function. This is reflected by greatly reduced endocytosis and killing of *Staphylococcus aureus.*

BLAD results from a point mutation in the *CD18* gene (Fig. 39.3). An aspartic acid is replaced by a glycine, and functional CD18 is not produced. In the absence of this chain, complete integrins cannot be assembled. Neutrophils cannot attach to vascular endothelial cells or emigrate from blood vessels. Healthy carriers have a single copy of the mutated gene and thus have abnormally low levels of CD18 (Fig. 39.4). Through the use of a PCR test, the presence of the altered gene can be demonstrated. In this way, it has been shown that one bull, Osborndale Ivanhoe, with thousands of registered sons and daughters, was a carrier of this gene. As a result, the defective gene was widespread and common among Holstein cattle in the United States (14% of bulls, 5.8% of cows). Fortunately, carrier animals can be rapidly detected and removed from breeding programs.

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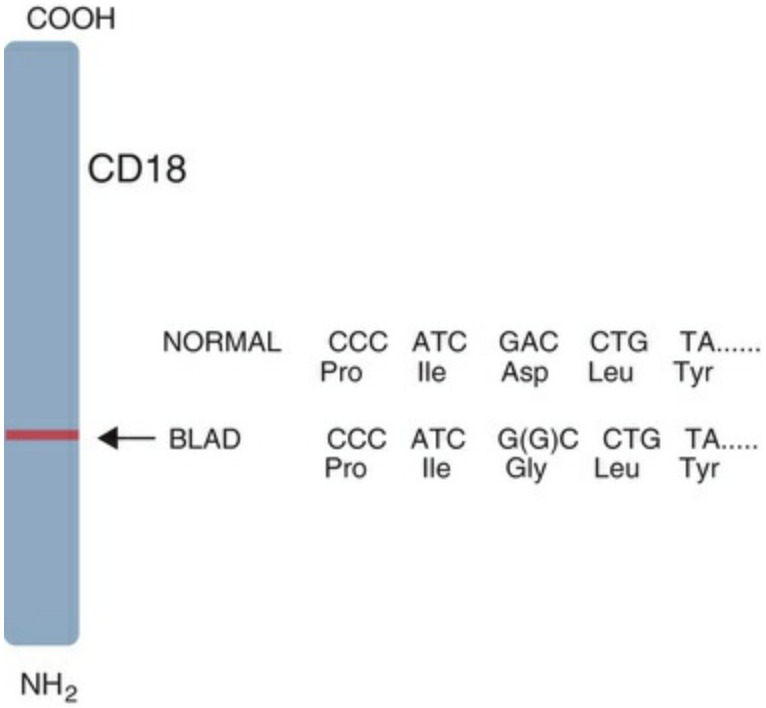
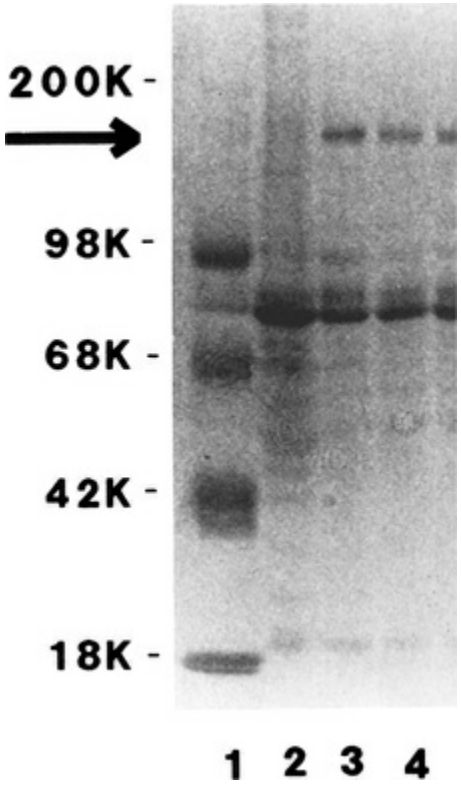


FIG. 39.3 The BLAD mutation. The mutation involves replacement of a cytosine by a guanosine in the *CD18* gene. As a result, an aspartic acid residue (A) is replaced by a glycine residue (G). The mutation occurs in a highly conserved region of the CD18 molecule and prevents formation of a biologically active molecule.



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FIG. 39.4 A Western blot of bovine Mac-1. An extract has been made from the neutrophils of a BLAD calf *(lane 2)* or from clinically normal calves *(lanes 3 and 4).* The extracts have been electrophoresed and blotted onto nitrocellulose. The bands are stained to show the presence of glycoproteins. Note that CD18 *(arrow)* is absent from the lysate of neutrophils from a BLAD calf. Lane 1 shows molecular weight standards (kDa). (From Kehrili ME, Schmalstieg FC, Anderson DC, et al: Molecular definition of the bovine granulocytopathy syndrome: identification of deficiency of the Mac-1 [CD11b/CD18] glycoprotein, *Am J Vet Res* 51:1826-1836, 1990.)

Canine Cyclical Neutropenia

Canine cyclical neutropenia (gray Collie syndrome) is an autosomal recessive disease of Border Collies. Affected dogs have diluted skin pigmentation, eye lesions, and regular cyclic fluctuations in leukocyte numbers. Their hair is a characteristic silver-gray color, and their nose is gray—a diagnostic feature. The loss of neutrophils occurs about every 11 to 12 days and lasts for about 3 days. It is followed by normal or elevated neutrophil counts for about 7 days. Severe neutropenia suppresses inflammation and increases susceptibility to bacterial and fungal infections. (Their neutrophils also have reduced myeloperoxidase activity, so the disease is not entirely due to a neutrophil deficiency.) In humans, this disease is due to a defect in the gene coding for neutrophil elastase, an enzyme found in azurophil granules. Affected dogs have severe enteric and respiratory infections, gingivitis, arthralgia, and lymphadenitis and rarely live beyond 3 years. Because platelet numbers also cycle, affected dogs may also have bleeding problems, including gingival hemorrhage and epistaxis. Immunoglobulin levels rise as a result of the recurrent antigenic stimulation, but complement levels cycle in conjunction with the neutropenia. The disease begins to express itself as maternal immunity wanes. Affected puppies are weak, grow poorly, and have wounds that fail to heal and high mortality. If they are kept alive by aggressive antibiotic therapy, chronic inflammation may lead to amyloidosis.

Treatment involves the repeated use of antibiotics to control the recurrent infections. If endotoxin is administered repeatedly, it can stimulate the bone marrow and stabilize neutrophil, reticulocyte,

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and platelet numbers. Lithium carbonate has a similar effect. Unfortunately, both endotoxin and lithium carbonate are toxic, and the disease recurs when the treatment is discontinued.

Other Examples of Defective Neutrophil Function

An inherited defect in neutrophil bactericidal activity has been reported in Dobermans. Dogs had bronchopneumonia and chronic rhinitis that developed soon after birth and persisted despite antimicrobial therapy. Although their chemotaxis and ingestion were apparently normal, their neutrophils were unable to kill *S. aureus.* Since these cells showed reduced reduction of nitroblue tetrazolium and superoxide production, it was suggested that they had a defect in the respiratory burst pathway.

Young Weimaraner dogs have been described as suffering from an immunodeficiency syndrome with clinical signs that include recurrent fevers, diarrhea, pneumonia, pyoderma, osteomyelitis, stomatitis, and osteomyelitis. They may have defective neutrophil respiratory burst, as shown by a depressed chemiluminescent response to phorbol ester. Their IgG levels may be significantly lower than normal and their IgM and IgA levels somewhat reduced; the other immunological parameters of these animals fall within normal ranges.

A persistent neutropenia attributable to a deficiency of granulocyte colony-stimulating factor (G-CSF) has been reported in a 3-year-old male Rottweiler. The animal had a fever due to multiple recurrent infections, especially a chronic bacterial arthritis in the presence of a persistent neutropenia. The animal was not making G-CSF. Its myeloid stem cells responded readily to additional G-CSF, suggesting that they were functionally normal. Bone marrow examination suggested that its neutrophil precursors had failed to mature.

A possible autosomal recessive neutropenia has been described in Border Collies. This disease, called "trapped neutrophil syndrome," results in recurrent bacterial osteomyelitis and gastroenteritis. Animals present with persistent fever and lameness due to lytic bone lesions. They have myeloid hyperplasia and dense

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accumulations of neutrophils in the marrow but few in the blood. The neutropenia apparently results from an inability of the neutrophils to escape from the bone marrow into the bloodstream. The causative mutation is a four base pair deletion in the canine *VPS13B* gene that affects vesicular transport and protein sorting within the cell.

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Inherited Defects in the Adaptive Immune System

Inherited immunological defects have served to confirm the overall arrangement of the immune system, as outlined in Fig. 39.5. For example, if both the cell- and antibody-mediated immune responses are defective, it may be assumed that the genetic lesion operates at a point prior to thymic and bursal cell processing. That is, a stem cell lesion. A defect that occurs only in thymic development leads to a failure to mount cell-mediated immune responses, although antibody production may be normal. Similarly, a lesion restricted to B cells is reflected by impaired antibody responses.

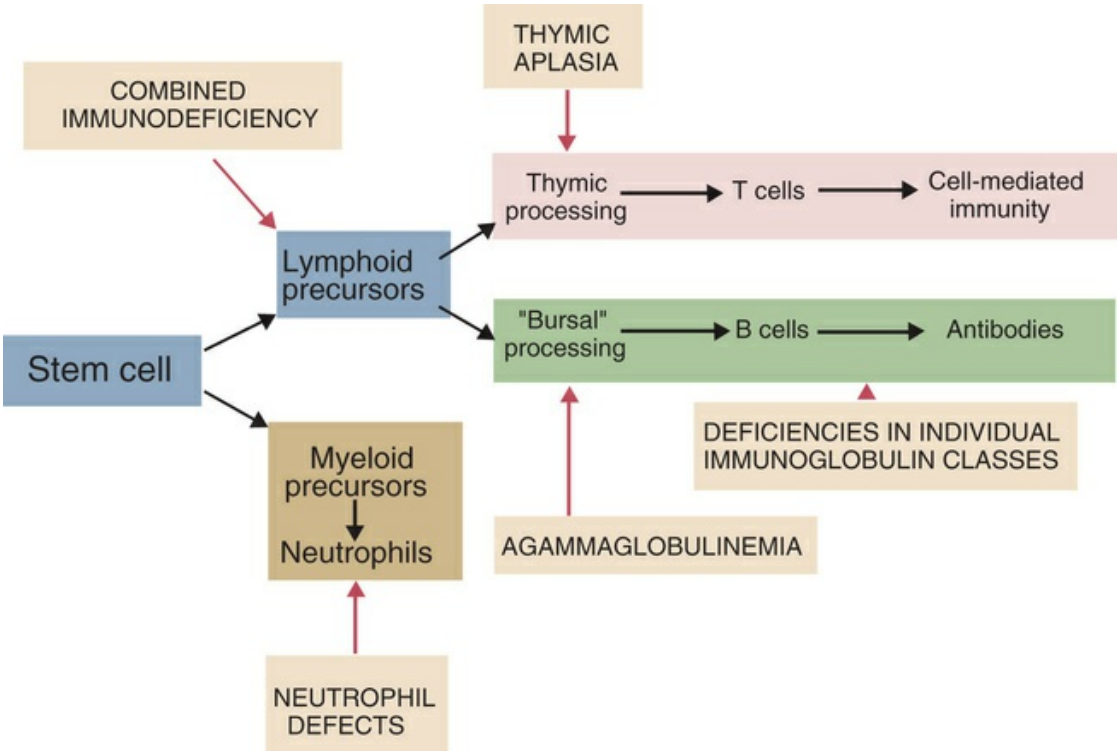


FIG. 39.5 The points in the immune system where development blocks may lead to immunodeficiencies.

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Immunodeficiencies of Horses

Horses are among the few domestic animals whose economic worth has permitted a thorough analysis of neonatal mortality. As a result, a significant number of primary immunodeficiency syndromes have been identified in this species (Fig. 39.6).

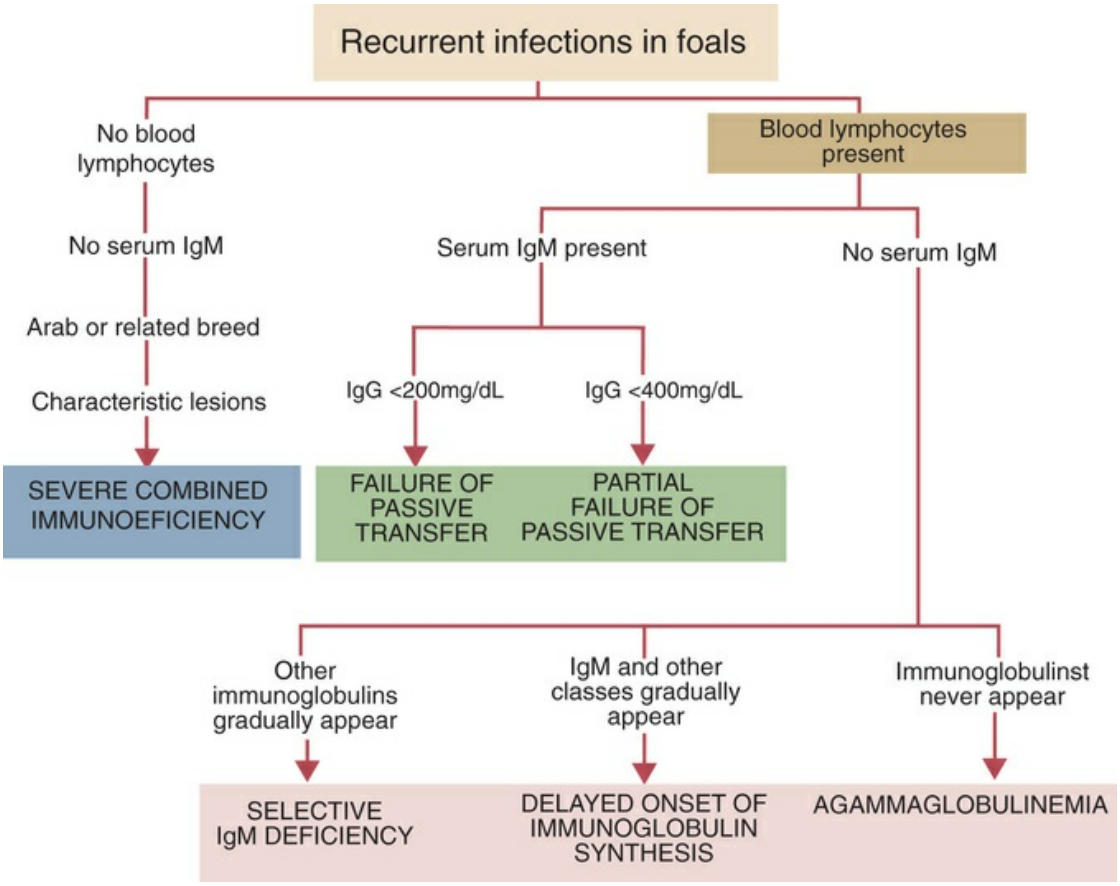


FIG. 39.6 The differential diagnosis of the equine immune deficiencies.

Severe Combined Immunodeficiency

The most important congenital equine immunodeficiency is severe combined immunodeficiency (SCID). Affected foals fail to produce functional T or B cells and have very few circulating lymphocytes. If they suckle successfully, they acquire maternal immunoglobulins. Once these have declined, however, these foals cannot produce

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their own antibodies and eventually become agammaglobulinemic. Affected foals are therefore born healthy but begin to sicken by 2 months of age. The precise time of onset depends on the quantity of colostral antibodies absorbed. All die by 4 to 6 months as a result of overwhelming infection. Severe bronchopneumonia is the predominant presenting sign. Organisms that have been implicated in this bronchopneumonia include equine adenovirus, *Rhodococcus equi,* and *Pneumocystis* (an opportunistic fungal pathogen). The disease is manifested by a nasal discharge, coughing, dyspnea, weight loss, and fevers. Affected foals may also develop enteritis, omphalophlebitis, and many other infections. *Cryptosporidium parvum* and many different bacteria have been implicated in the enteritis.

On necropsy, the spleens of these foals lack germinal centers and periarteriolar lymphoid sheaths. Their lymph nodes lack lymphoid follicles and germinal centers, and there are few cells in the paracortex. Their thymus may be difficult to find. Neutrophil, monocyte, and NK functions are normal.

SCID is an autosomal recessive disease, and its occurrence therefore indicates that both parents carry the mutation. Accurate diagnosis is of great importance since the presence of the mutation significantly reduces the value of the parent animals. Thus all suspected cases must be confirmed by postmortem examination. The clinical diagnosis of SCID requires that at least two of the following three criteria be established: (1) very low (consistently below 1000/mm^3) circulating lymphocytes; (2) histology typical of SCID; that is, gross hypoplasia of the primary and secondary lymphoid organs; and (3) an absence of IgM from presuckle serum. (The normal equine fetus synthesizes small amounts of IgM. As a result, IgM in normal newborn foals is about 160 μg/mL. If the foal successfully suckles, it will obtain immunoglobulins of all isotypes from the mare's colostrum. However, the half-life of IgM is only about 6 days, so maternal IgM will disappear within a few days of birth. Thus a normal foal will always have some IgM in its serum, but a SCID foal will have none.)

Pathogenesis

When B and T cell antigen receptors are produced, large segments

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of DNA are excised so that *V*, *D*, and *J* gene segments can be rejoined (Chapter 17). Several enzymes are involved in this recombination process. Some cut the DNA strands, and others join them. Studies on SCID foals show that there is a defect in the large multicomponent enzyme that rejoins the cut ends. The specific defect lies in the gene coding for the catalytic subunit of an enzyme called DNA-dependent protein kinase *(DNA-PK$_{cs}$)* (Fig. 39.7). In the mutant *DNA-PK$_{cs}$* gene, a loss of five nucleotides results in a frame-shift, premature termination of the peptide chain, and a deletion of 967 amino acids from the C-terminus of the molecule, including its entire kinase domain (Fig. 39.8). Functional *DNA-PK$_{cs}$* is totally absent from affected foals. Because of this deficiency, broken DNA strands cannot be rejoined, and neither T cells nor B cells can form functional V regions. In the absence of TCRs and BCRs, affected foals cannot respond to antigens. Since *DNA-PK$_{cs}$* is needed to rejoin broken strands of DNA, it also plays a key role in other DNA repair processes. Thus the cells from SCID foals are unable to repair DNA damaged by radiation.

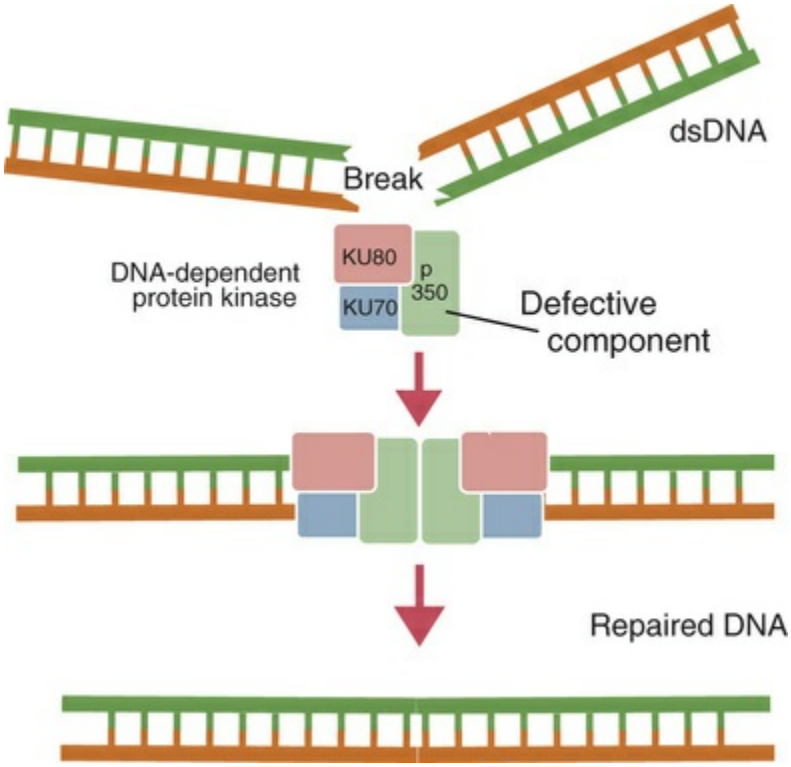


FIG. 39.7 The defect in DNA-dependent protein kinase (DNA-PK) that prevents DNA repair in SCID foals.

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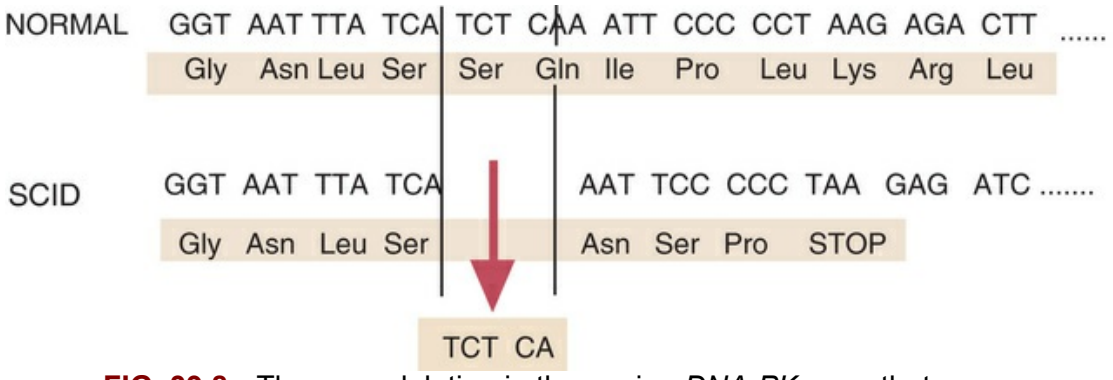


FIG. 39.8 The gene deletion in the equine *DNA-PK* gene that leads to premature termination of the molecule.

The presence of a mutant *CID* gene in horses can be detected by means of a PCR test (Chapter 42). A sample of DNA is obtained from horse skin cells. A primer set designed to amplify only DNA containing the five base pair deletion and another set designed to amplify only the normal gene sequence are used to determine whether the mutant gene is present. This test has demonstrated that the frequency of the *CID* gene in Arabian horses is 8.4%. Based on this, it would be expected that 0.18% of Arabian foals would be homozygous for the trait and hence clinically affected. Pedigree analysis suggests that the SCID trait was introduced to the United States by a single stallion in the 1920s.

Immunoglobulin Deficiencies

Primary agammaglobulinemia is a rare disease of foals. Affected animals have no identifiable B cells (cells with surface immunoglobulins) and have very low serum immunoglobulin levels. Their lymphoid tissues contain no primary follicles, germinal centers, or plasma cells. Nevertheless, their blood lymphocytes can respond to mitogens. Intradermal inoculation of the T cell mitogen phytohemagglutinin induces a typical type IV delayed hypersensitivity reaction. Affected foals experience recurrent bacterial infections but may survive for up to 18 months. The disease should be suspected in a foal having a normal lymphocyte count but lacking both IgM and IgG. It may be confirmed by

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showing normal responses to T cell mitogens and an absence of B cells.

Selective IgM deficiencies have been described in foals. Serum IgM levels in these animals are at least two standard deviations below normal, but IgG and IgA levels and B cell numbers are normal. In most cases, foals develop septicemia or recurrent respiratory tract infections, often involving *Klebsiella pneumoniae* or *R. equi*, and die by 10 months. Some affected foals live longer and respond to therapy but fail to grow, have recurrent respiratory infections, and usually die by 24 months. Most affected foals have been Arabians or Quarter Horses, suggesting that the disease has a genetic basis. IgM deficiency has also been described in adult horses over 2 years of age. In many cases, such horses also have a lymphoreticular neoplasm.

A single case of IgG deficiency has been described in a 3-month-old foal with salmonellosis. The animal had normal IgA and IgM but no germinal centers, lymphoid follicles, splenic follicles, or periarteriolar lymphoid sheaths. Serum IgG was extremely low.

Between 2 and 3 months of age, some foals experience a transient hypogammaglobulinemia as a result of a delayed onset of immunoglobulin synthesis. These animals may have recurrent infections during the period when their immunoglobulin levels are low. Lymphocyte numbers and responsiveness remain normal at this time.

Common Variable Immunodeficiency

Common variable immunodeficiency is the second most common primary immunodeficiency syndrome in humans (after selective IgA deficiency). It is a heterogeneous group of sporadic diseases all characterized by a late-onset B cell lymphopenia as a result of impaired B cell production in the bone marrow. It occurs in horses where four genes, *E2A, PAX5, CD19*, and *IGD*, have significantly reduced expression. Immunohistochemistry has confirmed the absence of *PAX5* in the bone marrow of these horses. *PAX5* and the other molecules are known to be transcription factors that regulate the early differentiation of B cells. Their B cell development seems to be blocked at the transition to pro-B cells. Mutations in genes

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coding for tumor necrosis factor (TNF-α) receptors and other co-stimulatory molecules also result in loss of helper T cell function.

Cases of common variable immunodeficiency have been recorded in horses. Although they resemble primary immunodeficiencies in their sporadic nature and severity, they usually occur in animals over 3 years of age. Typically, these horses present with recurrent infections that are not responsive to medical treatment. Bacterial meningitis may be a consistent feature. Their serum contains only trace levels of IgG and IgM, no detectable IgG3, and very low IgA levels. Sometimes individual IgG subclasses are deficient, whereas IgA levels are normal. T cell numbers are normal, but B cells are undetectable, and there is no response to the B cell mitogen lipopolysaccharide. On necropsy, there are no B cells in lymphoid organs, blood, or bone marrow. Some horses may have severe liver disease, a feature also seen in humans. It is suspected that these individuals have an underlying defect that is only expressed when the immune system is stressed by infection. Other cases have included horses between 2 and 5 years old with a selective IgM deficiency. Many develop a concurrent lymphosarcoma, and they may have excessive regulatory T cell function.

Foal Immunodeficiency Syndrome

This primary immunodeficiency syndrome was first described in the highly inbred Fell and Dales pony breeds. It presents as a B cell immunodeficiency accompanied by a profound anemia. Affected foals appear normal at birth but fail to thrive. Their hematocrit and B cell numbers decline over 4 to 12 weeks until clinical disease develops. Affected animals lack germinal centers and plasma cells. Their B cell numbers decline to less than 10% of normal and serum immunoglobulin levels drop rapidly once maternal antibodies are catabolized. The loss of these immunoglobulins coincides with the development of clinical disease. T cell numbers remain within the normal range. Animals develop severe respiratory disease caused by opportunistic pathogens such as adenoviruses and from diarrhea caused by *Cryptosporidium.* At the same time, they develop a profound progressive, nonregenerative anemia that alone may be sufficient to cause death. Foals inevitably die or are euthanized by 1

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to 3 months of age.

The syndrome is inherited as an autosomal recessive disease. It results from a mutation in the gene coding for the sodium/myoinositol co-transporter *(SLC5A3).* (The mutation switches a single amino acid from proline to leucine and is homozygous in affected foals.) This protein controls cellular osmotic regulation and is required for lymphoid cell survival and erythropoiesis. Because this mutation may occur in any horse breed, the original name, Fell pony Immunodeficiency Syndrome, has been changed to Foal Immunodeficiency Syndrome. A PCR-based test is available that can be used to determine whether a foal carries the mutant gene. Carrier rates are about 40% for Fell ponies and 20% for Dales ponies. The disease prevalence has been markedly reduced as carrier breeding has been avoided, although the carrier rate remains relatively high as might be expected within such a small breeding population.

Prevalence of Equine Immunodeficiencies

The most important immunodeficiency in foals is not inherited but results from a failure to absorb sufficient colostral antibodies from the mare (Chapter 23). This failure of passive transfer may affect up to 10% of all foals. SCID occurs in 2% to 3% of Arab foals and is 10 times more common than selective IgM deficiency. Selective IgM deficiency is, in turn, 10 times more common than agammaglobulinemia.

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Immunodeficiencies of Cattle

Severe Combined Immunodeficiency

A combined immunodeficiency has been recorded in an Angus calf. The animal was apparently normal when born and suckled normally. It became ill, however, at 6 weeks of age when it developed pneumonia and diarrhea. The animal was lymphopenic and severely hypogammaglobulinemic. It had undetectable IgM and IgA and a low level of IgG, which was believed to be due to residual maternal antibodies. The animal died within a week with systemic candidiasis. It had a hypoplastic thymus consisting of epithelial cells but no thymocytes. It had no detectable lymph nodes and a hypoplastic spleen that had no lymphocytes within its periarteriolar lymphoid sheaths. The syndrome thus closely resembled equine SCID.

Selective Immunoglobulin G2 Deficiency

IgG2 deficiency has been reported in Red Danish cattle. About 1% to 2% of this breed is completely deficient in this immunoglobulin subclass and as a result has an increased susceptibility to pneumonia and gangrenous mastitis. An additional 15% may also have low IgG2 levels, although this does not appear to cause any ill effects.

Hereditary Parakeratosis

Certain Black Pied Danish and Friesian cattle carry an autosomal recessive trait of thymic and lymphocytic hypoplasia (trait A-46). Affected calves are born healthy, but by 4 to 8 weeks they begin to experience severe skin infections. If untreated, they die within a few weeks, and none survive for longer than 4 months. Affected calves have exanthema, hair loss on the legs, and parakeratosis around the mouth and eyes. There is depletion of lymphocytes in the intestine and atrophy of the thymus, spleen, and lymph nodes. These animals are T cell deficient and have depressed cell-mediated immunity but normal antibody responses. Thus they mount a

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normal antibody response to tetanus toxoid but respond poorly to dinitrochlorobenzene or tuberculin, both of which induce cell-mediated reactions. If these calves are treated by oral zinc oxide or zinc sulfate, they recover the ability to mount normal cell-mediated responses. If, however, the zinc supplementation is stopped, the animals will relapse within a few weeks. It is probable that these animals have a reduced ability to absorb zinc from the intestine. Zinc is an essential component of the thymic hormone thymulin (Chapter 12) and is therefore required for a normal T cell response.

Other Immunodeficiencies

A transient hypogammaglobulinemia associated with a delayed onset of immunoglobulin synthesis has been recorded in a Simmental heifer, and a case of thymic aplasia with absence of hair has been described in calves. It resembles the "nude" mutation seen in mice and cats (discussed later).

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Immunodeficiencies of Pigs

Porcine SCID

A spontaneous form of inherited severe combined immunodeficiency has been recognized in inbred Yorkshire pigs. The piglets appeared normal while suckling but were gradually overcome by opportunistic infections resulting in reduced growth, skin lesions, and respiratory distress. They did not survive beyond 60 days. They suffered from pneumonitis, serositis, and dermatitis. Affected pigs were small with a rough hair coat. The thymus was not visible, while lymph nodes and ileal Peyer's patches were small and inconspicuous. Lymph nodes, tonsils, Peyer's patches, and spleen had reduced lymphocyte numbers and no lymphoid follicles. $CD3^+$ T cells were few in lymph nodes and absent from the spleen and bloodstream. The animals lacked both T and B cells in the bloodstream but had normal numbers of NK cells and neutrophils. They did not produce antibodies in response to viral infection (PRRS). There was no sex predilection. Subsequent matings of the parents of these pigs produced approximately 22% of affected piglets per litter, confirming that this is a primary immunodeficiency.

Further studies on these SCID pigs demonstrated two spontaneous mutations. Both mutations occurred in the *Artemis* gene. One mutation (H12) occurs in the splice donor site of exon 8. As a result, exon 8 is deleted resulting in a non-functional protein. The second mutation (H16) is a point mutation in exon 10. This results in a frame-shift and a stop codon is generated so that the protein is truncated. The phenotype of these two mutations is indistinguishable. *Artemis* encodes a small nuclear endonuclease that plays an essential role in *V(D)J* recombination. (The DNA between the *V* and *D* genes is excised and the two ends bind together to form a hairpin [Fig. 39.9]. *Artemis* then cleaves the hairpin at variable sites so that with nucleotide deletion and addition, when the ends are rejoined there are changes in the amino acid sequences in this area.) Thus it plays a key role in generating diversity in both immunoglobulins and T cells. It also makes cells

more sensitive to radiation-induced chromosome breakage.

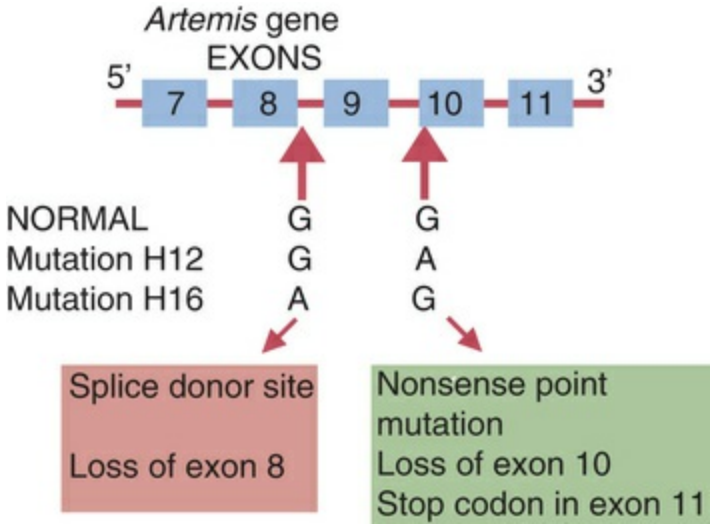


FIG. 39.9 The two identified mutations in the *Artemis* gene that lead to SCID in pigs.

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Immunodeficiencies of Dogs

The nature of dog breeding and the establishment of registered breeds with limited genetic diversity have resulted in the development of numerous inherited canine diseases. Many of these result in primary immunodeficiencies restricted to specific breeds. Few have been investigated thoroughly and the responsible genes have not been identified.

Combined Immunodeficiencies

A severe combined immunodeficiency resulting from a defect in the catalytic subunit of the DNA-dependent protein kinase ($DNA\text{-}PK_{cs}$) has been identified in Jack Russell terriers. From a single breeding pair of terriers, 12 of 32 siblings died from opportunistic infections between 8 and 14 weeks of age. These animals showed a SCID phenotype with lymphopenia, agammaglobulinemia, and thymic and lymphoid aplasia. The disease was an autosomal recessive condition. It resulted from a point mutation, leading to stop codon formation and premature termination of the peptide chain. Affected dogs showed severely diminished expression of $DNA\text{-}PK_{cs}$. As in equine and porcine SCID, the defect blocks gene splicing during *V(D)J* recombination in TCR and immunoglobulin variable regions. The carrier frequency of this gene is 1.1%.

An X-linked SCID has been recorded in Basset Hounds and Cardigan Welsh Corgis. The disease is characterized by stunted growth, increased susceptibility to infections, and absence of lymph nodes. Clinically, animals are healthy during the immediate neonatal period as a result of maternal antibodies. However, by 6 to 8 weeks, as the levels of maternal antibodies decline, the animals begin to develop infections. At first these are relatively mild, such as superficial pyoderma and otitis media. Eventually, they become more severe, and untreated animals die of pneumonia, enteritis, or sepsis by 4 months of age. Common infections include canine distemper, generalized staphylococcal infections, adenoviral and parvoviral disease, and cryptosporidiosis. This is an X-linked immunodeficiency since breeding of a carrier female to a normal

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sire results in approximately half the males in each litter being affected and all the females being phenotypically normal.

These dogs are lymphopenic (~1000/μL). Their CD4/CD8 ratio is, however, approximately 15 : 1, compared with normal dogs that have a ratio of 1.7 : 1, indicating a major drop in $CD8^+$ cell numbers. Their absolute number of T cells is less than 20% of normal. The dogs have normal numbers of B cells. The few lymphocytes in the blood are unresponsive to mitogens. The puppies have normal IgM levels but very low, or no, IgG and IgA. These dogs do not make antibodies against antigens such as tetanus toxoid.

On necropsy the thymus of affected dogs is approximately 10% of the normal weight and lacks a defined cortex (Fig. 39.10). Their lymph nodes and tonsils are very small and dysplastic and may be very difficult to find. When present, the nodes are disorganized and contain few small lymphocytes. Their spleens contain large periarteriolar lymphoid nodules with occasional small lymphocytes and few plasma cells. The bone marrow in these dogs appears normal.

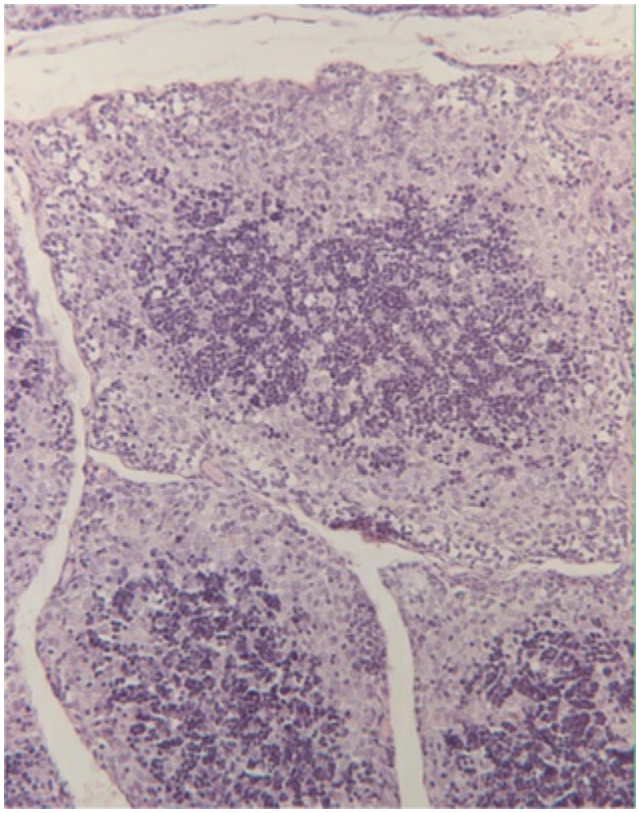


FIG. 39.10 Photomicrograph of the thymus of a Basset Hound with

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X-linked immunodeficiency. Note the lack of a defined cortex and the scattered foci of dark-staining lymphocytes. (H&E stain.) (From Snyder PW, Kazacos EA, Felsburg PJ: Histologic characterization of the thymus in canine X-linked severe combined immunodeficiency, *Clin Immunol Immunopathol* 67:55-67, 1993.)

The disease results from a mutation in the gene coding for the common γc chain of the IL-2R *(IL-2Rγ)*. The same chain is also a component of the IL-4, IL-7, IL-9, and IL-15 receptors.

In affected Basset Hounds, a loss of four bases in the *γc* gene causes a frame-shift. As a result, a stop codon is generated. Thus, instead of the complete protein, only a small peptide is produced, and no functional protein is made. A second SCID mutation has been described in Cardigan Welsh Corgis. In these animals a single cytosine residue is inserted into the *γc* gene so that a stop codon is generated before the transmembrane domain, resulting in a failure to synthesize the complete chain (Fig. 39.11). As a result this peptide is not expressed on the cell surface. In both cases, the mutation does not interfere with IL-2 production, but the lymphocytes of these animals are unresponsive to IL-2. In the absence of a γc chain, mature T cells will not develop.

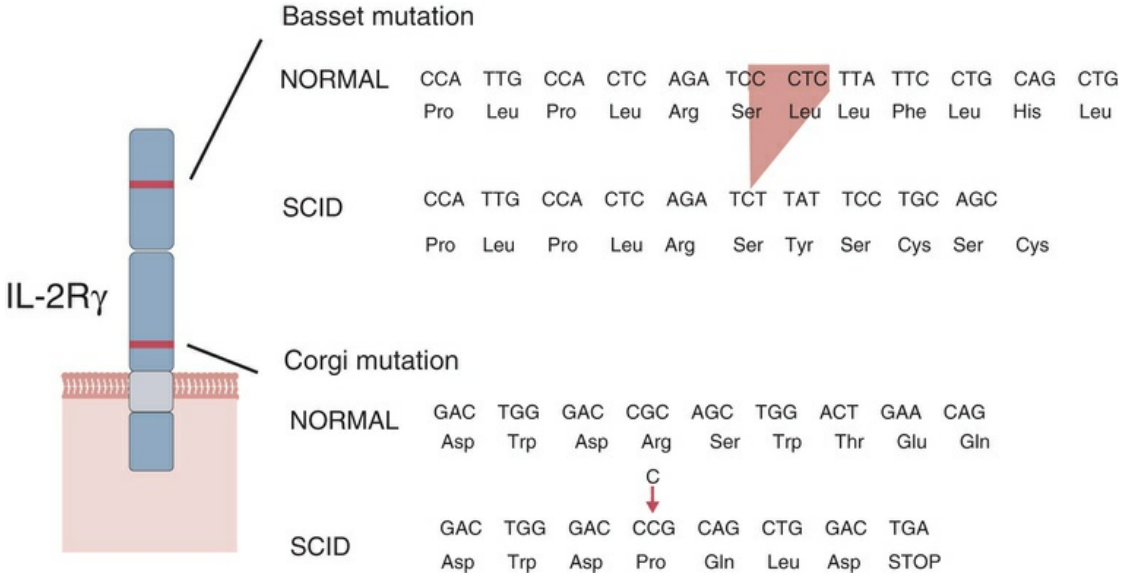


FIG. 39.11 The two defined canine X-linked SCID mutations in the *IL-2Rγ* gene. In the Corgi mutation, the insertion of a single cytosine residue into the gene leads to the generation of a stop codon and premature termination of peptide synthesis. In the Basset mutation, deletion of four bases causes a frame-shift mutation and also leads to the generation of a stop codon (not shown). (Data from Henthorn PS,

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Somberg RL, Fimiani VM, et al: IL-2R gamma gene microdeletion demonstrates that canine X-linked severe combined immunodeficiency is a homologue of the human disease, *Genomics* 23:69-74, 1994; and from Somberg RL, Pullen RP, Casal ML, et al: A single nucleotide insertion in the canine interleukin-2 receptor gamma chain results in X-linked severe combined immunodeficiency disease, *Vet Immunol Immunopathol* 47:203-214, 1995.)

Experimentally, affected dogs may be treated by bone marrow allografts. It is interesting to note, however, that SCID dogs kept alive by stem cell allografts began to age prematurely at 2 to 3 years. They developed intestinal malabsorption and neural cell tumors. Presumably the absence of *DNA-PK*$_{cs}$ prevents the repair of DNA in other cells, leading to premature aging.

Immunoglobulin Deficiencies

A selective IgM deficiency has been reported in two related Doberman Pinschers. One animal was asymptomatic, whereas the other had a chronic mucopurulent nasal discharge and bronchopneumonia. Both these animals had high IgA, low IgG, and very low IgM. They experienced only a chronic nasal discharge, so the clinical significance of this deficiency is in doubt.

Selective deficiencies of IgA have been observed in several breeds of dogs, but German Shepherd dogs and Shar-Peis are especially predisposed to a range of infectious disorders, including mycoses, anal furunculosis, deep pyoderma, and small intestinal bacterial overgrowth. This suggests that they have deficiencies in mucosal immunity. Consistent with this is the observation that German Shepherds in the United Kingdom have normal IgM and IgG levels but significantly reduced levels of IgA (~80 mg/dL, as opposed to 170 mg/dL in control dogs). Likewise, dogs of this breed have significantly lower concentrations of IgA in their tears compared with other breeds. They have normal numbers of IgA-producing plasma cells, implying that the deficiency may be due to defective synthesis or secretion of IgA. German Shepherds have significantly reduced median IgA levels in feces compared with control dogs of other breeds. Many have IgA concentrations below the 95% confidence limit of the control population, and some lack detectable fecal IgA. Their fecal IgG and albumin levels tend to be higher than controls. Other breeds with a significant proportion (>10%) of dogs

with low IgA include Hovawarts, Norwegian Elkhounds, Nova Scotia Duck Tolling Retrievers, Bull Terriers, Golden Retrievers, and Labrador Retrievers (Box 39.1).

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Box 39.1

IgA Deficiency in Wolves

IgA concentrations have been measured in the serum of 163 free-ranging and captive wolves in Scandinavia as well as from 33 Canadian wolves. The median IgA concentration for Scandinavian wolves was 0.054 g/L. The median value for Canadian wolves was 0.18 g/L. The median values in dogs range from 0.15 to 0.3 g/L depending on the breed. Thus relative to dogs and Canadian wolves, Scandinavian wolves are IgA deficient. Up to 60% of Scandinavian wolves had IgA levels as low as high-risk dog breeds such as Shar-Pei dogs. Whether this difference in serum levels is clinically significant, and whether the difference extends to IgA levels on body surfaces or in secretions such as milk, is unclear.

Frankowiack M, Hellman L, Zhao Y, et al: IgA deficiency in wolves, *Dev Comp Immunol* 40:180-184, 2013.

Frankowiack M, Olsson M, Cluff HD, et al: IgA deficiency in wolves from Canada and Scandinavia, *Dev Comp Immunol* 50:26-28, 2015.

Shar-Pei puppies with recurrent cough, nasal discharge, conjunctivitis, and pneumonia, as well as demodicosis and *Microsporum canis* infections, have been identified as having a selective IgA deficiency (<15 mg/dL). Likewise, abnormally low IgA concentrations are found in a high percentage of clinically normal Shar-Pei dogs. Atopic disease is common in these dogs, a feature also seen in IgA-deficient humans. In the German Shepherds, low IgA levels are also associated with the development of canine atopic dermatitis as well as pancreatic acinar atrophy.

A primary IgA deficiency has been described in inbred Beagles. The dogs had a history of parainfluenza and endemic kennel cough due to *Bordetella bronchiseptica.* Despite vaccination, these animals continued to experience recurrent respiratory tract infections and otitis. Immunoelectrophoresis and radial immunodiffusion showed

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normal serum IgG and IgM levels but very little IgA (<5 mg/dL). Phenotypically normal parent dogs had very low IgA levels. Four affected dogs had circulating anti-IgA antibodies. Their T and B lymphocyte numbers and lymphocyte responses to mitogens were normal, as was their response to tetanus toxoid. They had a normal number of plasma cells secreting IgG and IgM, but no plasma cells secreting IgA. When two affected animals were mated, four out of five pups in a litter were IgA deficient. The disease was not sex linked.

A transient hypogammaglobulinemia has been seen in two puppies from a litter of Spitz puppies that experienced recurrent upper respiratory tract infections between 8 and 16 weeks of age. These dogs had normal T cell numbers and mitogen responses. They had low immunoglobulin levels and low antibody titers to vaccine antigens at 16 weeks. The puppies responded very weakly to tetanus toxoid when it was administered at 4 months. By 6 months, however, immunoglobulins had risen to normal levels, and the puppies regained their health. It is believed that these puppies experienced a delayed onset of immunoglobulin synthesis. Symptomatic treatment is sufficient to support these animals until their immune system becomes functional.

Cavalier King Charles Spaniels with *Pneumocystis* pneumonia had IgG concentrations that were significantly lower in affected dogs (median, 3.2 mg/mL) than in breed- and age-matched control dogs (median, 8.5 mg/mL). IgM levels, in contrast, were significantly higher in the affected dogs. IgA levels were within the normal range. Lymphocyte counts in affected dogs were normal or high. This may well be an IgG-deficiency syndrome.

Pneumocystis pneumonia has been observed repeatedly in Miniature Dachshunds. The affected animals were usually less than 1 year old and appeared to be immunodeficient. Serum electrophoresis showed a marked reduction in IgM, IgG, and IgA. In addition, lymphocyte responses to both phytohemagglutinin and pokeweed mitogens were severely depressed. There was a reduction in B cell numbers. Although the *Pneumocystis* pneumonia responded to aggressive therapy, these animals did not do well and died young.

An inherited rhinitis/bronchopneumonia syndrome has been

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identified in Irish Wolfhounds and is presumed to result from an IgA deficiency.

T Cell Deficiencies

A family of inbred Weimaraners has been reported as having immunodeficiency and dwarfism. The animals appeared normal at birth, but at 6 to 7 weeks they developed a wasting syndrome characterized by emaciation and lethargy. The puppies began to experience recurrent infections that eventually killed them. On necropsy their thymuses were atrophied and lacked a cortex. These animals had normal immunoglobulin levels, their helper cell activity was unimpaired, and their secondary lymphoid organs appeared normal. Their lymphocytes were unresponsive to mitogens. Growth hormone treatment caused thymic cortical regeneration and a dramatic clinical improvement. However, growth hormone did not restore lymphocyte responsiveness to mitogens. The disease was almost certainly due to a deficiency of growth hormone as a result of a lesion in the hypothalamus and confirms that the thymus requires growth hormone to function.

Lethal acrodermatitis has been identified in Bull Terriers. This is a complex immunodeficiency syndrome associated with growth retardation, skin lesions (acrodermatitis, chronic pyoderma, paronychia), diarrhea, recurrent pneumonia, and abnormal behavior. The puppies were weak at birth and did not nurse well. Some showed a lighter pigmentation than their littermates. When weaned, they had difficulty eating and failed to grow. Small, crusted lesions developed between the digits, and a pustular dermatitis developed around the eyes and mouth at 6 to 10 weeks. The lesions developed into a severe pyoderma. Fungi such as *Malassezia* and *Candida* were readily isolated from the lesions. Diarrhea developed early in the disease, and respiratory tract infections were common. The puppies became depressed and sluggish and died by 15 months of age, with a median survival of 7 months. They had a neutrophilia, normal IgG and IgM levels but significantly lower IgA levels and hypercholesterolemia. Plasma zinc levels were unusually low. They showed depressed lymphocyte mitogen responses. On necropsy, there was a severe

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loss of T cells so that the puppies lacked a thymus, and the lymph nodes and spleen were very small. The disease was inherited as an autosomal recessive disease, and the parents of affected puppies could be traced to one common ancestor. Because of its similarities to trait A-46 of cattle, these dogs were treated with oral zinc. Very high doses resulted in some clinical improvement, but this could not be sustained.

German Shepherd pyoderma is, as its name implies, a chronic skin disease that occurs in middle-aged German Shepherds and is associated with infection with coagulase-positive staphylococci. These cases do not respond well to antibiotic therapy, and it is believed to reflect some form of underlying genetic or immunological defect. Although affected dogs appeared to mount normal humoral responses, limited studies have shown reduced lymphocyte responses to mitogens, an imbalance of lymphocyte subsets (CD4 cells are depressed, CD8 cells are increased), and a decline in the level of $CD21^+$ B cells. (The complement receptor CD21 plays a role in B cell activation.) When the number of $CD3^+$ T cells and B cells were examined in normal dog skin and in the skin of dogs with pyoderma, it was found that the B cell numbers were similar, but the number of T cells infiltrating the lesions in German Shepherds was significantly reduced, suggesting that T cell dysfunction may play a role in the pathogenesis of pyoderma in this breed.

Uncharacterized Immunodeficiencies

The veterinary literature contains several reports of dogs with severe recurrent infections caused by organisms that are not normally considered to be highly pathogenic. Protothecosis has been recorded in dogs. One-third of the cases have been in Collies, suggesting an inherited predisposition. Weimaraners are unusually susceptible to some systemic bacterial infections; German Shepherds are susceptible to generalized systemic *Aspergillus* infections, whereas some Rottweiler and Doberman families are unusually susceptible to parvovirus infection. None of these have been shown to be due to primary immunodeficiencies, and all require further investigation.

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Immunodeficiencies of Cats

Hypotrichosis With Thymic Aplasia

The nude mouse has long been accepted as an important mouse model of immunodeficiency. Nude mice are a strain of hairless mice that fail to develop a functional thymus. This disease has been described in rats, guinea pigs, and calves. A similar mutation has been described in Birman kittens. These kittens were born without any body hair (Fig. 39.12). On necropsy, they also had no thymus and had depletion of lymphocytes in the paracortex of lymph nodes, spleen, and Peyer's patches. Thus they were effectively T cell deficient. Analysis of the pedigree suggested that the disease was autosomal recessive.



FIG. 39.12 Kittens born with an autosomal recessive form of congenital hypotrichosis with thymic aplasia-nude kittens. (From Casal ML, Straumann U, Sigg C, et al: Congenital hypotrichosis with thymic aplasia in nine Birman kittens, *J Am Anim Hosp Assoc* 30:600-602, 1994. Courtesy Dr. M.J. Casal.)

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Immunodeficiencies of Mice

Many different genetic mutations have been shown to impair immunity in mice. Some of these mutant mice are widely employed in research investigating basic immunologic mechanisms.

Nude Mice

The best-known mouse model of immunodeficiency is the nude mouse. Nude mice are a strain of hairless mice whose thymic epithelial cells are nonfunctional as a result of a defect in the gene for the transcription factor FoxN1. (Similar mutations have been observed in rats, guinea pigs, calves, and cats.) Because their thymic epithelial cells fail to function, the primitive thymus in nude mice develops into cysts with walls of immature epithelial cells that do not produce mature T cells. They do possess a limited number of immature T cells and B cells so that a few lymphocytes may be found in peripheral blood. Normal thymus grafts, by restoring epithelial cell function, permit the T cells of nude mice to mature and develop immune competence. Nude mice are deficient in conventional cell-mediated immune responses, as reflected by prolonged allograft survival and lack of responses to T cell mitogens. Their IgG and IgA levels also are depressed, presumably as a result of a loss of helper T cells.

Although nude mice show enhanced susceptibility to virus-induced tumors, they fail to develop more than the normal level of spontaneous tumors. This observation was, for many years, a major objection to the immunological surveillance theory because if T cells destroy tumors, T cell–deficient animals should have an increased incidence of cancer. However, nude mice possess normal numbers of NK cells, which may protect them in the absence of T cells.

Severe Combined Immunodeficiency Mice

SCID mice have very low numbers of B cells and T cells as a result of a mutation that affects the differentiation of lymphoid stem cells. Development of B cells is halted before expression of cytoplasmic or

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cell membrane immunoglobulins. T cell development is also arrested at a very early stage, and those lymphocytes that do reach the bloodstream are $CD4^-$, $CD8^-$. They have no immunoglobulins and are unable to mount cell-mediated immune responses. SCID mice survive relatively well for about a year in specific-pathogen-free facilities but eventually die of *Pneumocystis* pneumonia. The defects in SCID mice result from an inability to rearrange their BCR or TCR V region genes correctly. Several different mutations in the DNA joining enzymes have been identified. As a result, the cells cannot produce functional receptors, and no functional T or B cells are produced. As in SCID horses, the mouse SCID mutations also increase sensitivity to ionizing radiation since these animals are unable to repair DNA damage. About 15% of SCID mice are "leaky"; they have low levels of immunoglobulins of limited heterogeneity and can reject allografts. Antigen-presenting cells, myeloid and erythroid cells, and NK cells are normal in SCID mice.

Moth-Eaten Mice

Moth-eaten mice have defective dendritic cell and neutrophil function as a result of a mutation in a protein-tyrosine phosphatase gene, *SHP1.* Their name comes from their appearance. Within a few days of birth, neutrophils invade their hair follicles and cause patchy loss of pigment. The deletion in neutrophils results in enhanced integrin signaling leading to cutaneous and lung inflammation. The deletion in dendritic cells is due to exaggerated MyD88-dependent signaling and results in severe autoimmunity. They produce excessive quantities of immunoglobulins and develop autoimmune disease. Mice that are *me/me* have a short life span and usually die as a result of lung damage. The B cell hyperactivity may be due to excessive production of some B cell–stimulating cytokines.

X-Linked Immunodeficiency

Xid mice have a recessive, X-linked B cell defect. The mutation affects a cytoplasmic tyrosine kinase. As a result, B cells cannot respond to certain T-independent carbohydrate antigens. They lack

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certain B cell subsets. Mice that are *bg*/*nu*/*xid* are severely immunosuppressed since they lack T, B, and NK cells. Lightly irradiated *bg*/*nu*/*xid* mice can accept human bone marrow xenografts.

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Immunodeficiencies of Humans

Many different immunodeficiency syndromes have been reported in humans. It is anticipated that investigators will succeed eventually in identifying most of these syndromes in domestic animals as well.

The most important phagocytic deficiency syndrome of humans is chronic granulomatous disease. This has not yet been reported as occurring in domestic animals, although it undoubtedly does. Children affected with chronic granulomatous disease have recurrent infections characterized by the development of septic granulomas in lymph nodes, lungs, bones, and skin. The neutrophils of these children are less capable than normal cells of destroying organisms such as staphylococci and coliforms. Their specific lesion is a defect in one of the subcomponents of the NADPH oxidase (NOX) complex.

Infants suffer from several different forms of combined immunodeficiency. The most severe is reticular dysgenesis, which results from a defect in the development of both myeloid and lymphoid stem cells. Other combined immunodeficiencies result from defects in the development of both T and B lymphoid stem cells. Some of these CID cases are due to a deficiency of the enzyme adenosine deaminase. In other cases, there is a defect in the genes coding for interleukin-2 (IL-2) or IL-7 receptors, for recombinase-activating gene proteins, for CD25, for the CD3γ chain, or for MHC class I or class II molecules. The standard treatment for all these diseases is a stem cell allograft.

T Cell Deficiencies

The DiGeorge anomaly results from a failure of the third and fourth thymic pouches to develop. In consequence, no thymic epithelial tissue develops, and few cells populate the T-dependent areas of the secondary lymphoid tissues. Since these infants have no functional T cells, they can neither mount a delayed hypersensitivity reaction nor reject allografts. The importance of T cells in protection against viruses is emphasized by the observation

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that infants with the DiGeorge anomaly generally die of virus infections but remain resistant to bacteria.

B Cell Deficiencies

The most severe of the human B cell deficiencies, called Bruton-type agammaglobulinemia, is an X-linked recessive disease. Affected infants are devoid of all immunoglobulin classes. They experience recurrent infections due to bacteria such as pneumococci, staphylococci, and streptococci but are usually resistant to viral, fungal, and protozoan infections. The disease results from a mutation in a receptor tyrosine kinase. Inherited deficiencies of individual immunoglobulin classes have also been recorded in humans. As might be anticipated, there are many possible combinations of deficiencies in IgG, IgM, IgA, and IgE, and a tendency to give each a specific name leads to confusion. One of the most important of these is Wiskott-Aldrich syndrome. In this disease, a selective IgM deficiency is associated with multiple infections, eczema, and thrombocytopenia. Another such syndrome is ataxia-telangiectasia, in which serum IgA and IgE levels are extremely low or absent and cerebellar and cutaneous abnormalities exist. Affected children, lacking an effective surface immune system, have recurrent bacterial respiratory tract infections. Ataxia-telangiectasia results from a defect in DNA repair mechanisms. In another disease called hyper-IgM syndrome, a defect in the CD40 ligand leads to a defect in late B cell development and a failure in the IgM class switch so that affected individuals have high levels of IgM but very low (or absent) IgG and IgA. Patients suffer from recurrent respiratory tract infections. The most common human primary immunodeficiency is an IgA deficiency that affects 1 in 600 Caucasians. Some of these individuals are asymptomatic; others suffer from an increased frequency of respiratory and gastrointestinal infections. The genetic defect appears to lie within the MHC complex.

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40

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Secondary Immunological Defects

CHAPTER OUTLINE

Virus-Induced Immunosuppression, 449
Canine Distemper, 450
Retrovirus Infections in Primates, 450
Type D Simian Retrovirus, 451
Retrovirus Infections in Cats, 451
Feline Leukemia, 451
Immunosuppression, 452
Immunity, 452
Diagnosis, 452
Feline Immunodeficiency Virus, 452
Immunosuppression, 453
Immunity and Diagnosis, 454
Retrovirus Infections in Cattle, 455
Retrovirus Infections in Dogs, 455
Circovirus Infections, 455
Other Causes of Secondary Immunodeficiency, 455
Bacterial and Parasite Infections, 455

Toxin-Induced Immunosuppression, 455
Malnutrition and Immunity, 455
Obesity, 456
Trace Elements, 456
Vitamins, 457
Exercise and Immunity, 459
Posttraumatic Immune Deficiency, 460
Age and Immunity, 460
Innate Immunity, 460
Lymphoid Organs, 461
B Cell Responses, 461
T Cell Responses, 461
Other Secondary Immunodeficiencies, 461

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Explain why defects in the immune systems resulting from many forms of damage (secondary immunodeficiencies) are not uncommon in domestic animals.
- Describe how the most significant causes of secondary immunodeficiencies are viral infections.
- Describe the pathogenesis of FeLV- and FIV-induced immunodeficiencies.
- Summarize the data indicating that some environmental toxins and drugs are immunosuppressive.
- Explain how malnutrition due to starvation, vitamin deficiency, or obesity can significantly impair immune functions.

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- Understand why stress of various types suppresses immunity.
- Explain the links between obesity, inflammation, and immunosuppression.
- Explain the basic features of immunosenescence.

The immune system, like any body system, is liable to destruction and dysfunction as a result of attacks by pathogenic and environmental agents. Among the most important of these agents are microorganisms, especially viruses, environmental toxins, stress of various types, malnutrition, and the progressive deterioration of old age.

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Virus-Induced Immunosuppression

Viruses that invade the immune system may be divided into those that affect primary lymphoid tissues and those that affect secondary lymphoid tissues. Both types of virus can cause immunodeficiencies. For example, in chickens, infectious bursal disease virus (IBDV) destroys lymphocytes in the bursa of Fabricius. IBDV is not completely specific for bursal cells; it also destroys cells in the spleen and thymus. These tissues usually recover, whereas the bursa atrophies. The resulting immunosuppression, as might be predicted, is most severe in young birds infected soon after hatching, at a time when the bursa is actively engaged in generating B cells.

Loss of lymphocytes is common in virus infections since viral survival and persistence may require immunosuppression. Thus lymphopenia occurs in feline panleukopenia, canine parvovirus-2 infection, feline leukemia, and African swine fever. Bovine viral diarrhea virus (BVDV) causes destruction of both B and T cells. Surviving B cells fail to make immunoglobulins and respond poorly to mitogens. BVDV destruction of Peyer's patches causes intestinal ulceration and leads to secondary bacterial invasion. Cattle infected with cytopathic BVDV have depressed neutrophil functions and impaired bacterial clearance from the blood.

Herpesviruses are also immunosuppressive. For example, equine herpesvirus-1 causes a drop in T cell numbers and depresses cell-mediated responses in foals. Bovine herpesvirus-1 (BHV-1) also causes a drop in T cells and in their responses to mitogens. Although BHV-1 stimulates bovine alveolar macrophages to increase expression of MHC class II and promotes phagocytosis, it also depresses macrophage-mediated cytotoxicity and IL-1 synthesis. Parainfluenzavirus-3 has long been known to interfere with alveolar macrophages. It inhibits phagosome-lysosome fusion paving the way for secondary infections with *Mannheimia hemolytica* in stressed calves. Porcine reproductive and respiratory syndrome (PRRS) virus in pigs causes destruction of alveolar macrophages and predisposes affected animals to severe enzootic pneumonia. It also kills dendritic cells, a feature that may account for the ability of

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PRRS virus to persist in pigs for up to 6 months.

The results of virus-induced lymphoid tissue destruction are usually obvious. Animals are lymphopenic and have reduced lymphocyte responses to mitogens. For example, responses to phytohemagglutinin are depressed in influenza, measles, canine distemper, Marek's disease, Newcastle disease, feline leukemia, and bovine virus diarrhea. Destruction of lymphoid tissues may also result in hypogammaglobulinemia or a reduced response to vaccine antigens (Fig. 40.1). Thymic atrophy and lymphopenia are common manifestations of many virus infections, and before any primary immunodeficiency syndrome is diagnosed, rigorous steps must be taken to exclude the possibility that it is, in fact, secondary to a virus infection.

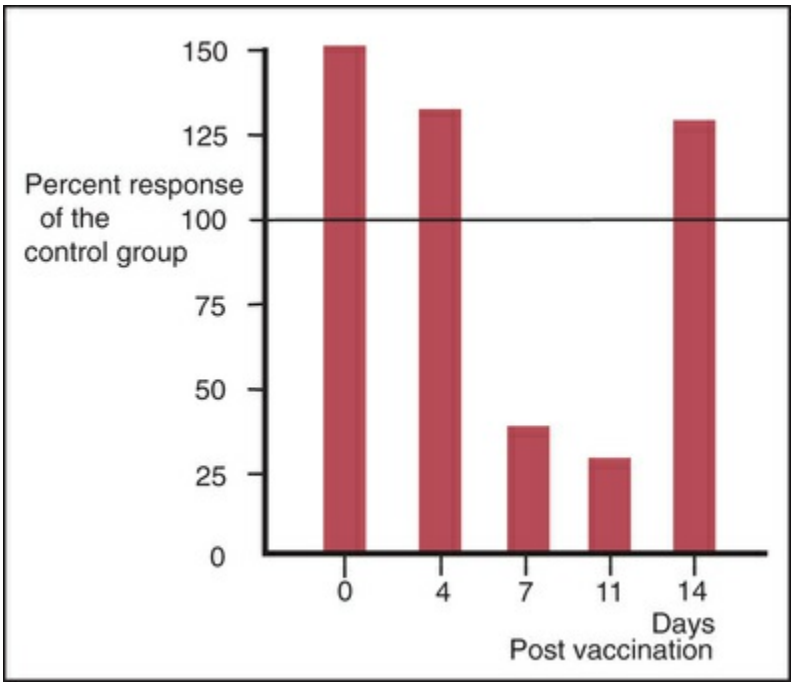


FIG. 40.1 The immunosuppressive effect of viruses. The effect of administering a mixed vaccine (containing canine distemper, canine adenovirus, canine parainfluenza, canine parvovirus-2, and leptospira) on the response of a puppy's lymphocytes to the mitogen phytohemagglutinin. Control levels were 100%. (From Phillips TR, Jensen JL, Rubino MJ, et al: Effects of vaccines on the canine immune system, *Can J Vet Res* 53:154-160, 1989.)

Canine Distemper

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One important virus that destroys secondary lymphoid organs is canine distemper. Canine distemper virus (CDV), although it can multiply in many different cell types, has a predilection for lymphocytes. Its major cellular receptor is CD150, expressed on activated B and T cells. CDV spreads from its initial invasion sites in the tonsils and bronchial lymph nodes to the bloodstream, where it kills both T and B cells and causes a lymphopenia. Subsequently it invades the thymus, spleen, lymph nodes, and mucosal lymphoid tissues, where it destroys yet more cells. Thymic atrophy occurs and lymphocytes are depleted in spleen, lymph nodes, and tonsils. There is a complete loss of secondary follicles. The bone marrow, in contrast, is minimally affected. The lymphocyte populations most affected are $CD4^+$ T, $CD8^+$ T, and $CD21^+$ B cells. CDV also suppresses production of interleukin-1 (IL-1) and IL-2, while stimulating prostaglandin release by macrophages. The CDV N-protein interacts with FcγR (CD32) to suppress IL-12 production and B cell maturation. As a result, lymphocyte responses to mitogens are depressed, immunoglobulin levels fall, and skin allograft rejection is suppressed. Subsequent regeneration of the lymphoid organs leads to a recovery of double-negative T cell subsets. The numbers of CD5- and immunoglobulin-positive cells remain low and recovered dogs remain profoundly immunosuppressed.

This immunosuppression accounts, in large part, for clinical disease. For example, many dogs with distemper develop *Pneumocystis* pneumonia. (*Pneumocystis* is a fungus that occurs in the lungs. It does not cause disease in immunocompetent animals but produces a severe pneumonia in animals with suppressed immune function. Indeed, the development of *Pneumocystis* pneumonia is evidence of a significant immunodeficiency.) If virulent distemper virus infects germ-free dogs, they develop a relatively mild disease, presumably because secondary infection does not occur.

CDV also causes a demyelinating leukoencephalomyelitis. This is a two-stage process. The initial lesion is probably due to direct viral activity, but this is followed by progression triggered by a strong Th1 response and the production of proinflammatory cytokines (IL-6, IL-8, IL-12, and TNF-α). Thus the damage may be secondary to

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excessive macrophage function and bystander mechanisms. Cytotoxic $CD8^+$ cells may also contribute to the loss of myelin.

Retrovirus Infections in Primates

More than 40 lentiviruses have been isolated from nonhuman primates, especially African species. These simian immunodeficiency viruses (SIV) selectively invade $CD4^+$ T cells. When SIV_{mac} infects rhesus macaques and other Asian species, it stimulates a strong but ineffective immune response. Viral replication continues and eventually causes an immunodeficiency syndrome similar to human acquired immunodeficiency syndrome (AIDS). These infections are believed to be transmitted sexually. Clinical disease progression is slow, but the animals eventually develop lymphadenopathy, severe weight loss, chronic diarrhea, lymphomas, neurological lesions, and opportunistic infections by organisms such as *Pneumocystis, Mycobacterium avium-intracellulare, Candida albicans,* and *Cryptosporidium parvum.* The macaques are depleted of $CD4^+$ T cells, macrophages, and dendritic cells. The virus invades both T cells and macrophages using two cellular receptors, CD4 and the chemokine receptors CCR5 or CXCR4. About 25% of infected animals do not mount a significant response to SIV and die within 3 to 5 months from severe SIV encephalitis. Macaques that mount an immune response usually die 1 to 3 years after infection. Spontaneous recovery does not occur.

Type D Simian Retrovirus

Immunodeficiencies also develop in primates infected with type D simian retroviruses (SRVs). These viruses, much more common than the lentiviruses, are transmitted by biting. SRVs have a much broader tissue tropism than the SIVs and, in addition to lymphocytes and macrophages, can also infect fibroblasts, epithelial cells, and the brain. The SRVs destroy both B and T cells, leading to death from opportunistic infections. The syndrome is associated with a profound drop in serum IgG and IgM and a severe lymphopenia. Monocyte function is unimpaired, but surviving lymphocytes do not respond to mitogens. Affected monkeys are also profoundly neutropenic. On necropsy, the monkeys have a

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generalized lymphadenopathy, hepatomegaly, and splenomegaly. There is a loss of lymphocytes from the T-dependent areas of the secondary lymphoid organs. B cell areas show an initial hyperplasia of the secondary follicles followed by the loss of these follicles and of plasma cells. These changes are very similar to those seen in AIDS in humans. In many cases, normally innocuous agents such as *Pneumocystis,* cytomegalovirus, *C. parvum,* and *Candida albicans* cause infection. Some affected monkeys develop tumors such as fibrosarcomas. About half of the infected animals develop neutralizing antibodies and survive the disease. The others die from septicemia or diarrhea with wasting.

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Retrovirus Infections in Cats

Feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV) are retroviruses. FIV causes an acquired immunodeficiency syndrome, neurological disease, and some tumors. It is not itself highly pathogenic, and FIV-infected cats may live for many years. FeLV is more pathogenic and causes diverse clinical diseases including lymphomas, leukemia, bone marrow suppression leading to anemia, and immunosuppression. Effective vaccines against FeLV have significantly reduced its prevalence.

Feline Leukemia

Feline leukemia virus (FeLV) is an oncogenic retrovirus that causes both proliferative and degenerative diseases in cats (Fig. 40.2). A unique surface protein, called feline oncornavirus cell membrane antigen (FOCMA), is expressed on FeLV-infected cells. It is coded for by endogenous retroviral genes within the cat genome. It is not expressed on normal cells but rather on cells infected with FeLV or feline sarcoma virus (FeSV). It was originally believed that the presence of FOCMA on a cell membrane identified the cell as a FeLV-induced tumor cell. Of those cats that fail to make neutralizing antibodies to FeLV and remain viremic, about 80% develop antitumor activity by making antibodies against FOCMA. A cat that makes antibodies to FOCMA can usually destroy virus-induced tumor cells. Unfortunately, antibodies to FOCMA do not confer protection against FeLV-induced degenerative diseases, and viremic cats that fail to produce anti-FOCMA antibodies are fully susceptible to all FeLV syndromes, including lymphosarcoma.

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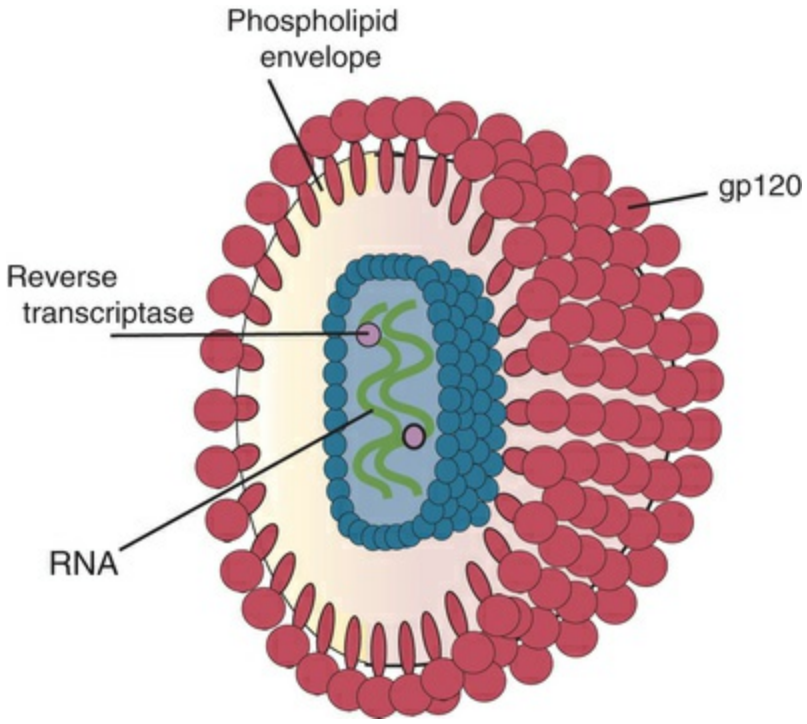


FIG. 40.2 The structure of a typical retrovirus such as feline leukemia virus or feline lentivirus.

On exposure to FeLV, about 70% of cats become infected, but the remaining 30% do not. Of the infected cats, about 60% become immune and 40% become viremic. Of the viremic cats, 10% cure spontaneously, whereas the remaining 90% remain infected for life. Of these persistently viremic animals, about 15% live normal healthy lives, but the remaining animals die within 3 to 5 years from FeLV disease. Lymphoid tumors develop in 15% to 20% of FeLV-infected cats. Persistently viremic cats have a half-life of 1 year.

Once FeLV infects a cat, the virus first grows in the lymphoid tissues of the pharynx and tonsils. This is followed by a transient viremia as it spreads throughout the body and infects the other lymphoid organs. A mild lymphopenia and neutropenia occur 1 to 2 weeks after infection. Antibodies develop between 7 and 42 days after the onset of infection, and the virus is cleared between 28 and 42 days. Virus can be found in the thymus at day 1, in blood between 2 and 145 days, and in lymphoid organs between 3 and 28 days. Some cats may become latently infected where the virus persists in the bone marrow and virus-neutralizing antibodies are present.

FeLV causes multiple cancers such as lymphosarcomas,

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reticulum cell sarcomas, erythroleukemias, and granulocytic leukemias. FeLV lymphosarcomas are usually of T cell origin. Some alimentary lymphomas are of B cell origin. In young cats, FeLV-induced tumors are mainly of T cell origin. In older cats, they originate from both T and B cells.

Immunosuppression

T cell defects.

FeLV develops T cell tropic variants (FeLV-T) as a result of mutations in the gene encoding the viral envelope glycoprotein, gp70. These variants replicate to high numbers in T cells.

The lymphopenia in FeLV-T–infected cats results from a loss of $CD4^+$ T cells. $CD8^+$ T cells may also drop in the early stages of the disease so that the CD4/CD8 ratio may remain within normal limits. (The CD4/CD8 ratio in normal cats ranges from about 0.4 to 3.5, with a median value of about 1.9.) $CD8^+$ T cell numbers eventually recover, and the CD4/CD8 ratio then drops. B cell numbers may also be depressed, but this depends on the severity of secondary infections. In cats without secondary infection, lymphoid atrophy is associated with loss of cells from the paracortex of lymph nodes. Changes in the spleen are less marked but may result in shrinkage of the entire white pulp. As a result of T cell loss, FeLV-infected cats have depressed cell-mediated immunity. This depression is probably due to the effects of p15e, the immunosuppressive envelope protein of FeLV, which is produced in very large quantities by dying cells. p15e suppresses the responses of cats to FOCMA and to lymphocyte mitogens and blocks the responses of T cells to IL-2. As a result, FeLV-infected cats may carry skin allografts for about twice as long as normal cats (24 days, compared with 12). As early as 9 weeks after infection, the $CD4^+$ T cells produce lower levels of B cell stimulatory cytokines. This is followed by a drastic drop in $CD4^+$ T cells, whereas $CD8^+$ T cell and B cell numbers remain normal. Leukocytes of infected cats produce significantly less IL-2 than leukocytes from normal cats. Bone marrow stem cells are also inhibited by p15e, preventing production of erythroid cells and resulting in a nonregenerative anemia.

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Kittens infected with FeLV-T develop a wasting syndrome associated with thymic atrophy and recurrent infections. Depending on the severity of the secondary infections, this may be associated with either lymphoid atrophy or lymphoid hyperplasia. In adult cats, this syndrome is characterized by progressive weight loss and lymphoid hyperplasia, followed by severe lymphoid depletion and chronic diarrhea. The immunosuppression also predisposes viremic cats to secondary infections such as feline infectious peritonitis, mycoplasmosis, toxoplasmosis, septicemia, and fungal infections.

B cell defects.

In contrast to the severe T cell dysfunction, B cell functions in FeLV-infected cats are only mildly impaired. There may be poor responses to antigen and reduced IgM production, but serum IgG levels remain normal. Because of this, chronically infected cats produce large quantities of antiviral antibodies. These antibodies combine with circulating virions or soluble proteins to form immune complexes. The immune complexes are deposited in the renal glomeruli and cause severe mesangioproliferative glomerulonephritis, leading to hypoproteinemia, edema, uremia, and death. Viral antigens binding to erythrocytes can also cause an antiglobulin-positive hemolytic anemia.

Immunity

About 40% of cats infected with FeLV become persistently infected. Persistently infected cats remain viremic. The remaining 60% of infected cats mount a strong immune response and develop virus-neutralizing antibodies to gp70. Immune cats also develop virus-specific cytotoxic T cells to some viral antigens. These prevent the virus from invading cells, and these cats become strongly immune.

Vaccines are currently available against FeLV. One type contains supernatant fluid from a cell line persistently infected with FeLV. This fluid contains several of the major protein antigens of FeLV. The second type of FeLV vaccine consists of inactivated whole virions from tissue culture, which are usually administered with a powerful adjuvant. The third type of FeLV vaccine is a live canarypox-vectored recombinant product that can be administered

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without an adjuvant. Widespread vaccination has significantly reduced the prevalence of this disease in the United States. These vaccines differ in their ability to prevent latent infections, although all are effective in preventing the development of clinical disease.

Diagnosis

The introduction of sensitive molecular diagnostic techniques to replace serologic assays has changed our views on persistent FeLV infections. Real-time and reverse-transcriptase PCR assays are much more sensitive and specific than virus isolation, antigen detection, or immunofluorescence. These tests have shown that many cats may have FeLV DNA integrated into their cells but never develop an antigenemia. Vaccines may be able to prevent the development of clinical disease but not proviral integration. These latent infections may persist for years, and viremia or disease develops occasionally. On the other hand, this integrated viral DNA may also be required for long-term protection. Other cats may have both detectable viral nucleic acids and antigenemia (i.e., active infections). FeLV antigenemia may be detected by an antigen-capture ELISA, by the membrane filter technique, or by rapid immunochromatography of blood or serum. A direct immunofluorescent test on a buffy coat smear using antibodies to group-specific antigen can detect cell-associated antigen and hence intracellular viremia (Fig. 40.3).

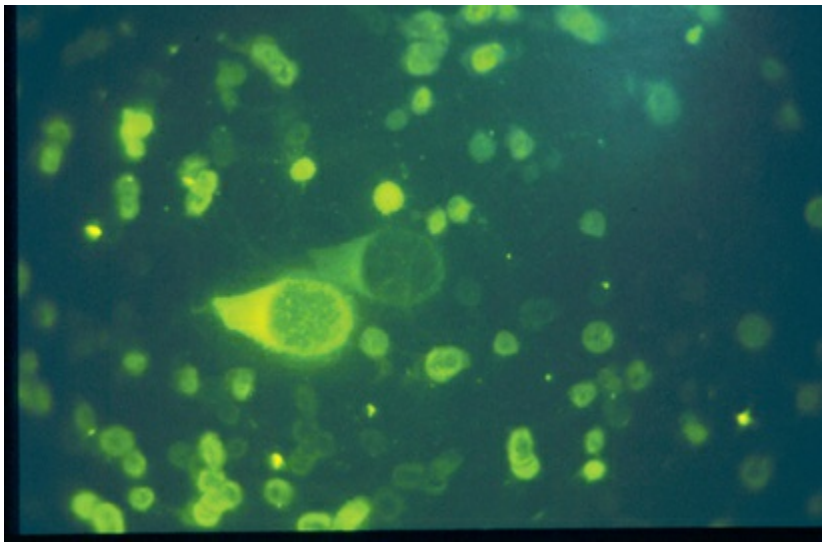


FIG. 40.3 A positive indirect immunofluorescence assay for FeLV in a peripheral blood smear from a cat. (Courtesy Dr. F.C. Heck.)

Feline Immunodeficiency Virus

Feline immunodeficiency virus (FIV) was originally isolated from cats with clinical immunodeficiency. It is an enveloped, single-stranded RNA virus belonging to the lentivirus subgroup of retroviruses. FIV is related to HIV, the cause of AIDS (Fig. 40.4). FeLV and FIV are distinctly different viruses. Nevertheless, approximately 12% to 33% of FIV-infected cats may also be infected with FeLV, an especially potent immunosuppressive mixture.

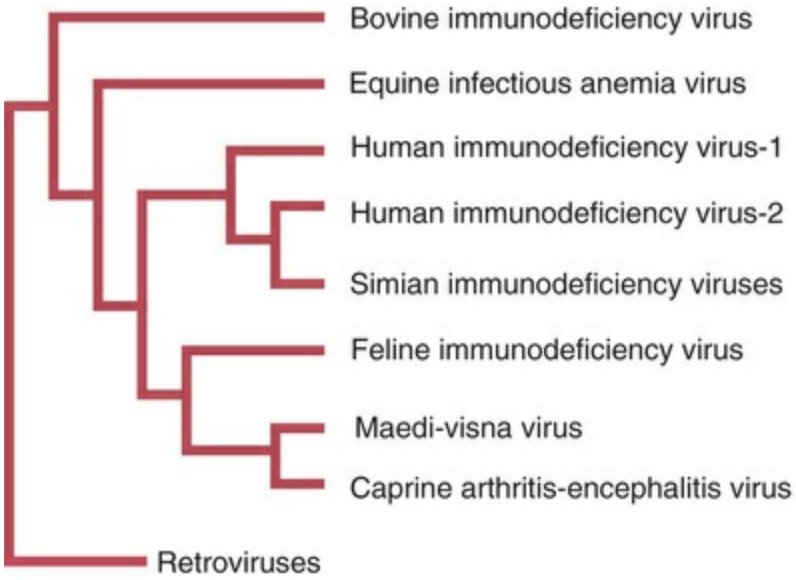


FIG. 40.4 A dendrogram showing the relationships of the major lentiviruses. Many of these destroy lymphocytes and so cause immunodeficiencies.

FIV is spread by territorial free-roaming male cats through aggressive biting but it can also be sexually transmitted.

Cats develop a fever about 3 to 10 weeks after exposure to FIV. The virus is carried to local lymph nodes, where it replicates in T cells. It then spreads to other lymph nodes throughout the body. Some cats may develop a generalized lymphadenopathy at this stage. They may also develop a mild lymphopenia as a result of a loss of $CD4^+$ T cells.

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This is followed by an asymptomatic stage that may last as long as 10 years. Cats appear healthy during this stage, but their $CD4^+$ T cell numbers drop progressively. Their lymph nodes show gradual hypoplasia, leading to aplasia. Cats may also develop bone marrow suppression, resulting in leukopenia and anemia. Thus this stage is marked by progressive impairment of immune function, but it may be many years before severe immunodeficiency and AIDS-like signs develop.

The gradual onset of progressive generalized lymphadenopathy marks the third stage of the disease. Lymph nodes develop follicular hyperplasia. As a result of the growing immunodeficiency, cats develop secondary but not opportunistic infections. These are mainly bacterial infections affecting the oral cavity, skin, and digestive tract.

The final stage is a severe disease that lasts for a few months until the cat dies. Secondary lymphoid tissues show follicular involution. Because of their severe immunodeficiency, opportunistic infections develop. These can include feline herpesvirus type I, rodent poxviruses, vaccine-induced rabies, FeLV, staphylococcal infections, anaerobic infections, tuberculosis *(Mycobacterium avium-intracellulare), Cryptococcus,* toxoplasmosis, mange, lungworms, and heartworms. Malignancies and ocular and neurological disease also occur. Clinical findings can include chronic fever, oral cavity disease (periodontitis, gingivitis, stomatitis), chronic upper respiratory tract disease, chronic enteritis leading to persistent diarrhea, and conjunctivitis, anemia, leukopenia, lymphosarcoma, and myeloproliferative disorders.

Immunosuppression

FIV replicates in $CD4^+$ and $CD8^+$ T cells, B cells, megakaryocytes, neuronal cells, and macrophages. Some strains only replicate well in lymphocytes, whereas others replicate well in both lymphocytes and macrophages. Primary targets of FIV infection are the lymphocytes. However, as infection persists, the virus increasingly affects macrophages. In clinically ill cats with a high viral load, macrophages are the major sites of viral replication. FIV-infected cats have fewer neutrophils, a lower proportion of T cells, and a higher proportion of B cells compared to uninfected animals.

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FIV binds specifically to CD134 expressed on a subset of $CD4^+$ T cells. This binding, in conjunction with binding to the chemokine receptor CXCR4 (CD184), is required for FIV to infect a cell. Most naturally infected cats suffer a critical loss of $CD4^+$ T cells. This loss is due to destruction of infected cells, decreased production, and premature apoptosis (Fig. 40.5). The surviving $CD4^+$ cells may show reduced responses to mitogens. FIV cats may show a shift away from a Th1 cytokine production pattern. They may also show an increase in $CD8^+$ T cells. As a result, the CD4/CD8 ratio of FIV-infected cats may drop to less than one. FIV chronically activates Treg cells, which further contributes to the immunosuppressive effect by downregulating IL-2 production and inhibiting $CD4^+$ T cell proliferation. Affected cats show decreased IL-2 and IL-12 production as well as increased IL-10. Thus the rise in the IL-10/IL-12 ratio is especially immunosuppressive.

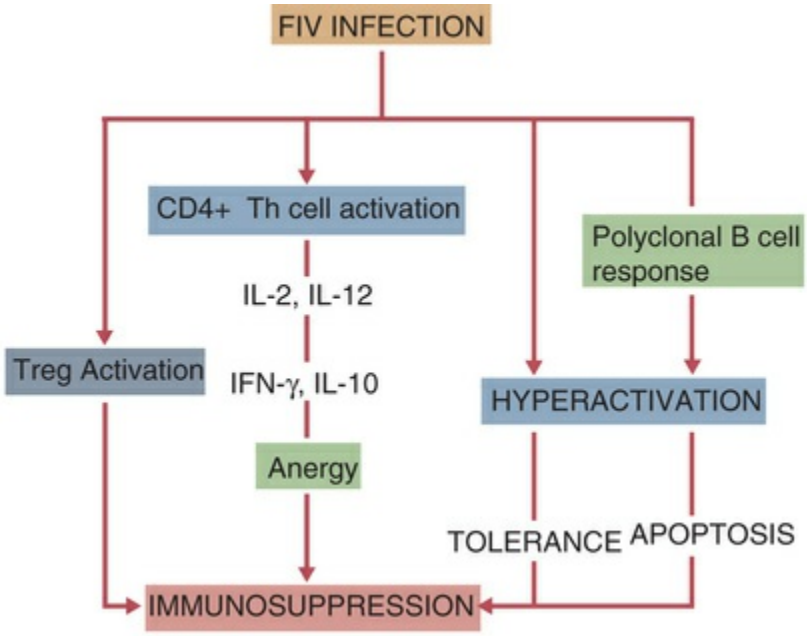


FIG. 40.5 The major immunosuppressive pathways activated in feline immunodeficiency virus infections.

During the acute phase of FIV, Treg cells are infected and activated by the virus. The virus induces both Foxp3 and membrane-bound TGF-β production and as a result suppresses IL-2 production by $CD4^+$ Th1 cells. The TGF-β converts Th1 cells into Treg cells. The Treg cells also suppress $CD8^+$ T cell responses by

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inducing cell cycle arrest. The Treg cells induce their targets to produce Foxp3 that then binds to the IL-2 promoter and effectively inhibits IL-2 production and effector cell function. This Treg activation severely limits the development of an effective antiviral response.

The lymphopenia that develops in both FeLV and FIV infections is due to a loss of T cells (Fig. 40.6). $CD4^+$ T cells are depressed in both, but the depression is much greater in FIV-infected animals. FIV-infected cats show a rapid drop in T cell numbers, whereas their B cells are unaffected. Their $CD8^+$ T cells recover, but their $CD4^+$ T cells fail to do so. Within 6 months of FIV infection, there is a measurable drop in $CD4^+$ T cells. The response to thymus-dependent and -independent antigens initially remains unchanged. By 2 to 3 years after the onset of infection, however, the drop in $CD4^+$ T cells continues, and the response to thymus-dependent antigens is profoundly depressed, while the response to thymus-independent antigens is unchanged. FIV-infected cats may have normal numbers of $CD8^+$ T cells and B cells and normal levels of IgM and IgA. Indeed, more than 25% of FIV-infected cats may be hypergammaglobulinemic as a result of polyclonal B cell activation. Affected cats may also have immune complexes in their blood and renal glomeruli.

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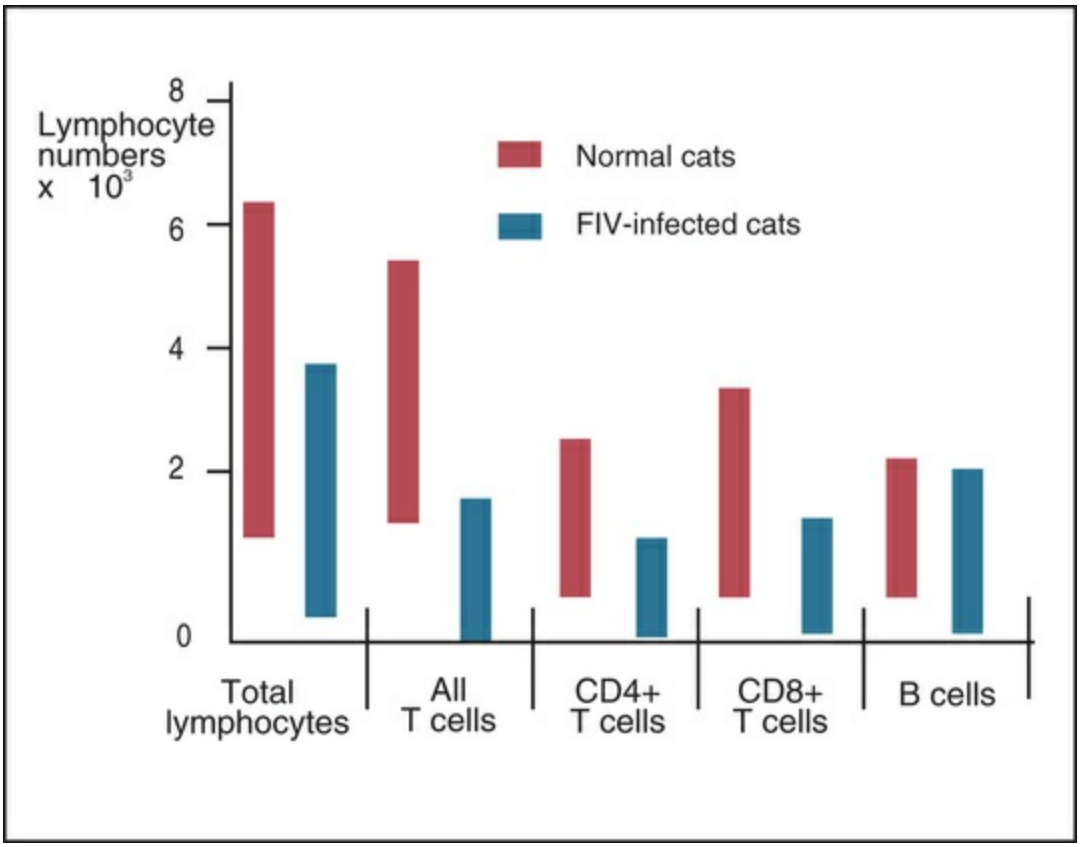


FIG. 40.6 The numbers of cells in different lymphocyte populations (pan T, CD4, CD8, B cells) for 11 normal cats and 11 cats infected with feline immunodeficiency virus. (From Novotney C, English RV, Housman J, et al: Lymphocyte population changes in cats naturally infected with feline immunodeficiency virus, *AIDS* 4:1213-1218, 1990.)

Immunity and Diagnosis

FIV infection may be diagnosed by testing for antibodies by ELISA or immunochromatography and confirmed by Western blotting or PCR. Antibodies appear by 2 weeks after infection, and most cats are positive by 60 days. These antibodies persist for the life of the animal, although they may become undetectable in terminal disease. Maternal antibodies persist in most kittens born to FIV-positive queens for the first 8 to 12 weeks of life regardless of whether the kitten is infected. Some may remain seropositive for up to 16 weeks. These antibodies afford protection, and kittens that receive high levels of antibodies from vaccinated or infected queens are protected.

Envelope glycoproteins stimulate strong cell-mediated and humoral immunity in cats. Good results have been obtained using inactivated whole FIV and certain DNA vaccines. An adjuvanted,

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inactivated vaccine against FIV clades A and D is commercially available.

Retrovirus Infections in Cattle

Bovine immunodeficiency virus (BIV) is a lentivirus originally isolated from a cow with lymphosarcoma. This animal showed lymph node hyperplasia, lymphocytosis, central nervous system lesions, loss of weight, and weakness. BIV-infected calves develop transient lymphocytosis, lymphadenopathy, and a nonsuppurative meningoencephalitis. BIV infection may also cause minor changes in the response of lymphocytes to mitogens and may suppress some neutrophil functions such as antibody-dependent cell-mediated cytotoxicity. BIV can also infect sheep. In this species, experimental infection is associated with an increase in $CD2^+$ and $CD4^+$ T cells, as well as in the CD4/CD8 ratio, between 6 and 8 months after inoculation. The sheep showed no signs of illness by 1 year after inoculation and appeared to have normal immune function.

Retrovirus Infections in Dogs

Several different retroviruses have been isolated from dogs, although the existence of a "canine immunodeficiency virus" has not been established. Examples include a lentivirus isolated from the mononuclear cells of a leukemic German Shepherd. On inoculation into newborn Beagles, it caused pronounced lymphadenopathy. A C-type retrovirus has been isolated from a dog with anemia, neutropenia, lymphopenia, and thrombocytopenia, as well as depressed humoral and T cell–mediated immune responses. On necropsy, the dog showed depletion of lymphoid organs and bone marrow hypoplasia, yet it also had plasma cell infiltrates in many organs as well as multiple secondary infections. A lentivirus has also been isolated from a dog with hemorrhagic gastroenteritis. This animal showed a lymphopenia and agammaglobulinemia with lymphoid and bone marrow hypoplasia. The virus could grow in canine lymphocytes and thymocytes and was present in bone marrow, intestine, and

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lymph nodes. It reduced synthesis of IL-2 and reduced responsiveness to IL-2, and it was cytotoxic for lymphocytes.

Circovirus Infections

Circoviruses are small, nonenveloped DNA viruses that damage lymphoid tissues. They include the chicken anemia agent, which infects hemocytoblasts in the bone marrow and precursor T cells in the thymus; beak-and-feather disease virus, which can cause lymphoid atrophy in psittacine birds; and porcine circovirus-2 (PCV2), which is associated with postweaning multisystemic wasting syndrome (PMWS). PMWS is an acquired immunodeficiency syndrome of piglets characterized by wasting, lymphadenopathy, and respiratory disease. Some affected piglets have profound lymphocyte depletion, initially involving $CD4^+$, $CD8^+$, and double-positive T cells. T cell areas in tonsils and lymph nodes are depleted, and there is an absence of follicles in the cortex. Some pigs develop a necrotizing lymphadenitis as a result of hypertrophy and hyperplasia of their high endothelial venules, leading to thrombosis and necrosis. IgM-positive B cells are also reduced in chronic cases. Lymphoid depletion is directly related to viral load in lymphoid organs. Infected piglets suffer from diverse secondary and opportunistic infections. Although PCV2 is the most likely causative agent, it has proved difficult to reproduce the disease consistently, and other factors including environment and other infectious agents are also involved.

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Other Causes of Secondary Immunodeficiency

Bacterial and Parasite Infections

Immunosuppression generally accompanies infestation with *Toxoplasma* or trypanosomes, helminths such as *Trichinella spiralis,* arthropods such as *Demodex,* and bacteria such as *M. hemolytica,* the actinobacilli, and some streptococci (Chapters 26 and 28).

Toxin-Induced Immunosuppression

Many environmental toxins such as polychlorinated biphenyls, polybrominated biphenyls, dieldrin, iodine, lead, cadmium, methyl mercury, and DDT are immunosuppressive. $CdCl_2$ and $HgCl_2$ both inhibit phagocytosis by bovine leukocytes at very low concentrations. Higher concentrations are required to inhibit NK cell function and cell proliferation.

Mycotoxins are important immunosuppressants in cattle, pigs, or poultry fed moldy grain. The most prevalent of these are derived from *Fusarium* species. These include the trichothecenes (deoxynivalenol and T-2 toxin) and the fumonisins. When deoxynivalenol was administered to pregnant sows it resulted in low levels of colostral IgA and reduced IgA and IgG in their piglets. It can also significantly reduce the response of pigs to some vaccines. Deoxynivalenol causes immunosuppression, greater susceptibility to mastitis in dairy cattle, and high somatic cell counts in milk. T-2 toxin depresses the response of calf lymphocytes to mitogens and decreases the chemotactic migration of neutrophils. T-2 toxin also reduces IgM, IgA, and C3 levels in cattle. Trichothecenes are also immunosuppressive in pigs and birds. Fumonisin B1 inhibits division of both T and B cells in piglets, increases interferon-γ (IFN-γ) production while suppressing IL-4 production, and increases susceptibility to *E. coli* infections.

Aflatoxins from *Aspergillus* increase the susceptibility of chickens to *Salmonella* as a result of depressed phagocytosis. They also increase the susceptibility of dairy cattle to mastitis. They depress

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piglet growth and reduce immune responses to *Mycoplasma.* Toxin-induced immunosuppression may be especially important in wild carnivores situated at the top of the food chain. A good example of this is seen in seals feeding on environmentally contaminated fish. These animals show depressed responses to vaccines, impaired mitogenic responses, lowered delayed hypersensitivity responses, and reduced NK cell numbers. This immunosuppression may decrease their resistance to phocine morbillivirus.

Malnutrition and Immunity

It has long been recognized that famine and disease are closely associated, and we tend to assume that malnutrition leads to increased susceptibility to infection. The effects of malnutrition on immune functions are, however, complex. Malnutrition includes not only deficiencies but also excesses or imbalances of individual nutrients.

In general, severe nutritional deficiencies reduce T cell function and therefore impair cell-mediated responses, at the same time sparing B cell function and humoral immunity. Thus starvation rapidly induces thymic atrophy. The number of circulating T cells drops, and cells are lost from the T cell areas of secondary lymphoid organs. Delayed hypersensitivity reactions are reduced, allograft rejection is delayed, and IFN-γ production is impaired. Protein starvation selectively suppresses Th2 responses such as IL-4 and IgE production, leading to increased susceptibility to parasite invasion.

Severe starvation has little effect on B cell functions. The B cell areas in lymphoid tissues and the number of circulating B cells remain unchanged. Serum immunoglobulins of all classes may remain normal or even rise. Secretory IgA levels commonly drop, but secretory IgE may rise, suggesting abnormal immunoregulation. Starvation, however, depresses complement levels and impairs neutrophil and macrophage chemotaxis, the respiratory burst, release of lysosomal enzymes, and microbicidal activity.

Obesity

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Adipose tissue was long considered a resting tissue where fat reserves were stored until needed. We now know, however, that adipose tissue plays an active role in both innate and adaptive immunity. The major cell types found in adipose tissue are adipocytes and macrophages. Both produce multiple cytokines. For example, adipocytes produce two cytokines (also called adipokines): leptin and adiponectin. Leptin levels increase in the obese and this suppresses appetite. Adiponectin, in contrast, counteracts the activities of leptin and its level drops in the obese. Leptin levels are elevated in obese dogs and adiponectin levels are elevated in lean dogs. Serotonin also controls appetite. Its levels are higher in lean dogs and it may play a role in controlling appetite. All of these molecules, leptin, adiponectin, and serotonin influence the immune system.

Leptin is a 16-kDa protein produced by adipocytes. While first described as an antiobesity hormone, it also influences both innate and adaptive immunity and promotes inflammatory responses. It binds to receptors in the hypothalamus and suppresses the appetite. The amount of leptin in blood is proportional to the amount of fat in the body. The fatter an animal, therefore, the more leptin is produced, and appetite is correspondingly suppressed. Conversely, caloric restriction and weight loss lead to a loss of adipocytes, a drop in leptin levels, and an increase in appetite (Fig. 40.7). Breed-specific differences in leptin levels occur in dogs. The significance of this is unknown. In cats, leptin levels rise after spaying, which may account for the weight gain commonly observed in these animals. In horses, leptin levels in colostrum are two to three times those in blood, and it has been suggested, therefore, that this leptin may be is required for intestinal development.

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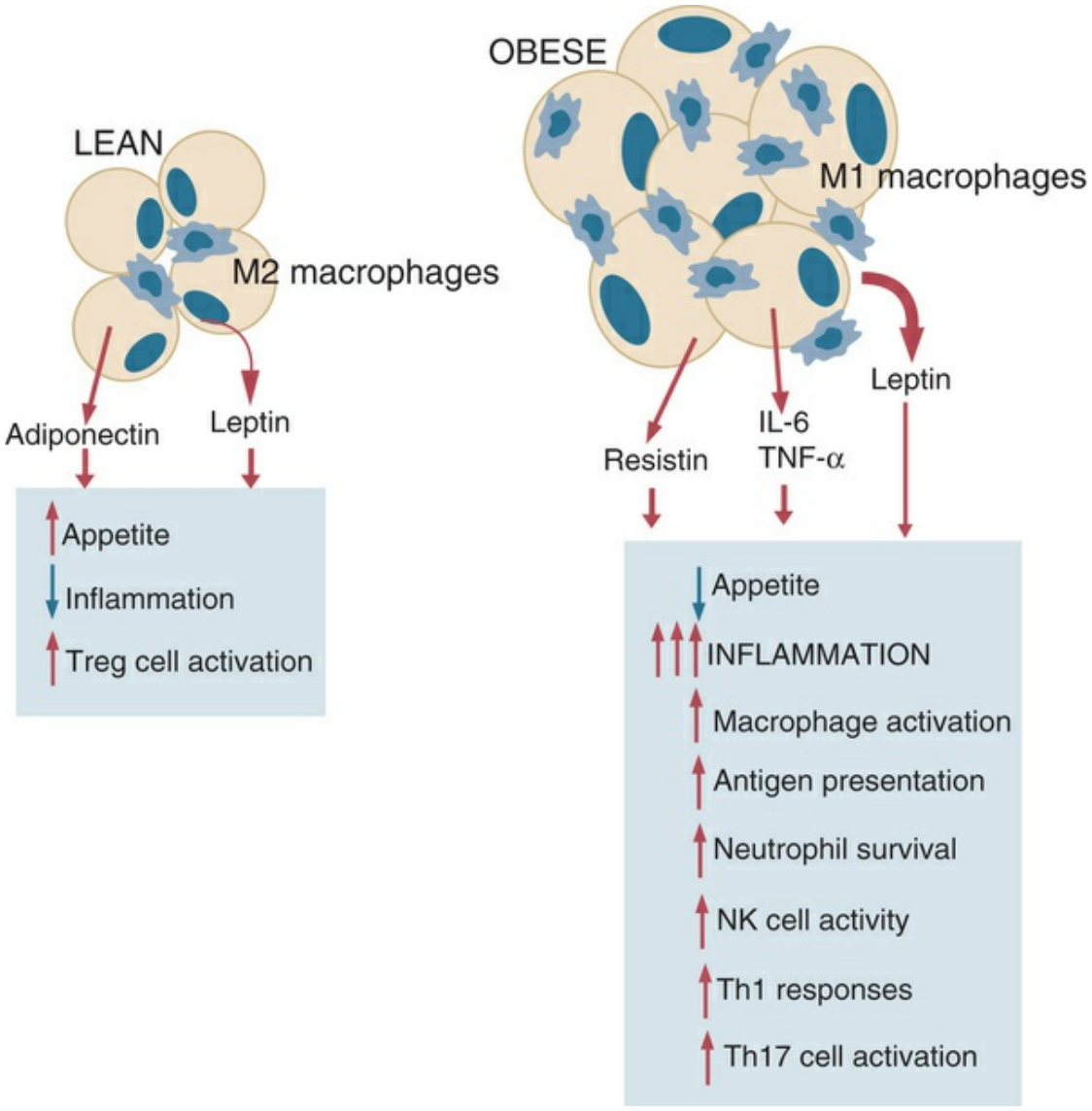


FIG. 40.7 Obese adipose tissue is rich in classically activated (M1) macrophages that, in association with large quantities of leptin, generate proinflammatory cytokines, resulting in enhanced inflammation throughout the body. Lean adipose tissue, in contrast, contains small numbers of alternatively activated (M2) macrophages and produces little leptin, tending to suppress inflammatory reactions. Adiponectin contributes to this antiinflammatory effect, whereas resistin is proinflammatory.

Adipose tissue in lean humans contains about 10% macrophages, whereas in the obese, it may contain 50% macrophages. The adipose tissue macrophages in obese individuals are M1 cells activated by leptin. Leptin increases their tumor necrosis factor-α (TNF-α), IL-6, and IL-1 production and upregulates MHC class II expression. This increased IL-6 production also promotes Th17-mediated responses. Leptin also enhances Th17 responses by upregulating RORγt and suppressing Treg differentiation.

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In obese animals, this widespread macrophage activation predisposes to inflammatory diseases such as atherosclerosis, arthritis, type 2 diabetes mellitus and autoimmunity, as well as to cancer. There is thus a clear link between obesity and chronic low-grade inflammation. Given the increasing obesity of pets as well as some of the effects of rapid growth in food animals, veterinarians would be well advised to take this link into account.

Leptin also enhances NK cell development and activation. It promotes Th1 cell production of IFN-γ, TNF-α, and leptin itself, thus generating an autocrine feedback loop. In lean animals where leptin levels are low, macrophage activation is suppressed, inflammatory responses are reduced, and there is a shift from Th1 to Th2 responses while increasing Treg cells.

Adiponectin counteracts the activities of leptin. Its concentration is inversely related to body weight, and it has strong antiinflammatory activity. In lean animals, it decreases production of IL-8, IFN-γ, IL-6, and TNF-α while increasing the production of IL-1RA and IL-10. It induces maturation and activation of dendritic cells. As a result, it reduces Th1 and Th17 responses. Adiponectin also regulates glucose and fatty acid metabolism. In obesity, adiponectin production drops, and adipose tissue macrophages then produce a cytokine called resistin that increases insulin resistance leading to diabetes.

Trace Elements

Several trace elements are required for optimal functioning of the immune system. The most important are zinc, copper, selenium, and iron. Deficiencies of any of these are immunosuppressive. Zinc is especially critical to the proper functioning of the immune system since it acts as an ionic signaling messenger to promote T cell activation. Zinc-deficient pigs have reduced thymus weight, depressed cytotoxic T cell activity, depressed B cell activity, depressed NK cell activity, and decreased antibody production. If pregnant animals are deprived of zinc, their offspring are immunosuppressed. Neutrophils from zinc-deficient animals have reduced chemotaxis and phagocytosis. Mild zinc supplementation may promote immunity. On the other hand, because many bacteria utilize zinc, the body also sequesters it to limit its availability

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during infections. Copper deficiencies are also immunosuppressive. Thus a copper deficiency reduces neutrophil numbers and function by depressing superoxide production. It also reduces lymphocyte responsiveness to mitogens; reduces T, B, and NK cell numbers; and enhances mast cell histamine release. Selenium deficiency depresses the function of most immune cells, reducing neutrophil, T, and NK cell responses, and IgM production. Supplementation with selenium upregulates the expression of IL-2R and prevents oxidative damage to immune cells. Iron deficiency is immunosuppressive for cell-mediated responses. However, the effects of this on resistance to infection are complex since many pathogens require iron to replicate (Chapter 7). Magnesium deficiency reduces immunoglobulin levels.

Vitamins

Three vitamins, A, D, and E, are critical for proper immune function. If a pregnant animal is vitamin A deficient, its lymphoid tissue development is impaired. Deficiencies of vitamin A reduce lymphocyte proliferation, NK cell activity, and cytokine and immunoglobulin production (Fig. 40.8). Some vitamin A metabolites such as retinoic acid enhance T cell proliferation and cytotoxicity. Retinoic acid is especially important in promoting Th2 and dendritic cell differentiation in the intestine and in the homing of IgA-positive B cells to mucosal surfaces. It also maintains Treg and ILC3 levels in the intestinal mucosa and thus regulates tolerance to food antigens (Chapter 21).

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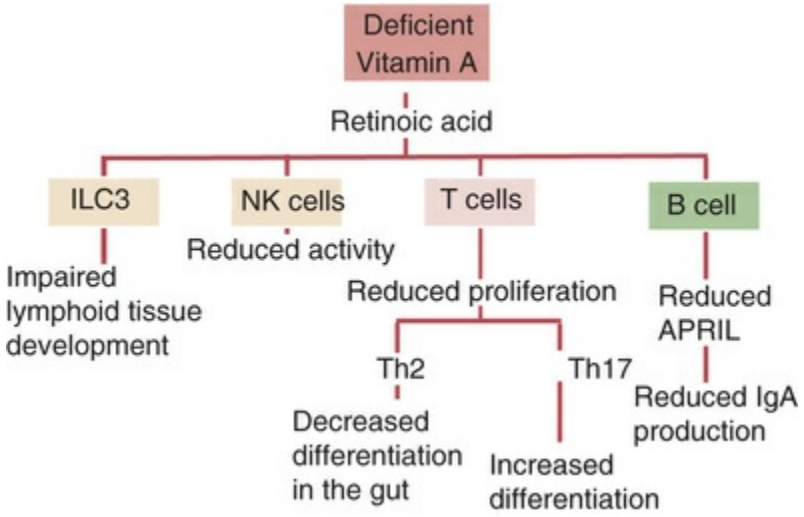


FIG. 40.8 The effects of a vitamin A deficiency on the immune system.

A prenatal vitamin A deficiency affects the responses of piglets to rotavirus vaccine. Deficient piglets shed the virus at 350% of the level of vitamin A sufficient piglets. Only 25% of the deficient piglets were protected as compared to 100% of the sufficient piglets. Deficient piglets had fewer IgG secreting cells in the ileum and fewer IgA secreting cells in the duodenum. Intestinal IgA titers were 11-fold less than in vitamin A sufficient animals. Deficient animals did, however, have higher levels of IL-8 and IFN-γ than sufficient piglets.

Calves fed a low vitamin A diet and immunized with an inactivated bovine coronavirus vaccine had reduced IgG1 antibody levels against bovine coronavirus, suggesting that Th2 responses may be impaired in these animals.

Vitamin E is a major antioxidant in cell membranes and is important in regulating the oxidants produced by phagocytic cells. Vitamin E deficiency depresses immunoglobulin levels through its effects on Treg cells and decreases lymphocyte responses to mitogens. Animals deficient in vitamin E also show reduced IL-2 and transferrin receptor expression and depressed phagocytic function. Vitamin E is one of the few vitamins for which supplementation has been shown to enhance immune responses and disease resistance. The importance of vitamin E for proper functioning of the immune system has been seen in an inbred population of donkeys whose foals were dying from overwhelming bacterial infections at 3 to 5 months of age. Investigations revealed

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that these foals were agammaglobulinemic but also lacked detectable vitamin E in their serum. Vitamin E supplementation by injection caused an immediate clinical improvement and within 2 months immunoglobulin levels were within normal limits. It was suggested that affected foals lacked a vitamin E transport protein. All subsequent foals in this herd received supplemental vitamin E and remained healthy. Supplemental vitamin E given to cats appeared to enhance T cell responsiveness to mitogens and leukocyte phagocytic activity.

When *Mycobacterium tuberculosis* interacts with toll-like receptor 1 (TLR1) or TLR2 on macrophages, it upregulates many different genes and enhances their antimicrobial activity. One such gene is that coding for the vitamin D receptor (Fig. 40.9). This receptor is present on most immune system cells, including neutrophils, macrophages, and T cells. Binding of vitamin D to its receptor on T cells downregulates IFN-γ and IL-2 expression and promotes Th2 responses. It also promotes Treg cell differentiation in the skin. Binding to the receptor on macrophages promotes their activation, NF-κB and MAPK signaling and the production of cathelicidin and β-defensin 2. It is no coincidence that resistance to tuberculosis and other respiratory diseases is directly related to serum vitamin D levels and that humans with a deficiency of vitamin D have significantly decreased resistance to this infection. It has been suggested that mice use nitric oxide rather than vitamin D as an intermediate in innate signaling because they are nocturnal, whereas humans acquire vitamin D from sunshine on exposed skin. It is unclear whether similar mechanisms operate in domestic mammals. Vitamin D levels decline with age. Th2 cytokines such as IL-13 enhance vitamin D–mediated expression of cathelicidins in bronchial epithelial cells.

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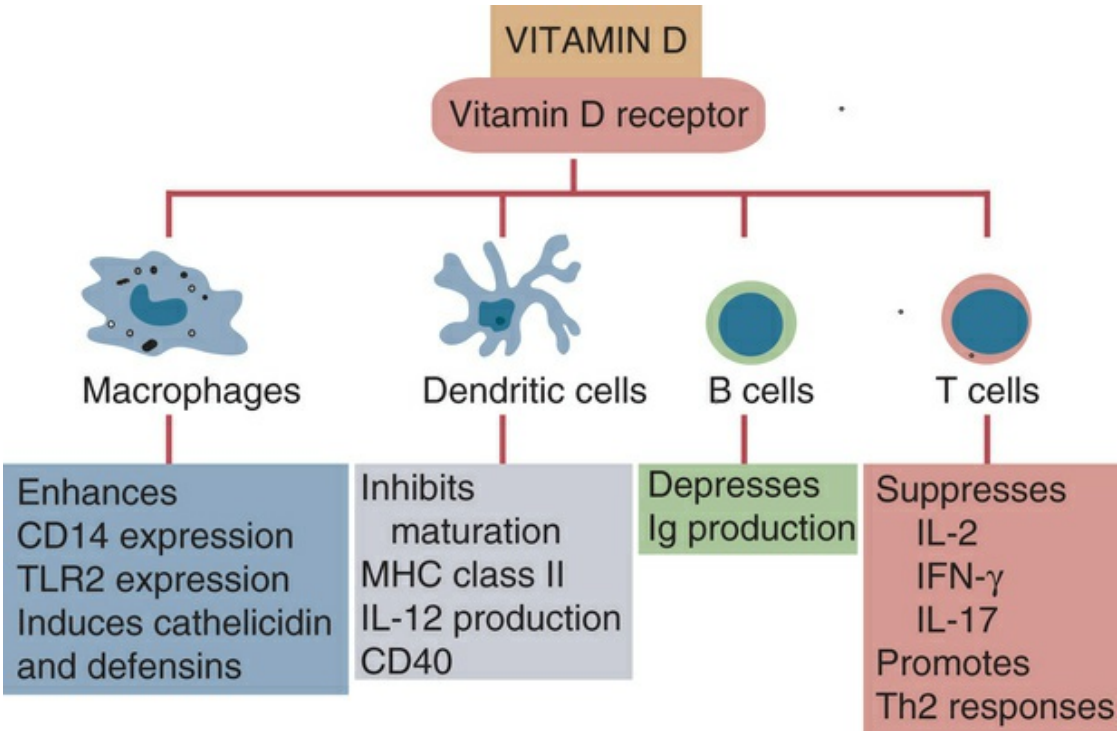


FIG. 40.9 The importance of vitamin D in immunity. Note that vitamin D is a potent stimulator of innate immunity by enhancing production of antimicrobial peptides. It is, however, somewhat suppressive of adaptive immune responses.

Taurine deficiencies in cats can result in a neutropenia, although mononuclear cell numbers may rise. The neutrophils of taurine-deficient cats show decreased respiratory burst activity and phagocytosis. Although these cats may show a hypergammaglobulinemia, germinal centers shrink, suggesting a loss of B cell activity.

The effects of malnutrition may be reflected in altered resistance to infectious diseases. Because bacteria can readily survive and multiply in body tissues despite malnutrition of the host, starvation commonly increases the severity of bacterial infections such as pneumonia. Viruses, in contrast, require healthy host cells in which to grow. Malnutrition, by rendering host cells unhealthy, may therefore increase resistance to viruses. Over-nutrition can also influence susceptibility to viruses. For example, overfed dogs show an increased susceptibility to canine distemper and canine adenovirus-1.

Exercise and Immunity

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Regular moderate exercise boosts immune function. For example, increased antibody responses are seen in mice that get moderate exercise, compared with unexercised control mice. Exercise also raises blood neutrophil counts, enhances NK cell activity, promotes lymphocyte responses to mitogens, and increases blood levels of IL-1, IL-6, and TNF-α. Although mild exercise is good for immune function, high-intensity exercise, prolonged exhaustive exercise, or overtraining may induce a functional immunodeficiency. In horses, blood lymphocytes show a decreased proliferative response for up to 16 hours after a race. Acute exercise in the unfit animal can be especially stressful. Unfit horses subjected to strenuous exercise showed significantly raised steroid levels, resulting in reduced lymphocyte responses to mitogens or influenza virus antigens and reduced neutrophil chemotactic responsiveness and respiratory burst activity (Fig. 40.10). These animals show a decline in their CD4/CD8 ratio as well as in both the number and activity of their NK cells. The age of an animal may moderate the effect of exercise on immune responses. For example, strenuous exercise significantly reduces lymphocyte proliferative responses in young horses, yet has much less effect on older animals. This resistance of older horses to exercise-induced immunosuppression may be due to their reduced steroid production.

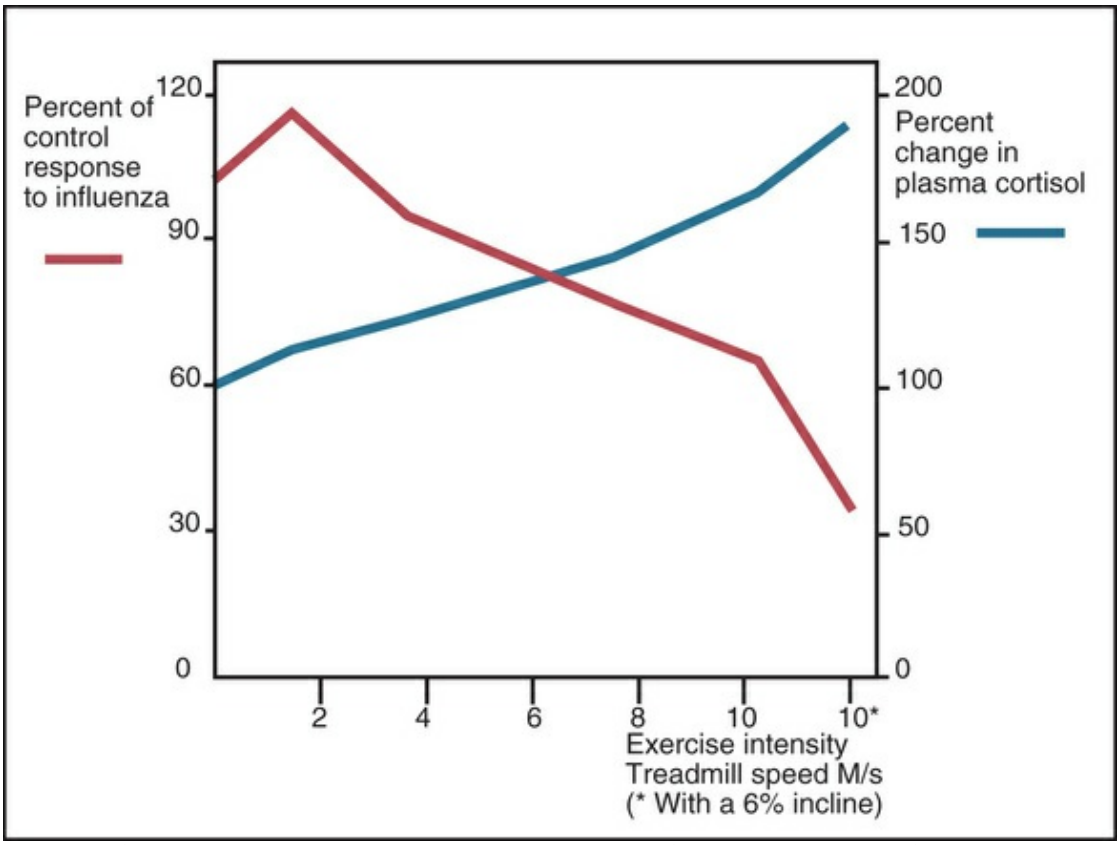


FIG. 40.10 Although a moderate amount of exercise is good for the immune system, excessive exercise causes severe stress that can be immunosuppressive. In this example, six thoroughbred horses were subjected to a treadmill-based exercise challenge of various intensities (speed and incline). Blood samples were assayed for plasma cortisol levels by radioimmunoassay, and influenza virus–specific lymphocyte proliferation was assayed by thymidine incorporation. A clear relationship exists among exercise intensity, the stress response, and immune responsiveness. (From data provided by Drs. S.G. Kamerling, P.A. Melrose, D.D. French, and D.W. Horohov.)

The complex effects of extreme exercise on the immune system are well seen in dogs undergoing long endurance sled races. The proportion of dogs with low total globulin immediately after racing was significantly greater than before racing. In some of these dogs it remained low 4 months after the race. IgG was also lower after racing than before racing. Likewise, serum IgM and IgE were higher before racing, although IgA was higher after racing. These changes in immunoglobulins might well affect resistance to infectious diseases. There is also evidence of increased inflammation in these dogs after racing.

Transportation stress is well recognized as predisposing to the development of respiratory disease in horses and cattle. Prolonged

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transportation of calves has been reported to increase leukocyte and neutrophil counts and NK cell activity and to reduce T cell numbers. Transportation of horses induced an acute-phase response and reduced lymphocyte responses to mitogens.

Posttraumatic Immune Deficiency

Trauma and massive tissue destruction initiate a response triggered by alarmins such as HMGB1 and DAMPs as well as enzymes escaping from damaged cells. Inflammasomes are generated, inflammatory cytokines are released, and macrophages are activated. Th1 cell activity is suppressed, while Th2 responses may be enhanced. This suppressive effect on Th1 responses may be mediated, in large part, by activated Treg cells and macrophages. Large amounts of IL-10 and other immunosuppressive cytokines are produced. Corticosteroids, prostaglandins from damaged tissues, and a small protein called suppressive active peptide, which appears in serum following a burn, all have immunosuppressive properties. The deficiency develops within minutes or hours and recovers as wounds heal. It affects T cell, macrophage, and neutrophil function, but B cell function appears to be normal. As a result, delayed hypersensitivity reactions, allograft rejection, and T-dependent antibody responses are all impaired. IL-2 and IL-2R production are reduced. $CD8^+$ T cells are increased in injured individuals, suggesting that regulatory cell function may be enhanced. Macrophages lose antigen-presenting ability as they express decreased levels of MHC class II. Neutrophil and macrophage phagocytosis and respiratory burst activities are both impaired. Although surgery may result in some suppression of lymphocyte responses to mitogens, evidence suggests that routine surgery has no significant effect on the response of healthy animals to vaccination.

Age and Immunity

Innate Immunity

Innate, cell-mediated, and humoral immune responses all deteriorate with advancing age, a phenomenon called immune

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senescence (Fig. 40.11). It is characterized by chronic activation, decreased responsiveness to stimulation, and reductions in antigen processing and presentation. For example, neutrophils and macrophages from the aged have an impaired ability to produce a respiratory burst and to produce reactive nitrogen species. As a result, they are less able than cells from the young to kill ingested bacteria. Macrophage numbers decline in aged animals and they express lower levels of TLRs. Aging is associated with impaired PRR signaling. When stimulated with TLR ligands, aged macrophages secrete reduced amounts of IL-6 and TNF-α. Aged macrophages show reduced responses to activators such as IFN-γ and produce less nitric oxide. Aged Beagles (>8 years of age) show decreased neutrophil phagocytosis (a decline of 39% in their ability to kill *Lactococcus lactis*). Young dogs (<1 year of age) had significantly higher levels of messenger RNA for IL-8R, L-selectin, and IL-1β-converting enzyme compared with older dogs.

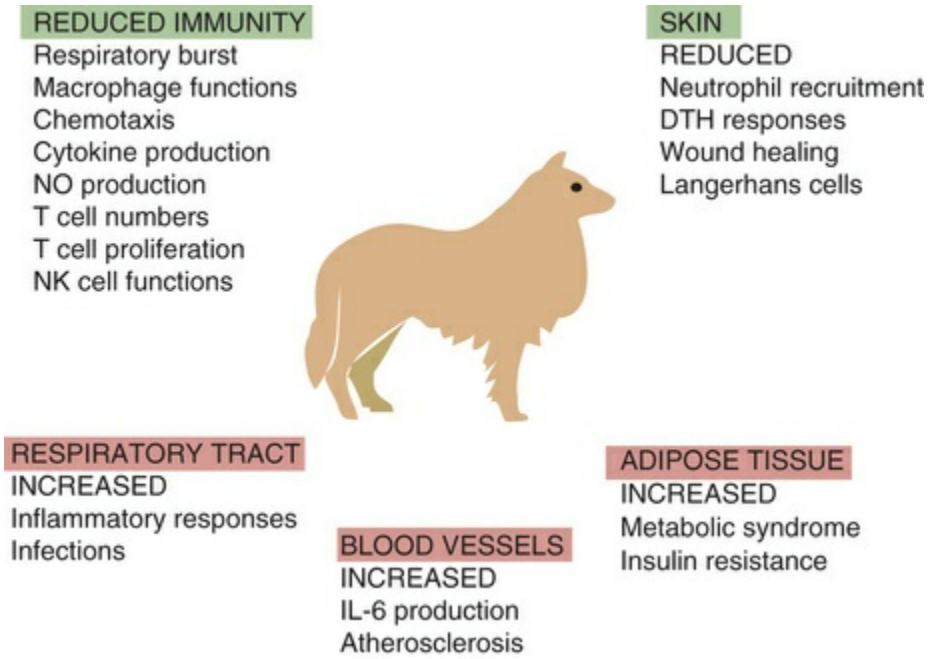


FIG. 40.11 The changes in the immune system that occur with aging.

Dendritic cells from aged individuals are less effective at antigen presentation. Their reduced ability to present antigen results from changes in surface antigen expression and cytokine production. Their reduced ability to stimulate B cells is due to reduced immune

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complex binding. NK cells from elderly individuals produce less IFN-γ and are less effective at killing tumor cells. These defects in innate immunity may be more profound than defects in adaptive immunity in the aged. Thus there may be a link between low-grade inflammation in the aged and geriatric syndromes such as the frailty of old age, a phenomenon called inflammaging. This low-grade inflammatory state has been identified in aged horses, where it may be due to pituitary pars intermedia dysfunction (PPID) secondary to neurodegeneration in the hypothalamus. Aged horses with PPID have increased blood levels of IL-8. Their neutrophils have decreased oxidative burst activity and decreased adhesion. The peripheral blood mononuclear cells from old horses produce more inflammatory cytokines than those from young horses. Neutrophils from these horses have decreased respiratory burst activity and adhesion. Likewise, the cells from fat old horses produce more cytokines than cells from thin old horses. Reducing body weight and fat in old horses significantly reduces the percentage of IFN-γ-negative and TNF-α-positive lymphocytes and monocytes and serum levels of TNF-α. The reverse was the case when weight and fat increased. Thus age-related obesity plays a role in the changes in the inflammatory response seen in older animals.

Lymphoid Organs

Aging is associated with progressive dysregulation and structural changes in primary and secondary lymphoid organs. Thymic involution is the most obvious of these changes. Blood lymphocyte numbers decrease significantly with age in dogs. Thus their CD4:CD8 ratio declines ($CD4^+$ T cell numbers drop, while $CD8^+$ T cells climb). Ileal Peyer's patches involute after sexual maturity in dogs. Old dogs have a decreased white pulp in the spleen. Lymph node changes vary depending on their location but include cortical atrophy and medullary fibrosis. Cats between 10 and 14 years of age had lower white cell, lymphocyte, and eosinophil counts than cats between 2 and 5 years of age. Absolute numbers of T cells, B cells, and NK cells were also lower in aged animals. Serum IgA and IgM, however, were higher in the aged group. There were no differences in their complement activity or their acute-phase

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responses. In Labrador Retrievers, absolute numbers of leukocytes, lymphocytes, monocytes, granulocytes and $CD3^+$, $CD4^+$, $CD8^+$, and $CD21^+$ lymphocytes decreased significantly with increasing age.

B Cell Responses

The bone marrow is relatively unaffected by old age, and an aged bone marrow can reconstitute the body as well as a young one. If aged B cells are mixed with young T cells, the response is relatively normal. If the reverse is attempted (i.e., mixing young B cells with aged T cells), the B cells respond poorly. Somatic mutation in immunoglobulin V region genes ceases in old animals so that antibody affinity tends to be lower than in young animals. Nevertheless, immunoglobulin concentrations do not decline in old age. Old dogs show little decline in antibody responses, although old horses have reduced antibody responses to influenza vaccination.

T Cell Responses

The greatest impact aging has on the immune system is the decline of cell-mediated responses. The relative percentages of lymphocytes and $CD4^+$ T cells decrease, whereas the percentage of granulocytes and $CD8^+$ T cells increases. As a result, there is a decline in the CD4/CD8 ratio. There is significant thymic involution, leading to a decline in the numbers of $CD4^+$ T cells and in the export of cells from the thymus. In addition, the lymphocyte population of the aged changes from a naïve population to a memory cell population. T cells from aged animals lose their ability to progress through the cell cycle. As a result, early events in the T cell response to antigens, such as activation of protein kinase C and the rise in intracellular calcium, are impaired. Even after expressing IL-2 receptors and being exposed to IL-2, aged T cells may not respond effectively to antigens. Analysis shows that some aged T cells continue to produce normal amounts of IL-2, but many do not. Thus aged T cell populations are mixtures of fully functional and impaired cells. In old horses (>20 years), there is a significant decrease in the proportion of $CD8^+$ T cells and a rise in the CD4/CD8 ratio compared with young animals. Horses more than 20 years of age have reduced lymphocyte responses to mitogens, and this

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deficiency cannot be overcome by exposure to additional IL-2.

Although aged animals may mount poorer primary responses to vaccines, their memory responses tend to remain unaffected. Elderly animals generally have persistent protective antibody levels and do respond by elevations in titer upon boosting. There is, however, a difference with novel antigens. A study of dogs receiving rabies vaccine for the first time showed that a significant decrease in antibody titers and a corresponding increase in vaccine failures occurred in older dogs (Chapter 25).

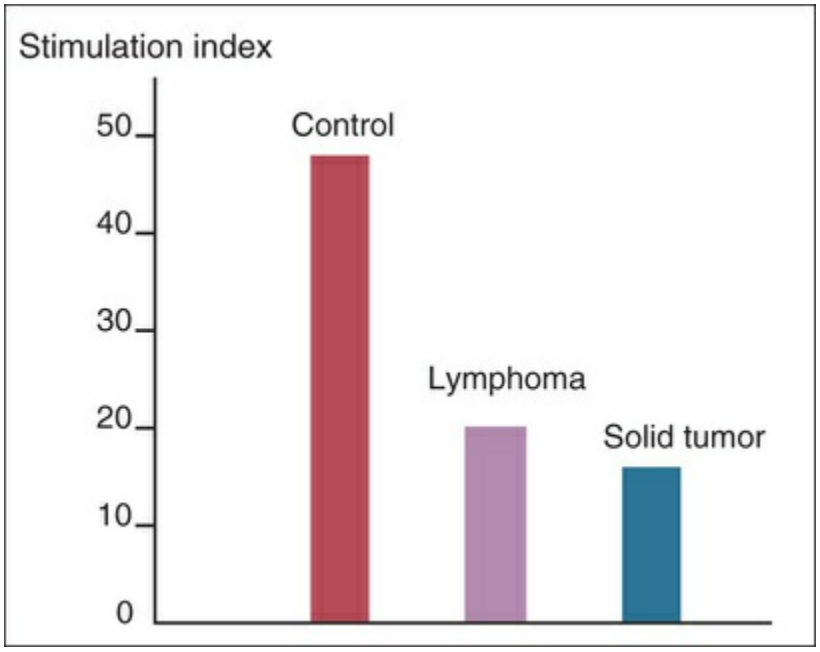
Feeding a low-calorie diet has been shown to extend the life span of dogs. One possible reason for this is the prevention of immunosenescence. Prolonged calorie restriction in dogs retarded age-related declines in lymphoproliferative responses, in absolute numbers of lymphocytes, and in the $CD4^+$, and $CD8^+$ T cell subsets. This may result from low circulating leptin levels. Calorie restriction appears to have no effect on neutrophil phagocytic activity, antibody production, or NK activity.

Despite the previous comments, young animals may show poorer resistance to some invaders than mature animals. This appears to be especially important in sheep. Lambs are more susceptible than mature sheep to parasitic and infectious diseases during the first year of life. Older sheep tend to show greater resistance to internal parasites such as *Haemonchus, Trichostrongylus,* and *Ostertagia.* Sheep younger than 1 year of age are more susceptible than mature sheep to virus diseases such as bluetongue and contagious ecthyma. Young sheep, 4 to 8 months of age, have a lower proportion of $CD4^+$ T cells in their blood than mature sheep. Lymphocytes from young sheep produce less IFN-γ than those from adult sheep. Older sheep produce more antibodies to *Brucella abortus* lipopolysaccharide and responded more intensely to the contact sensitizer dinitrochlorobenzene. However, the two age groups did not differ in B cell or $WC1^+$ T cell numbers and mounted comparable responses to diphtheria toxoid and tetanus. This mild immunodeficiency in lambs presumably reflects the immaturity of their immune system during the first year of life.

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Other Secondary Immunodeficiencies

Immunodeficiencies may result from a wide variety of insults to the body. For example, immunoglobulin synthesis is generally reduced in individuals with absolute protein loss (patients with the nephrotic syndrome, heavily parasitized or tumor-bearing individuals, and patients who have experienced severe burns or trauma). Stress may result in immunodeficiencies. For example, it is possible to provoke a combined immunodeficiency syndrome by chilling newborn puppies for 5 to 10 days. Diverse stressors such as rapid weaning, sleep deprivation, general anesthesia, prolonged transportation, and overcrowding are all effective immunosuppressants. Physical destruction of lymphoid tissues can result in immunodeficiencies. For example, loss of lymphoid tissue leading to immunosuppression may occur in tumor-bearing animals, especially if the tumors themselves are lymphoid in origin (Fig. 40.12). Adult horses with chronic diarrhea are immunosuppressed, as reflected by reduced IgA and reduced lymphocyte responses to mitogens. Some endocrine diseases such as thyrotoxicosis and diabetes mellitus may also result in immunosuppression.



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FIG. 40.12 Immunosuppression in dogs with lymphomas or solid tumors compared with normal control dogs. The stimulation index is a measure of the response of lymphocytes to the mitogenic lectin phytohemagglutinin. (Data taken from Weiden PL, Storb R, Kolb HJ, et al: Immune reactivity in dogs with spontaneous malignancy, *J Natl Cancer Inst* 53:1049-1056, 1974.)

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41

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Drugs and Other Agents That Affect the Immune System

CHAPTER OUTLINE

Suppression of the Immune System, 463
Nonspecific Immunosuppression, 463
Radiation, 463
Corticosteroids, 464
Cytotoxic Drugs, 465
Alkylating Agents, 465
Folic Acid Antagonists, 465
DNA Synthesis Inhibitors, 466
Selective Immunosuppression, 466
Calcineurin Inhibitors, 466
Target of Rapamycin Inhibitors, 467
Inosine Monophosphate Dehydrogenase Inhibitors, 468
Leflunomide, 468
Intravenous Immunoglobulin Therapy, 468
Stimulation of the Immune System, 468
Bacteria and Bacterial Products, 468

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Complex Carbohydrates, 469
Vitamins, 469
Cytokines and Monoclonal Antibodies, 469

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Explain how corticosteroids suppress the immune and inflammatory responses and hence are the most widely employed immunosuppressants.
- Explain how immunosuppressive drugs used to prevent allograft rejection or to treat autoimmune disease may either be nonspecific inhibitors of cell division or block the activation of T cells.
- Describe the mode of action of cyclosporine and related immunosuppressive drugs.
- Briefly describe some drugs employed to stimulate the immune system and how they are believed to work.
- Explain how specific monoclonal antibodies can block key points in immune or pathological processes.
- Understand why cytokine therapy may eventually be useful in veterinary medicine.
- Discuss how intravenous immunoglobulin therapy probably works.
- Define alkylating agent, folic acid antagonist, calcineurin inhibitor, and target of rapamycin.
- Discuss the role of vitamin treatment in enhancing disease resistance.

Many clinical situations exist in which it is desirable to either stimulate or suppress the adaptive immune system. Many different drugs and techniques are available to do this. Indeed, this area of immunology is a discipline in its own right, called immunopharmacology.

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Suppression of the Immune System

The methods available for suppressing adaptive immune responses may be classified into two main groups. Older techniques generally involved treatment that, by inhibiting all cell division, reduced the response of T and B cells to antigens. This approach is crude and dangerous since other rapidly proliferating cell populations, such as intestinal epithelium and hematopoietic stem cells, may also be severely damaged with potentially disastrous consequences. Alternatively, it is now possible to selectively eliminate selected immune cell populations by the use of specific antisera or monoclonal antibodies or by the use of highly selective immunosuppressive drugs.

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Nonspecific Immunosuppression

Radiation

Electromagnetic radiation is immunosuppressive because it prevents cell division. It affects cells by several different mechanisms. The simplest of these is through ionizing rays hitting an essential, unique molecule, such as DNA, within the cell. A loss of even one nucleotide results in a permanent mutation of a gene, with potentially lethal effects on the progeny of the affected cell. Radiation also causes ionization of water and the formation of highly reactive free oxygen and hydroxyl radicals within the cell. The hydroxyl radicals react with dissolved oxygen to form toxic peroxides that destroy DNA and inhibit cell division. Although radiation is of some use in prolonging graft survival in experimental animals, especially laboratory rodents, the amount of radiation required for effective prolongation of graft survival in dogs is so high that it is lethal.

Corticosteroids

Corticosteroids are among the most commonly used immunosuppressive and antiinflammatory agents. Their effects, however, differ among species. Mammals may be classified as corticosteroid sensitive or resistant depending on the ease by which they can be depleted of lymphocytes. Laboratory rodents and humans are much more sensitive to the immunosuppressive effects of corticosteroids than are the major domestic mammals, and care should be taken not to extrapolate laboratory animal results directly to other species.

Corticosteroids are absorbed directly into cells, where they bind to receptors in the cytosol (Fig. 41.1). The corticosteroid-receptor complexes are then transported to the nucleus, where they stimulate the synthesis of IκBα, the inhibitor of NF-κB. In a resting cell, NF-κB is inactive since its nuclear binding site is masked by IκBα. When a lymphocyte is stimulated, the two molecules dissociate, the IκBα is degraded by proteasomes, and the released

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NF-κB moves to the nucleus and activates genes involved in inflammation and immunity. However, because corticosteroids stimulate the production of IκBα, it continues to block NF-κB–mediated processes, including cytokine synthesis and T cell responses. As a result, corticosteroids suppress both immunological and inflammatory processes.

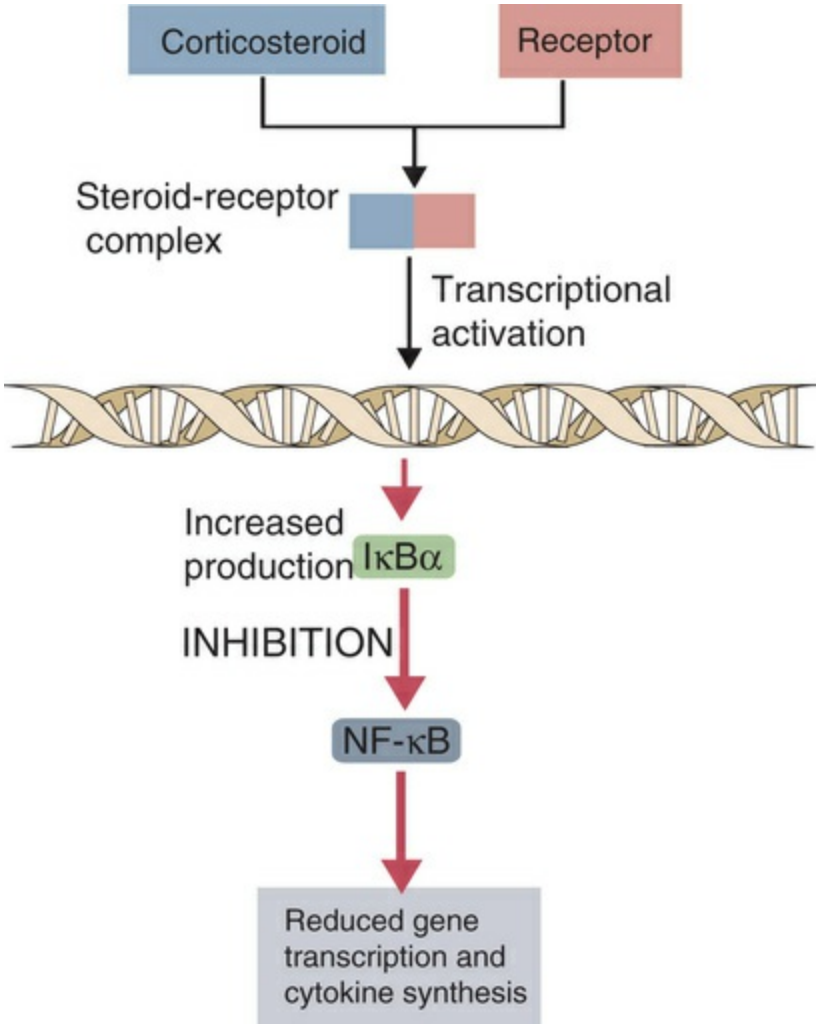


FIG. 41.1 A schematic diagram showing the mode of action of corticosteroids. Normally, signal transduction and cytokine synthesis occur when the transcription factor NF-κB dissociates from its inhibitor IκBα. The released IκBα is rapidly degraded. Corticosteroids stimulate the synthesis of excessive amounts of IκBα that binds to NF-κB and continues to prevent its activation.

Corticosteroids influence immunity in four areas (Box 41.1): they affect leukocyte production and circulation; they influence the effector mechanisms of lymphocytes; they modulate the activities of

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inflammatory mediators; and they modify protein, carbohydrate, and fat metabolism.

Box 41.1

Effects of Corticosteroids on the Immune System

Neutrophils

Neutrophilia

Depressed chemotaxis

Depressed margination

Depressed phagocytosis

Depressed ADCC

Depressed bactericidal activity

Stabilization of membranes

Inhibition of phospholipase A2

Macrophages

Depressed chemotaxis

Depressed phagocytosis

Depressed bactericidal activity

Depressed IL-1 and IL-6 production

Depressed antigen processing

Lymphocytes

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Depressed proliferation

Depressed T cell responses

Impaired T cell–mediated cytotoxicity

Depressed IL-2 production

Depressed lymphokine production

Immunoglobulins

Minimal decrease

Complement

No effect

The effects of corticosteroids on leukocytes vary among species. In horses and cattle, the number of circulating eosinophils, basophils, and lymphocytes declines within a few hours of corticosteroid administration as a result of sequestration in the bone marrow. The numbers of neutrophils, on the other hand, increase as a result of decreased adherence to vascular endothelium and reduced emigration into inflamed tissues. Neutrophil, monocyte, and eosinophil chemotaxis are suppressed by corticosteroids, but neutrophil random migration is enhanced. Corticosteroids suppress the cytotoxic and phagocytic abilities of neutrophils in some species, but in others, such as the horse and goat, they have no effect on phagocytosis. Macrophage production of prostaglandins and cytokines such as interleukin-1 (IL-1), as well as antigen processing, is reduced in some species.

Corticosteroids cause apoptosis of thymocytes and thus induce thymic atrophy. They also suppress the ability of T cells to produce cytokines. The most important exception to this is IL-2, which is not regulated by NF-κB (Chapter 8). Lymphocyte proliferation in response to foreign cells is suppressed, suggesting that there is interference with the recognition of MHC class II molecules. Corticosteroids also block production of lymphotoxin. Natural

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killer (NK) cells and some antibody-dependent cellular cytotoxicity (ADCC) reactions may be refractory to corticosteroid treatment. In cattle, corticosteroids may increase serum interferon levels. The effects of corticosteroids on antibody responses are variable and depend on timing and dose. In general, B cells tend to be corticosteroid resistant, and enormous doses are usually required to suppress antibody synthesis. It is interesting to note, however, that in horses, moderate doses of dexamethasone suppress IgG1 and IgG4 responses while having no apparent effect on IgG3 responses. Corticosteroids also upregulate the expression of CD121b. This is a decoy receptor that can bind active IL-1 but will not transduce a signal, effectively blocking IL-1 activity.

Synthetic corticosteroids suppress acute inflammation. They inhibit increased vascular permeability and vasodilation and thus prevent edema formation and fibrin deposition. At the same time, they block the emigration of leukocytes from capillaries. They inhibit the release of lysosomal enzymes and impair antigen processing by macrophages. Corticosteroids can also inhibit phospholipases and so prevent the production of leukotrienes and prostaglandins. In the later stages of inflammation, they inhibit capillary and fibroblast proliferation (perhaps by blocking IL-1 production) and enhance collagen breakdown. As a result, corticosteroids delay wound and fracture healing.

When systemic corticosteroid therapy is initiated, prednisolone or methylprednisolone are usually the agents selected for small animal treatment, and betamethasone and dexamethasone are commonly employed in large animal practice. Cats may require significantly higher doses than dogs to achieve a significant clinical response. This treatment is not without risks since it has the potential to suppress the pituitary-adrenal axis and induce Cushing's syndrome. By suppressing inflammation and phagocytosis, corticosteroids may render animals highly susceptible to infection. Once a response has been induced, therefore, the dose of corticosteroids should be gradually reduced by lengthening the dose interval and then decreasing the amount given (Box 41.2).

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Box 41.2

Immunosuppressive Fatty Acids

Inflammation is mediated by many different molecules, including lipids such as the leukotrienes and prostaglandins. Certain polyunsaturated fatty acids are the precursors of these prostanoids and so can regulate their production. Omega-6 fatty acids such as arachidonic acid tend to be proinflammatory, whereas the omega-3 fatty acids such as eicosapentaenoic acid and docosahexaenoic acid tend to have antiinflammatory effects since they suppress eicosanoid production. The omega-3 fatty acids also promote production of resolvins and protectins. These fatty acids suppress NF-κB signaling and inhibit the production of inflammatory cytokines such as IL-1 and TNF-β. They tend to be immunosuppressive as well, suppressing B, total T, and Th cells in cat skin. They do not appear to influence Tc or NK cell populations, IL-2 responses, delayed hypersensitivity, or immunoglobulin levels. The feeding of oils containing omega-3 fatty acids such as fish oil, evening primrose oil, and flaxseed oil may therefore reduce skin inflammatory responses and be of clinical benefit in the treatment of allergic skin diseases, especially atopic dermatitis. They may also be of benefit in the treatment of inflammatory bowel disease.

Rutherfurd-Markwick KJ, Hendriks WH, Morel PCH, Thomas DG: The potential for enhancement of immunity in cats by dietary supplementation, *Vet Immunol Immunopathol* 152:333-340, 2013.

Cytotoxic Drugs

Cytotoxic drugs inhibit cell division by blocking nucleic acid synthesis and activity. The major cytotoxic drugs currently in use are alkylating agents, folic acid antagonists, and DNA synthesis inhibitors.

Alkylating Agents

Alkylating agents cross-link DNA helices, preventing their separation, and thus block cell division. The most important of these is cyclophosphamide (Fig. 41.2). Cyclophosphamide is toxic

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for resting and dividing cells, especially for dividing immunocompetent cells. It impairs both B and T cell responses, especially the primary immune response. It blocks mitogen- and antigen-induced cell division and the production of IFN-γ. It prevents B cells from renewing their antigen receptors. Early in therapy, cyclophosphamide tends to destroy more B cells than T cells. In long-term therapy it affects both cell populations. It also suppresses macrophage function. Cyclophosphamide may be administered parenterally or orally and is inactive until biotransformed in the liver. It has a half-life of about 6 hours and is largely excreted through the kidney. It is of interest to note that corticosteroids enhance the metabolism of cyclophosphamide and so reduce its potency. The main toxic effect of cyclophosphamide is bone marrow suppression, leading to leukopenia with a predisposition to infection. Other adverse effects may include thrombocytopenia, anemia, and bladder damage. Cyclophosphamide may be of benefit in the treatment of lymphoid tumors and in the treatment of immune-mediated skin diseases, although its potential toxicity suggests that other, less-toxic alternatives be considered first.

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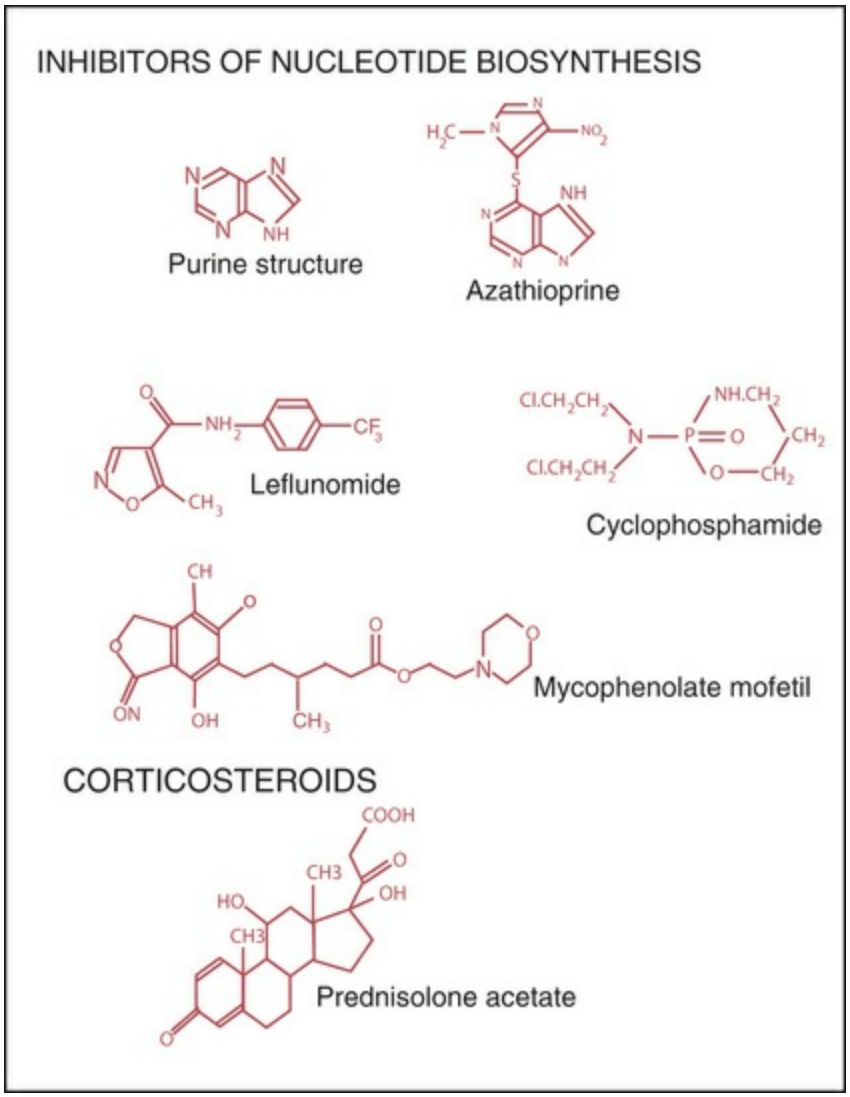


FIG. 41.2 The structure of some commonly employed immunosuppressive drugs and the normal compounds with which they compete. Cyclophosphamide acts by cross-linking DNA chains.

Folic Acid Antagonists

Methotrexate is a folic acid antagonist that binds to dihydrofolate reductase and blocks the synthesis of tetrahydrofolate, inhibiting the synthesis of thymidine and purine nucleotides. As a result, it can suppress antibody formation. Its side effects are similar to those caused by cyclophosphamide. It is used for the treatment of rheumatoid arthritis in humans.

DNA Synthesis Inhibitors

Azathioprine is a nucleoside analog that suppresses lymphocyte

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activation and mitosis. Under the influence of thiopurine methyltransferase, it is converted in the liver to 6-mercaptopurine, which inhibits both DNA and RNA synthesis. T and B cells are especially susceptible to this effect. It can suppress both primary and secondary antibody responses if given after antigen exposure. Azathioprine has significant antiinflammatory activity since it inhibits the production of macrophages. It has no effect on the production of cytokines or immunoglobulins by lymphocytes but tends to suppress T cell–mediated responses to a greater extent than B cell responses. Its major toxic effects include bone marrow depression (affecting leukocytes rather than platelets or red cells), acute pancreatitis, and gastroenteritis. Azathioprine is useful in the control of allograft rejection. It is favored by many clinicians for the treatment of immune-mediated skin diseases because of its combination of antiinflammatory and immunosuppressive activity. It is commonly used in association with corticosteroids. If azathioprine is used in dogs, marrow function should be monitored and the dose adjusted if necessary. There are breed-related variations in azathioprine metabolism in dogs that may affect its effectiveness and toxicity.

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Selective Immunosuppression

Calcineurin Inhibitors

Perhaps the single most important step in the development of routine, successful organ allografting has been the development of very potent but selective immunosuppressive agents. Of these, cyclosporine has been by far the most successful. Cyclosporine (also called ciclosporin) is an immunosuppressive polypeptide derived from a soil fungus, *Tolypocladium inflatum.* This fungus yields several natural forms of cyclosporine, of which the most important is cyclosporin A, a circular peptide of 11 amino acids (Fig. 41.3). As a result, cyclosporine has two distinct surfaces that allow it to bind two proteins simultaneously. When it enters the T cell cytosol, one surface binds to an intracellular receptor called cyclophilin, whereas the other binds and blocks the intracellular transmitter calcineurin, a serine-threonine phosphatase (Fig. 41.4). Cyclosporine therefore inhibits signal transduction and blocks production of IL-2 and IFN-γ by T cells. The primary effect of cyclosporine treatment is therefore the blocking of Th1 responses. It has indirect suppressive effects on macrophages, B cells, NK cells, neutrophils, eosinophils, and mast cells.

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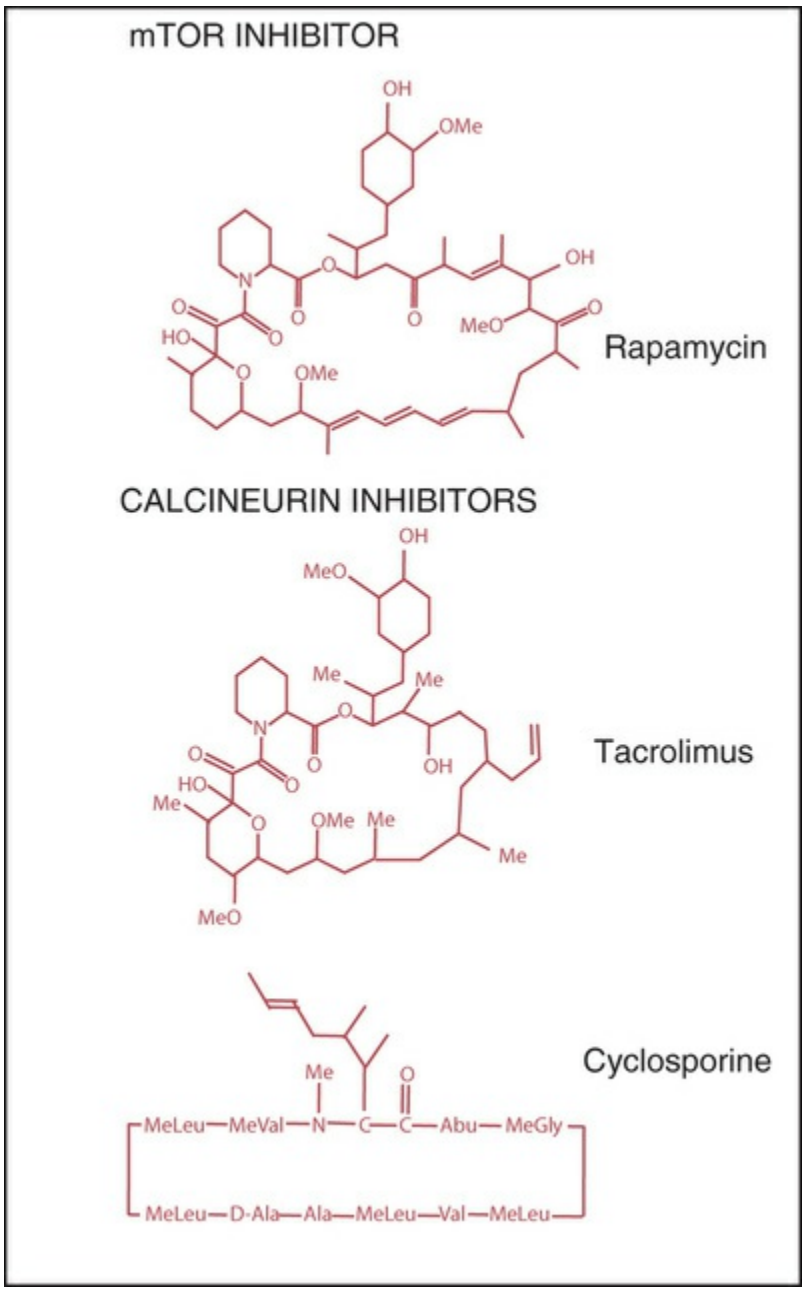


FIG. 41.3 The structure of the immunosuppressive drugs rapamycin, tacrolimus, and cyclosporine.

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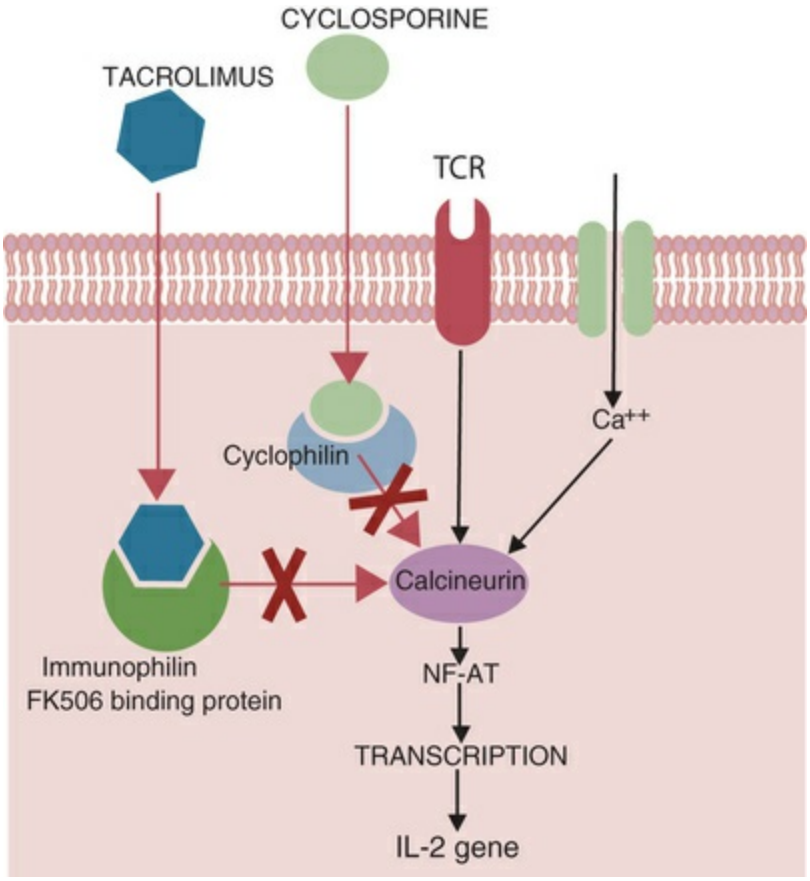


FIG. 41.4 The mode of action of cyclosporine and tacrolimus. Both prevent activation of the signaling molecule calcineurin. As a result the transcription factor NF-AT is inhibited, and activation of genes such as those for IL-2 production are prevented.

Because cyclosporine inhibits IFN-γ production by activated T cells, it blocks MHC class I expression on allografts. Since corticosteroids have a similar effect, the combination of corticosteroids and cyclosporine is especially potent and can enhance allograft survival while leaving other immune functions intact. This is a significant advantage over other older immunosuppressants. The use of cyclosporine has made tissue transplantation a routinely successful and safe procedure. In cats that received renal allografts from unrelated blood group–compatible donors and were treated with cyclosporine and prednisolone, mean survival was greater than 12 months. Cyclosporine also inhibits hypersensitivity reactions. It is useful in a variety of immunologically mediated dermatological diseases and appears to have a wide safety margin in dogs. The major adverse effect reported is gastroenteritis.

Tacrolimus is a macrolide antibiotic that acts as a calcineurin-

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blocking agent in a manner similar to cyclosporine (see Fig. 41.4). It inhibits the production of several key cytokines, including IL-2, IL-3, IL-4, IL-5, IFN-γ, and TNF-α. Tacrolimus is much more potent than cyclosporine in inhibiting T and B cell responses. It is also superior to cyclosporine in preventing or reversing allograft and xenograft rejection in humans and can prevent graft vascular disease (Chapter 34). Unfortunately, it causes severe intestinal toxicity in dogs, resulting in ulceration, vasculitis, anorexia, and vomiting. Topical tacrolimus has been used successfully to treat atopic dermatitis, discoid lupus erythematosus, and pemphigus erythematosus in dogs.

Target of Rapamycin Inhibitors

The macrolide antibiotic rapamycin (sirolimus) and the related molecule everolimus specifically inhibit a multifunctional serine kinase known as mechanistic target of rapamycin (mTOR). mTOR plays a critical role in regulating T cell activation by integrating the signals received from specific antigen, co-stimulatory receptors, and cytokines, and directing the T cell differentiation into effector, regulatory, or memory pathways (Fig. 41.5). mTOR also acts in nondividing macrophages and dendritic cells by associating with MyD88, activating IFN regulatory factors, and inhibiting caspase-1. Rapamycin acts on macrophages and dendritic cells, enhancing IL-12 and nitric oxide production but inhibiting IL-10. This in turn promotes Th1- or Th17-mediated inflammation. Rapamycin inhibits B and T cell proliferation by blocking stimulatory signals from IL-2, IL-4, and IL-6. It enhances regulatory T (Treg) cell production and promotes tolerance. It acts synergistically with calcineurin inhibitors and is much superior to cyclosporine in preventing or reversing allograft and xenograft rejection in humans. Because it blocks endothelial cell and fibroblast proliferation, rapamycin can prevent graft vascular disease (Chapter 34), although it also inhibits wound healing. When given to aged mice, rapamycin significantly increases their life span. It is believed to act as a dietary restriction mimetic. Unfortunately, it also induces severe intestinal toxicity in dogs, causing ulceration, vasculitis, anorexia, and vomiting.

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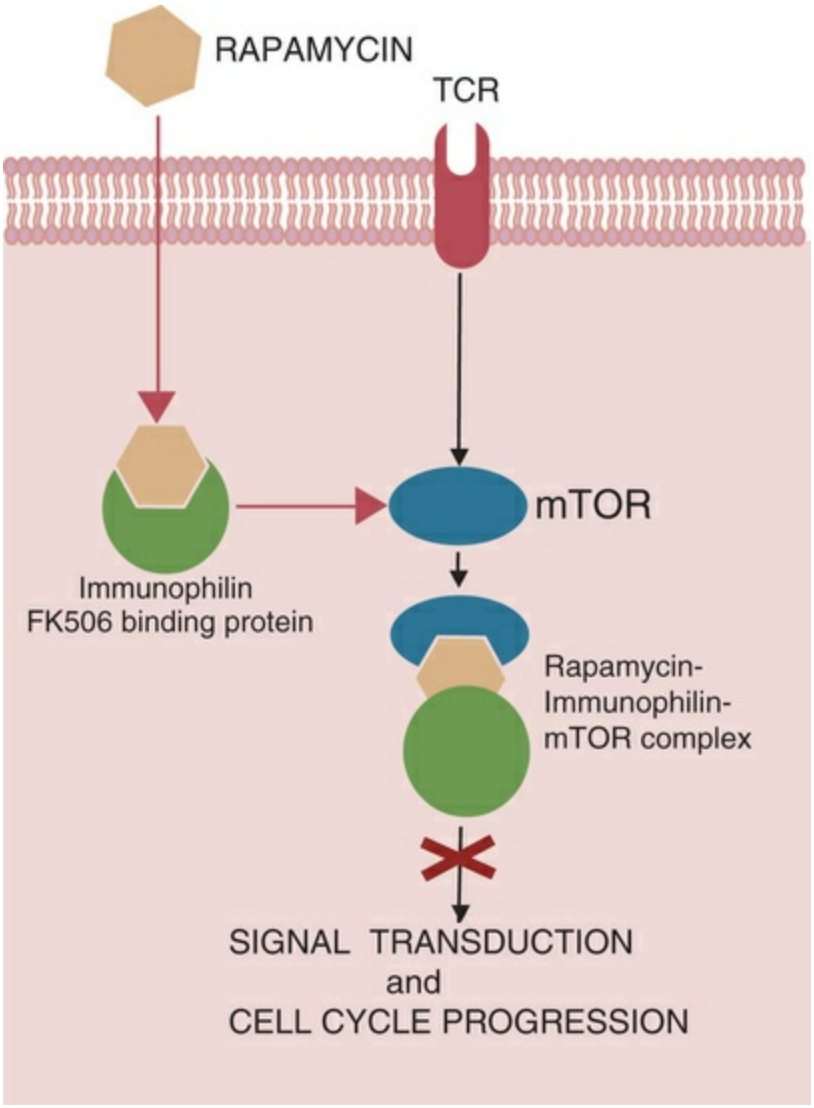


FIG. 41.5 The mode of action of rapamycin. This drug blocks activation of mTOR (mechanistic target of rapamycin). As a result numerous cell functions are blocked, including gene activation pathways and cell cycle progression.

Inosine Monophosphate Dehydrogenase Inhibitors

Mycophenolate mofetil prolongs canine allograft survival. It acts selectively on activated lymphocytes since it inhibits the purine pathway enzyme inositol monophosphate dehydrogenase found in activated but not resting lymphocytes. This leads to reduced production of guanosine monophosphate and prevents synthesis of DNA. It therefore blocks both B and T cell proliferation, T cell differentiation, antibody formation, and dendritic cell maturation.

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When given with cyclosporine, mycophenolate mofetil prevents renal allograft rejection between unrelated mongrel dogs. It has been reported to be effective in controlling canine diseases such as immune-mediated thrombocytopenia and hemolytic anemia, meningoencephalitis, polymyositis, and pemphigus foliaceus, as well as systemic histiocytosis (Chapter 10). It is well tolerated by dogs.

Leflunomide

Leflunomide is an antiinflammatory agent that inhibits pyrimidine synthesis. It may induce production of Treg cells. It has been used for the prevention of allograft rejection in dogs. It has also been applied to several canine autoimmune and inflammatory diseases, especially in cases refractory to corticosteroid treatment or in which corticosteroids are contraindicated.

Intravenous Immunoglobulin Therapy

Although immunoglobulin replacement is appropriate for animals with antibody deficiencies, intravenous immunoglobulin (IVIG) therapy is immunosuppressive and antiinflammatory. Human IVIG has been used to treat autoimmune and inflammatory diseases in domestic animals. This is a pooled IgG preparation derived from a large number of healthy donors. Administered intravenously, its beneficial effects are probably mediated by IgG molecules with sialic acid on their Fc region. These bind to the integrin DC-SIGN on myeloid cells. This in turn stimulates IL-33 production, which then promotes IL-4 production, which in turn upregulates the inhibitory receptor FcγR2b on effector macrophages and dendritic cells. This then inhibits the activities of autoantibodies. In addition, administration of IVIG has been shown to increase production of transforming growth factor-β (TGF-β) and IL-10 by Treg cells and numerous other activities (Fig. 41.6). In dogs, it may act by saturating Fc receptors such as CD16 and CD32 on monocytes. IVIG may also interfere with CD95-mediated apoptosis.

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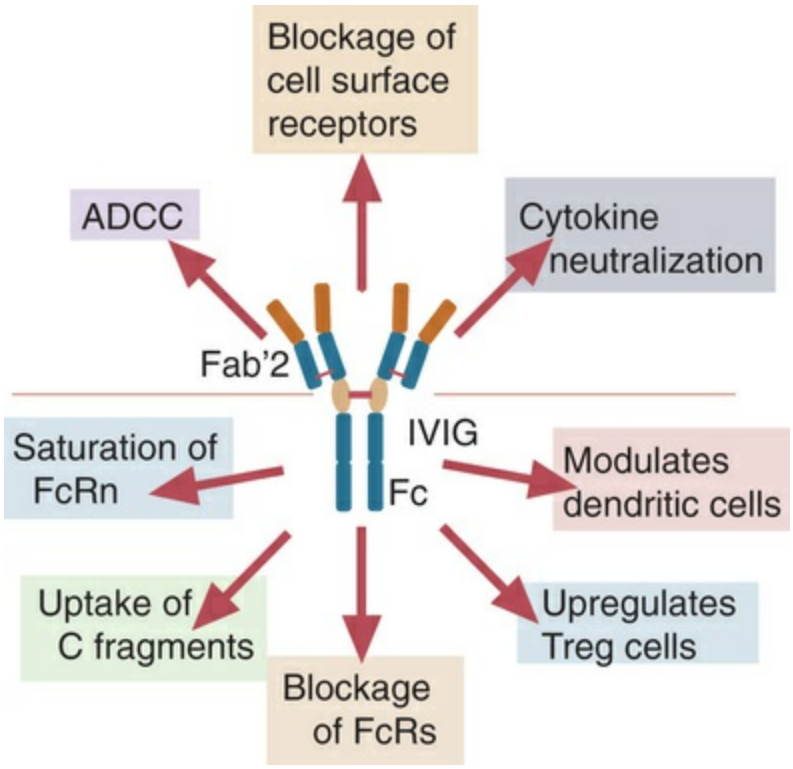


FIG. 41.6 Some of the many postulated activities mediated by intravenous immunoglobulins (IVIG). In addition, the sialyl groups bind to SIGN and trigger immunomodulatory pathways.

When administered to dogs, IVIG causes a mild thrombocytopenia, leucopenia, increased total plasma protein, increases in fibrin degradation products, thrombin-antithrombin complexes, and C-reactive protein. In effect it enhances blood coagulation and some inflammatory responses. It also binds to canine monocytes and lymphocytes ($CD4^+$T, $CD8^+$T, and B) and inhibits antibody-mediated phagocytosis by monocytes. IVIG has been used to successfully treat immune-mediated hemolytic anemia, thrombocytopenia, and pemphigus, as well as severe cutaneous drug reactions such as erythema multiforme and Stevens-Johnson syndrome (Chapter 33). Most authors report positive clinical responses with minimal adverse reactions.

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Stimulation of the Immune System

There are many situations in veterinary medicine in which it is desirable to enhance innate or adaptive immunity, for example the enhancement of resistance to infection and the treatment of immunosuppressive diseases. Immunostimulants vary according to their origin, their mode of action, and the way in which they are used. In contrast to adjuvants, immunostimulants need not be administered together with an antigen to enhance an immune response.

Bacteria and Bacterial Products

A wide variety of bacteria have been employed as immunostimulants. These usually act as sources of pathogen-associated molecular patterns and stimulate one or more TLRs. As a result, they activate macrophages and dendritic cells and stimulate cytokine synthesis. The most potent of these cytokine synthesis enhancers is bacille Calmette-Guérin (BCG), the live attenuated vaccine strain of *Mycobacterium bovis.* BCG generally enhances B and T cell–mediated responses, phagocytosis, allograft rejection, and resistance to infection. Unfortunately, whole BCG induces tuberculin hypersensitivity in treated animals and is therefore unacceptable for use in farm animals. To prevent sensitization, purified cell wall fractions of BCG have therefore been developed. These have been used to treat equine sarcoid tumors, equine endometritis, bovine colibacillosis, and ocular squamous cell carcinoma. They are also of benefit in the treatment of upper respiratory tract infections in horses. Several active constituents have been identified. One of these is trehalose dimycolate, which promotes nonspecific immunity against several bacterial infections and may provoke regression of some experimental tumors. Another is muramyl dipeptide (MDP), a simple mycobacterial glycopeptide that enhances antibody production, stimulates polyclonal activation of lymphocytes, and activates macrophages. Because MDP is rapidly excreted in the urine, its biological activity is enhanced by incorporation into liposomes. Polymerization and conjugation with

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glycopeptides or synthetic antigens can also enhance the immunostimulating effects of MDP. MDP prolongs survival time and decreases metastases in dogs with osteosarcoma.

Killed anaerobic corynebacteria, such as *Propionibacterium acnes,* also promote antibody formation. These bacteria are phagocytosed by macrophages and presumably stimulate cytokine synthesis through TLRs. *P. acnes* has a complex activity since it stimulates macrophages and the antibody response to thymus-dependent antigens, but it has a variable effect on the response to thymus-independent antigens. Killed *P. acnes* has been of benefit in the treatment of staphylococcal pyoderma, malignant oral melanoma in dogs, feline leukemia, and respiratory disease in horses. Other bacterial components, such as staphylococcal cell walls (especially staphylococcal phage lysate), some streptococcal components, and components of *Bordetella pertussis, Brucella abortus, Bacillus subtilis,* and *Klebsiella pneumoniae,* all have immunostimulating activity.

Immunomodulators have never been widely employed in veterinary medicine, largely because of the biological variability of the results obtained by their use. *Parapoxvirus ovis* (orf), mycobacterial cell wall fractions, and killed *Propionibacterium acnes* have, however, been employed in equine medicine. They improve immune defenses nonspecifically and provide support for other antimicrobial therapy. While they do not appear to protect against respiratory infection, they may reduce respiratory disease severity, reduce the frequency of complications, and accelerate recovery.

Complex Carbohydrates

Certain complex carbohydrates derived from yeasts, namely zymosan, glucans, aminated polyglucose, and lentinans, can also activate macrophages. These may function as adjuvants and potentiate resistance to infectious agents. Fish such as trout, salmon, and catfish appear to respond especially well to these immunostimulants when incorporated in the diet. As a result, immunostimulation by complex carbohydrates, especially glucans, is routine in aquaculture.

Vitamins

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Some vitamins, most notably A, D, and E, play a key role in regulating immunity.

Vitamin A metabolites, especially retinoic acid, enhance cytotoxicity and T cell proliferation by stimulating IL-2 production. Conversely, vitamin A deficient mice have defective helper T cell activity. Retinoic acid can inhibit B cell proliferation and inhibit B cell apoptosis. Retinoic acid also enhances dendritic cell antigen presentation and maturation. Vitamin A metabolites can modulate the Th1-Th2 balance as well as the differentiation of Treg and Th17 cells (Chapter 21). Retinoic acid also regulates the gut-homing abilities of T and B cells, and a vitamin A deficiency is associated with impaired gastrointestinal immune responses and increased susceptibility to gastrointestinal and respiratory diseases (Chapter 40). If a pregnant mouse lacks vitamin A, her offspring have small lymph nodes and have an impaired immune response as adults.

Vitamin D, as described previously (Chapter 40), also plays a key role in immunity. The most important form, vitamin D_3, is synthesized within the skin or the liver, kidneys, and lymphoid tissues. Macrophages and dendritic cells require vitamin D for the production of the antimicrobial peptide cathelicidin. The vitamin D receptor is upregulated by IL-15 triggered by T cell receptor (TCR) activation.

Vitamin E and selenium affect immune responses and disease resistance in poultry, pigs, and laboratory animals. A deficiency of vitamin E ([dl]-α-tocopheryl-acetate) results in immunosuppression and reduced resistance to disease. On the other hand, supplementation of diets with vitamin E can enhance certain immune responses. Lymphocyte responses to pokeweed mitogen are higher in pigs with high vitamin E levels. Vitamin E supplementation given to cows for several weeks before calving prevents the decline in neutrophil function and macrophage function that normally occurs in the immediate post-parturient period. Vitamin E promotes B cell proliferation; the effect is most marked in the primary immune response. It can act as an adjuvant when administered with *Brucella ovis* vaccine, clostridial toxoid, and *Escherichia coli* J5 vaccine. In some cases, this increased antibody production may lead to increased disease resistance. Vitamin E can reduce age-related declines in immune function by a direct action

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on T cells and by suppressing macrophage prostaglandin E_2 production. Supplemental vitamin E may enhance immune function in adult cats and elderly humans.

Cytokines and Monoclonal Antibodies

Allograft rejection intensity can be minimized by reducing T cell numbers through administration of an antiserum specific for T lymphocytes produced in rabbits or horses. Thus polyclonal anti-lymphocytic serum (ALS) suppresses the cell-mediated immune response and leaves the humoral immune response relatively intact. In practice, ALS is of variable efficiency and specificity and may cause severe side effects as a result of global immunosuppression. ALS-treated mice have been shown to accept rat xenografts, whereas clinical use of ALS in humans has not been universally accepted as useful. As a result of these issues with polyclonal antibodies, much more specific mouse monoclonal antibodies have been produced. The first to be employed was monoclonal anti-CD3. Anti-CD3 is directed only against T cells and is effective in reversing allograft rejection in humans. An even more specific monoclonal antibody is anti-CD25. This binds to the α chain of the IL-2 receptor and so prevents lymphocyte activation. Anti-CD25 helps prevent acute renal allograft rejection and, since it does not cause T cell depletion, has fewer adverse effects and results in fewer opportunistic infections than crude ALS.

Monoclonal antibodies against canine CD4 and CD8 have been used to control rejection of canine renal allografts. They are very effective, even with highly mismatched mongrel dogs. Both anti-CD4 and anti-CD8 must be used together, and their immunosuppressive effect lasts for about 10 days. (The dogs eventually develop neutralizing antibodies against these mouse antibodies.) These are especially effective in combination with cyclosporine.

In some diseases, especially those due to excessive immune function, it may be beneficial to neutralize excessive cytokine activity using monoclonal antibodies against a cytokine or against its receptor. Monoclonal antibodies directed against these targets are widely employed in humans. The original monoclonal

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antibodies were of mouse origin, and as a result had a short half-life and were relatively ineffective. Subsequently, these antibodies have been "humanized" and their effectiveness has increased significantly. Thus anti-TNFR (Enbrel) and anti-TNFα (Humira) have been used to treat rheumatoid arthritis. Monoclonal antibodies against the checkpoint molecules CTLA4 and PD-1 are being used for cancer treatment with remarkable success (Chapter 35). Anti-CD20 may be effective in treating canine B cell lymphomas, while anti-CD52 may help treatment of canine T cell lymphomas. Anti-nerve growth factor is being tested for its ability to reduce pain in canine osteoarthritis. Anti-IgE (Xolair) has been successfully used in the treatment of canine atopic dermatitis. Anti–IL-31 (Lokivetmab) is marketed for the relief of itch in canine atopic dermatitis.

The successful use of monoclonal antibodies against cytokines and tumors may be contrasted with the disappointing effects of administering cytokines to animals. Since purified cytokines produced by recombinant DNA techniques are available, many investigators have investigated their use in the treatment of disease. By administering additional cytokines, it has been assumed that the amount of these molecules in the normal animal is rate-limiting and that administering additional cytokines in pure form will somehow promote disease resistance or healing. It has also been assumed that by administering a single new cytokine, one will not trigger mechanisms that will regulate its activity or even neutralize its effects. None of these assumptions may be valid. The major cytokines (IL-1, IL-2, IL-12, colony-stimulating factors, and the IFNs) have all been tested on animals *in vivo.* Unfortunately, the administration of these cytokines has usually had minimal effects on disease processes and has been accompanied by significant adverse effects.

For example, administration of interferons should inhibit virus replication as well as stimulate cellular functions such as macrophage activity, thereby promoting disease resistance. This has proved to be an oversimplification. High doses of interferons are very toxic and cause severe fever, malaise, and appetite loss. They inhibit hematopoiesis and cause thrombocytopenia and granulocytopenia. They can also cause liver, kidney, and neural toxicity. In addition, IFNs seem to be relatively poor antiviral

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agents.

Recombinant human IFN-α (rHuIFN-α) has been used to treat rhinopneumonitis caused by bovine herpesvirus-1 (BHV-1) and rotavirus-induced diarrhea in calves. Recombinant bovine interferons (rBoIFN-α or rBoIFN-γ) have also been used to treat BHV-1, *Mannheimia hemolytica, Histophilus somni,* vesicular stomatitis, coliform mastitis, brucellosis, and salmonellosis in calves, and transmissible gastroenteritis in piglets. Recombinant porcine IFN-γ has been used on *Actinobacillus pleuropneumoniae* infections in pigs. Porcine IFN-α (PoIFN-α) is a powerful adjuvant for foot-and-mouth disease vaccine in swine. Both human and bovine IFN-α have been used for the treatment of feline leukemia. Recombinant feline IFN-ω has also been tested in feline leukemia virus and feline immunodeficiency virus infections. In almost all cases, high-dose IFN treatment of infectious diseases has produced some positive responses. These are not impressive, however, and the treatments may have toxic side effects, such as fever, inappetence, and malaise.

A modified form of granulocyte colony-forming factor (pegbovigrastim) has been used to elevate neutrophil numbers in dairy cattle around the time of parturition. It is given by injection just before and after calving. This is effective in decreasing the prevalence of mastitis in these animals. (This G-CSF molecule is "pegylated." That is, linked to the hydrophilic polymer polyethylene glycol [PEG]. This modification significantly increases its half-life and stability.)

Recombinant IL-2 has been administered to pigs at the same time that they were vaccinated against *A. pleuropneumoniae* or pseudorabies and to calves vaccinated against BHV-1. Although it enhances immunity, IL-2 is very toxic. It causes severe side effects, including malaise, a capillary leak syndrome, diarrhea, and fever. It is interesting to note, however, that relatively low doses of rHuIL-2, when injected directly into papillomas or carcinomas of the vulva in cattle, induced a positive response in more than 80% of cases, and some complete regressions were observed. Despite this, clinical trials employing purified cytokines have generally produced disappointing results.

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42

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Immunodiagnostic Techniques

CHAPTER OUTLINE

Reagents Used in Serological Tests, 472

Serum, 472

Antiglobulins, 472

Monoclonal Antibodies, 472

Specific Antibodies, 472

Primary Binding Tests, 472

Radioimmunoassays, 472

Immunofluorescence Assays, 473

Direct Fluorescent Antibody Tests, 473

Indirect Fluorescent Antibody Tests, 473

Particle Concentration Fluorescence Immunoassays, 474

Immunoenzyme Assays, 474

Microwell Enzyme-Linked Immunosorbent Assay Tests, 474

Western Blotting, 476

Immunohistochemistry, 478

Disposable Immunoassay Devices, 478

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Bidirectional Flow ELISA Techniques, 478
Immunochromatography, 479
Antibody Labels, 479
The Flow Cytometer, 480
Secondary Binding Tests, 481
Precipitation Tests, 481
Immunodiffusion, 482
Radial Immunodiffusion, 482
Immunoelectrophoresis and Related Techniques, 483
Titration of Antibodies, 484
Agglutination, 484
Antiglobulin Tests, 484
Passive Agglutination, 484
Viral Hemagglutination and Its Inhibition, 485
Complement Fixation, 485
Cytotoxicity Tests, 485
Assays in Living Systems, 486
Neutralization Tests, 486
Protection Tests, 486
Molecular Methods, 487
Diagnostic Applications of Immunological Tests, 487

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LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Explain how the detection of antibodies in serum may assist in the diagnosis of infectious diseases.
- Define antiglobulin, monoclonal antibodies, complement fixation, ELISA, Western blot, immunoelectrophoresis, radioimmunoassay, immunochromatography, lateral flow assays, immunofluorescence, immunohistochemistry, radial immunodiffusion, immunofiltration, viral hemagglutination, sensitivity and specificity, and ROC curves.
- Understand the principles behind the major serologic assays as well as their advantages and disadvantages.
- Describe why the most sensitive and specific tests are those that directly detect the antigen or antibody of interest.
- Understand why secondary binding tests tend to be the easiest to perform but are less sensitive than primary binding tests.
- Understand that tertiary tests directly measure protection but are often complex, slow, and expensive.
- Explain how serological tests are judged by the number of false-positive results they generate (their *specificity*) and by the number of false-negative test results they generate (their *sensitivity*).
- Understand why, in general, highly sensitive tests tend to have low specificity, and vice versa. Thus in selecting a test, a veterinarian must assess the relative importance of false-positive and false-negative results.
- Describe the principles of the polymerase chain reaction assay.
- Describe how a flow cytometer works.

The exquisite specificity of antibody-mediated immune responses may be exploited in two ways. First, specific antibodies may be used to detect or identify an antigen of interest. These antigens can be associated with an infectious agent, or can simply be molecules that need to be located or measured. Second, by detecting specific antibodies in serum, it is possible to determine if an animal has been exposed to an infectious agent. This may establish a diagnosis or determine the degree of immunity of the animal to that agent.

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The measurement of antigen-antibody interactions for diagnostic purposes is called serology.

Serological techniques can be classified into three broad categories. Primary binding tests directly measure the binding of antigen to antibody (Table 42.1). Secondary binding tests measure the results of antigen-antibody interaction *in vitro.* These tests are usually less sensitive than the primary binding tests but may be simpler to perform or require less complex technology. The third category, *in vivo* tests, measure the actual protective effect of antibodies in an animal.

TABLE 42.1

Smallest Amount of Antibody Protein Detectable by Selected Immunological Tests

Tests	Protein (μg/mL)
Primary Binding Tests	
ELISA	0.0005
Competitive radioimmunoassay	0.00005
Secondary Binding Tests	
Gel precipitation	30
Ring precipitation	18
Bacterial agglutination	0.05
Passive hemagglutination	0.01
Hemagglutination inhibition	0.005
Complement fixation	0.05
Virus neutralization	0.00005
Bactericidal activity	0.00005
Antitoxin neutralization	0.06
***In Vivo* Test**	
Passive cutaneous anaphylaxis	0.02

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Reagents Used in Serological Tests

Serum

The most common source of antibodies is serum obtained from clotted blood. Serum may be stored frozen and tested when convenient. If necessary, the serum can be depleted of complement activity by heating to 56° C for 30 minutes.

Antiglobulins

Immunoglobulins are antigenic when injected into an animal of a different species. For example, purified dog immunoglobulins can be injected into rabbits. The rabbits respond by making specific antibodies called antiglobulins. Depending on the purity of the injected immunoglobulin, it is possible to make nonspecific antiglobulins against immunoglobulins of all classes, or very specific antiglobulins directed against single classes. Antiglobulins are essential reagents in many immunological tests.

Monoclonal Antibodies

Hybridoma-derived monoclonal antibodies are pure and specific, can be used as standard chemical reagents, and can be obtained in almost unlimited amounts (Chapter 15). As a result, monoclonal antibodies frequently replace conventional antiserum as reagents in immunodiagnostic tests.

Specific Antibodies

When detecting antigens in tissues or body fluids, the first steps may involve the use of a specific antibody against the antigen of interest. Although these antibodies are usually made by immunizing goats or rabbits, there is a growing interest in using chicken IgY antibodies. Birds may react very strongly against mammalian antigens. Chickens produce large amounts of IgY antibodies that become concentrated in the egg yolk. It may be much more convenient to harvest large amounts of antibody from

egg yolks than have to bleed animals repeatedly. Antiglobulins may then be used to detect the bound IgY.

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Primary Binding Tests

Primary binding tests are performed by allowing antigen and antibody to combine and then measuring the immune complexes formed. In order to measure these reactions, one of the reactants must be chemically labeled. Radioisotopes, fluorescent dyes, plastic beads, colloidal metals, and enzymes have all been used as labels in these tests.

Radioimmunoassays

Assays that use radioisotopes as labels have the advantage of being incredibly sensitive. On the other hand, isotope detection systems are expensive. This expense, combined with the hazards of radioactivity and the need to dispose of radioactive material in a safe manner, has ensured that radioimmunoassays are only used when highly sensitive assays are required to detect small amounts of antigens such as performance-enhancing drugs.

Competitive immunoassays are based on the principle that unlabeled antigen will displace radiolabeled antigen from immune complexes (Fig. 42.1). These tests are commonly used to detect trace amounts of drugs. The antigen (or drug) is labeled with a radioactive isotope such as tritium (H^3), carbon-14, or iodine-125. When radiolabeled antigen is mixed with its specific antibody, the two combine to form immune complexes that can be precipitated out of solution. Any radioactivity remaining in the supernatant fluid is due to the presence of unbound antigen. If unlabeled antigen is added to the mixture, before adding the antibody, it will compete with the radioactive antigen for antibody-binding sites. As a result, some labeled antigen will be unable to bind, and the amount of radioactivity in the supernatant will increase. If a standard curve is first constructed based on the use of known amounts of unlabeled antigen, the amount of antigen in a test sample may be measured by reference to this standard curve.

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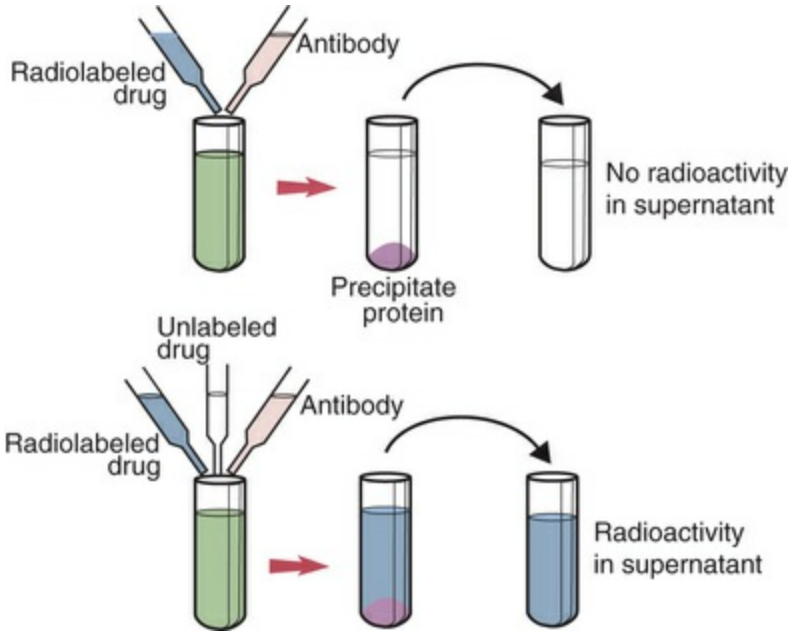


FIG. 42.1 The principle of competitive radioimmunoassay. Unlabeled antigen in the test solution displaces labeled antigen from immune complexes. The amount of labeled antigen released will be proportional to the amount of unlabeled antigen added.

Immunofluorescence Assays

Fluorescent dyes are commonly employed as labels in primary binding tests, the most important being fluorescein isothiocyanate (FITC). FITC is a yellow compound that can be chemically linked to antibodies without affecting their reactivity. When radiated with invisible ultraviolet or blue light at 290 and 145 nm, FITC re-emits visible green light at 525 nm. This green light can be readily seen under a fluorescent microscope. FITC-labeled antibodies are used in the direct and indirect fluorescent antibody tests.

Direct Fluorescent Antibody Tests

Direct fluorescent antibody tests are used to identify the presence of antigen in a tissue sample. Antibody directed against a specific antigen such as a bacterium or virus is first labeled with FITC. A tissue section or smear containing the organism is fixed to a glass slide, incubated with the labeled antiserum, and then washed to remove any unbound antibody (Fig. 42.2). When examined by dark field illumination under a microscope with an ultraviolet light source, the organisms that bind the labeled antibody will fluoresce

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brightly. This test can identify the presence of small numbers of bacteria in a sample. For example, it can be used to detect *Mycobacterium avium* subspecies *paratuberculosis* in feces, or to detect bacteria such as *Dichelobacter nodosus, Listeria monocytogenes,* or clostridia in diseased tissues (Fig. 42.3). It may also be employed to detect viruses in tissue culture or in tissues from infected animals. Examples include the detection of rabies virus in the brains of infected animals or feline leukemia virus in infected cat leukocytes (see Fig. 40.3).

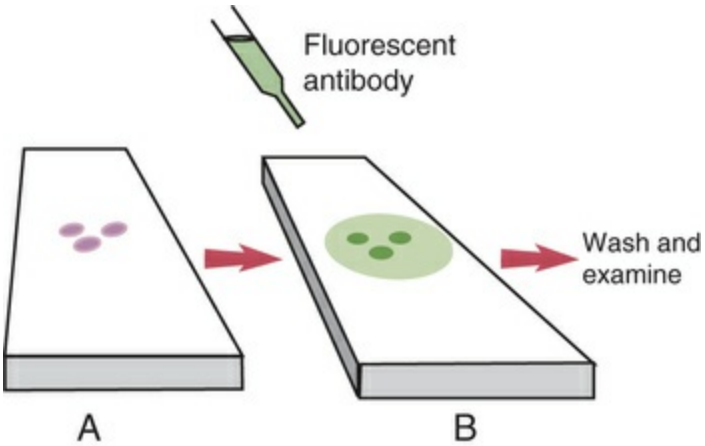


FIG. 42.2 The direct fluorescent antibody assay. This technique is used to detect antigen by means of FITC-labeled antibody.

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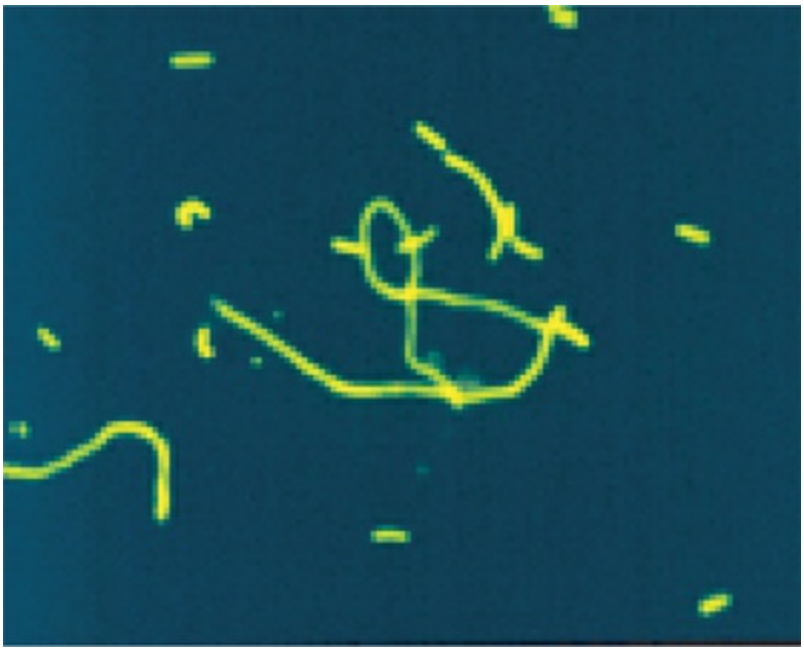


FIG. 42.3 Direct immunofluorescence of a smear of *Clostridium septicum.* (See also Figs. 22.10 and 38.4.) (Courtesy Dr. J. Huff.)

Indirect Fluorescent Antibody Tests

Indirect fluorescent antibody tests can be used to measure antibodies in serum or to identify specific antigens in tissues or cell cultures. When measuring antibody levels, antigen is employed as a tissue smear, section, or cell culture on a slide or coverslip. This is incubated in serum suspected of containing antibodies to that antigen. The serum is then washed off, leaving only specific antibodies bound to the antigen (Fig. 42.4). These bound antibodies may then be visualized by incubating the smear in FITC-labeled antiglobulin. When the unbound labeled antiglobulin is removed by washing and the slide examined, the presence of fluorescence indicates that antibody was present in the test serum. The quantity of antibody in the test serum may be estimated by testing increasing dilutions of serum on different antigen preparations.

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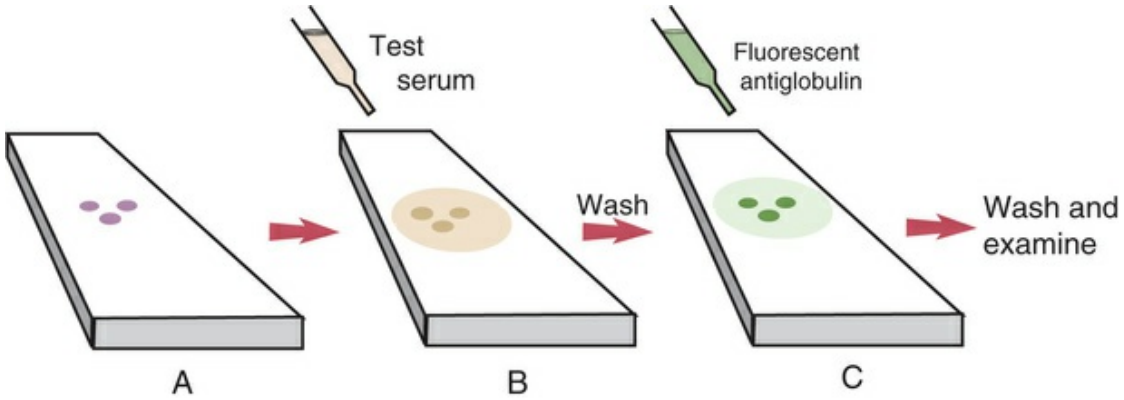


FIG. 42.4 The indirect fluorescent antibody test may be used to detect either antigen or antibody. The antigen, in a section smear or culture, will bind antibody from serum. After washing, this antibody may be detected by binding to FITC-labeled antiglobulin.

The indirect fluorescent antibody test has two advantages over the direct technique. Since several labeled antiglobulin molecules will bind to each antibody molecule, the fluorescence will be considerably brighter than in the direct test. Similarly, by using antiglobulins specific for each immunoglobulin class, the class of the specific antibody may also be determined.

Particle Concentration Fluorescence Immunoassays

Immunofluorescence assays can be automated and quantitated by means of particle immunoassays (Fig. 42.5). For example, antigen-coated, sub-micrometer polystyrene particles (beads) can be mixed with test serum. After incubation, the particles are recovered by vacuum filtration, washed to remove unbound antibody, and exposed to a fluorescent antiglobulin. After filtering the suspension again and washing to remove unbound antiglobulin, the particle suspension can be placed in a spectrofluorometer and the intensity of particle-bound fluorescence measured. This provides a measure of the level of antibodies in the test serum. A very useful variation on this is the competitive assay used as a rapid test for antibodies to *Brucella abortus* in cattle. In this case, *Brucella* antigen-coated polystyrene particles are mixed with a standard amount of fluorescent anti-*Brucella* serum and the serum under test. If positive, the unlabeled test serum blocks the binding of fluorescent antibodies to the particles. The more antibodies in the test serum, the greater is the inhibition of fluorescent antibody binding.

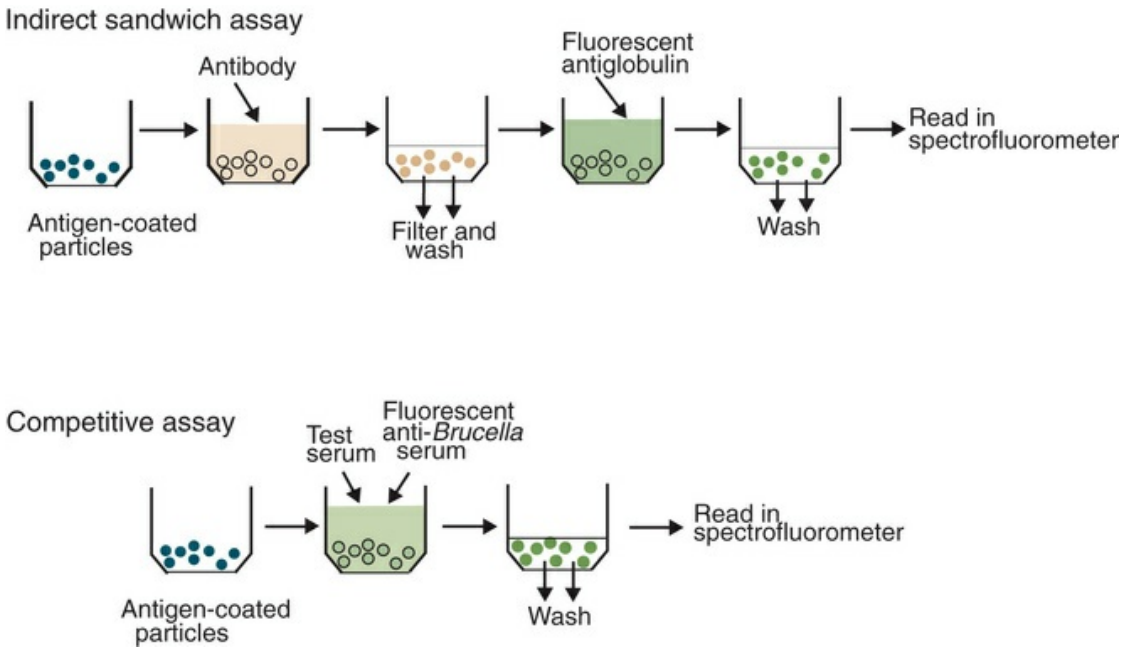


FIG. 42.5 The principle of the particle concentration fluorescence immunoassay.

Immunoenzyme Assays

Among the most widely employed immunoassays in veterinary medicine are the enzyme-linked immunosorbent assays (ELISAs). As with other primary binding tests, ELISAs may be used to detect and measure either antibodies or antigens. They have good sensitivity and specificity, and can be performed in many different formats ranging from individual animal testing to automated high throughput screening of large numbers of samples. Unlike fluorescent antibody tests, they do not require specialized equipment to read.

Microwell Enzyme-Linked Immunosorbent Assay Tests

The most common form of ELISA is used to detect and measure specific antibodies. In order to perform this assay, it is usual to use microwells in polystyrene plates (the commonly used plate uses 96 wells). The wells are first filled with an antigen solution (Fig. 42.6). Proteins bind firmly to polystyrene surfaces so that after unbound antigen is removed by vigorous washing, the wells remain coated with a layer of antigen. These coated plates can be stored until required. The serum under test is added to the wells. Any

antibodies in the serum will bind to the antigen layer. After incubation and washing to remove unbound antibody, the presence of bound antibodies can be detected by adding a solution containing an antiglobulin chemically linked to an enzyme. This labeled antiglobulin binds to the antibody and, following incubation and washing, can be detected and measured by adding a solution containing the enzyme substrate. The enzyme and substrate are selected to ensure that a colored product develops in the tube. The intensity of the color that develops is therefore proportional to the amount of enzyme-linked antiglobulin that is bound, which in turn is proportional to the amount of antibody present in the serum under test. The color intensity may be estimated visually or by spectrophotometry. The sensitivity of the assay depends on the affinity of the antibodies and the detection system used. It is also affected by nonspecific binding of antibodies to the plastic surface.

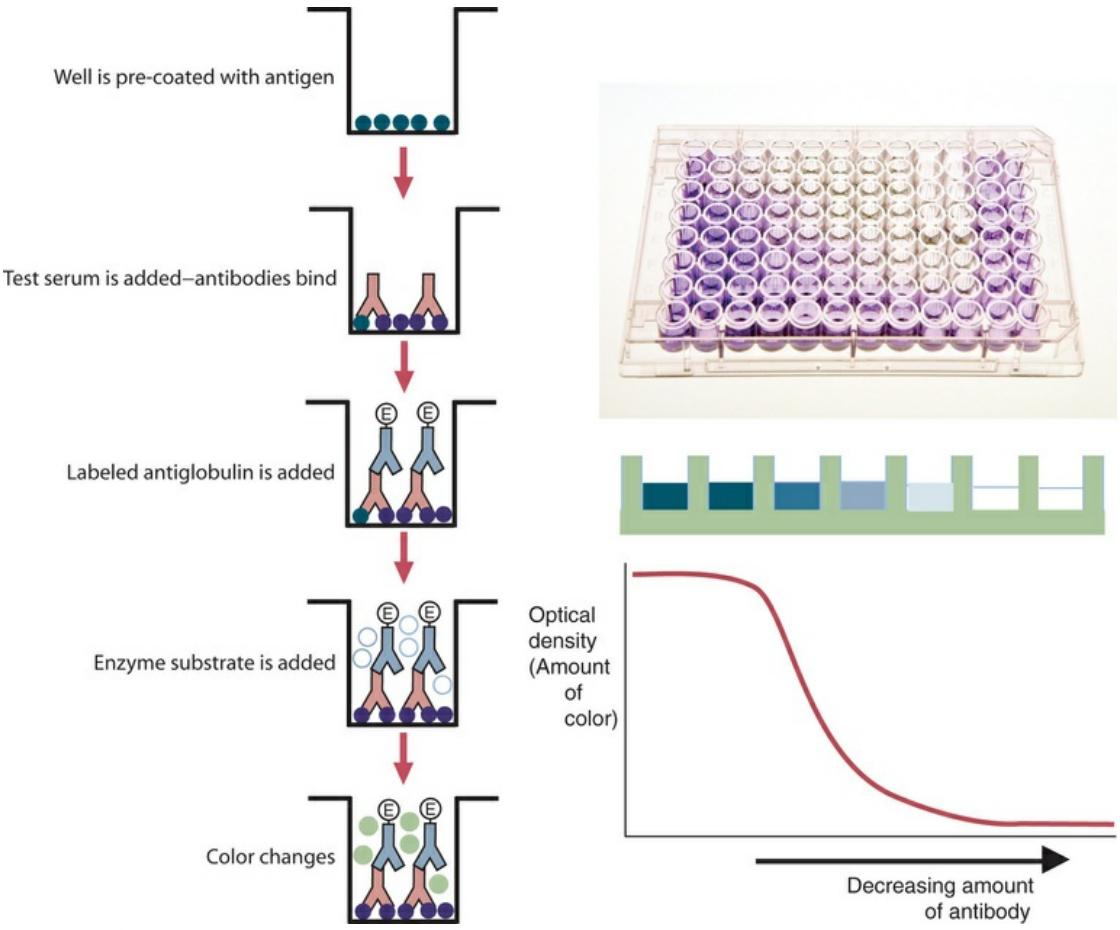


FIG. 42.6 The indirect ELISA technique. Antigen is bound to the

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wells in a styrene plate. The presence of bound antibody is detected by means of an enzyme-labeled antiglobulin. Addition of the enzyme substrate leads to a color change proportional to the amount of bound antibody. This color change can be estimated visually or read in an ELISA reader (a specially adapted spectrophotometer).

One modification of this technique is the antibody sandwich ELISA, which can be used to detect and measure a specific antigen (Fig. 42.7). The wells in polystyrene plates are coated with specific antibody (capture antibody) before testing. To conduct the test, the antigen solution to be tested is added to each well. The capture antibody will bind any antigen present in the test solution. This step is followed, after washing, by specific antibody, which also binds the antigen (the detection antibody). After washing to remove unbound antibody, enzyme-labeled antiglobulin and substrate (as described for the indirect technique) are added. (It is important that the capture antibody and the detection antibody are from a different species and that a species-specific antiglobulin is used for visualization of the detection antibody. This will avoid false-positive results caused by binding of the antiglobulin to the capture antibody in the absence of antigen.) In this assay, the intensity of the color reaction is related directly to the amount of bound antigen. Because these tests involve the formation of antibody-antigen-antibody layers, they are called sandwich ELISAs. They are used, for example, to detect circulating virus in blood from cats with feline leukemia.

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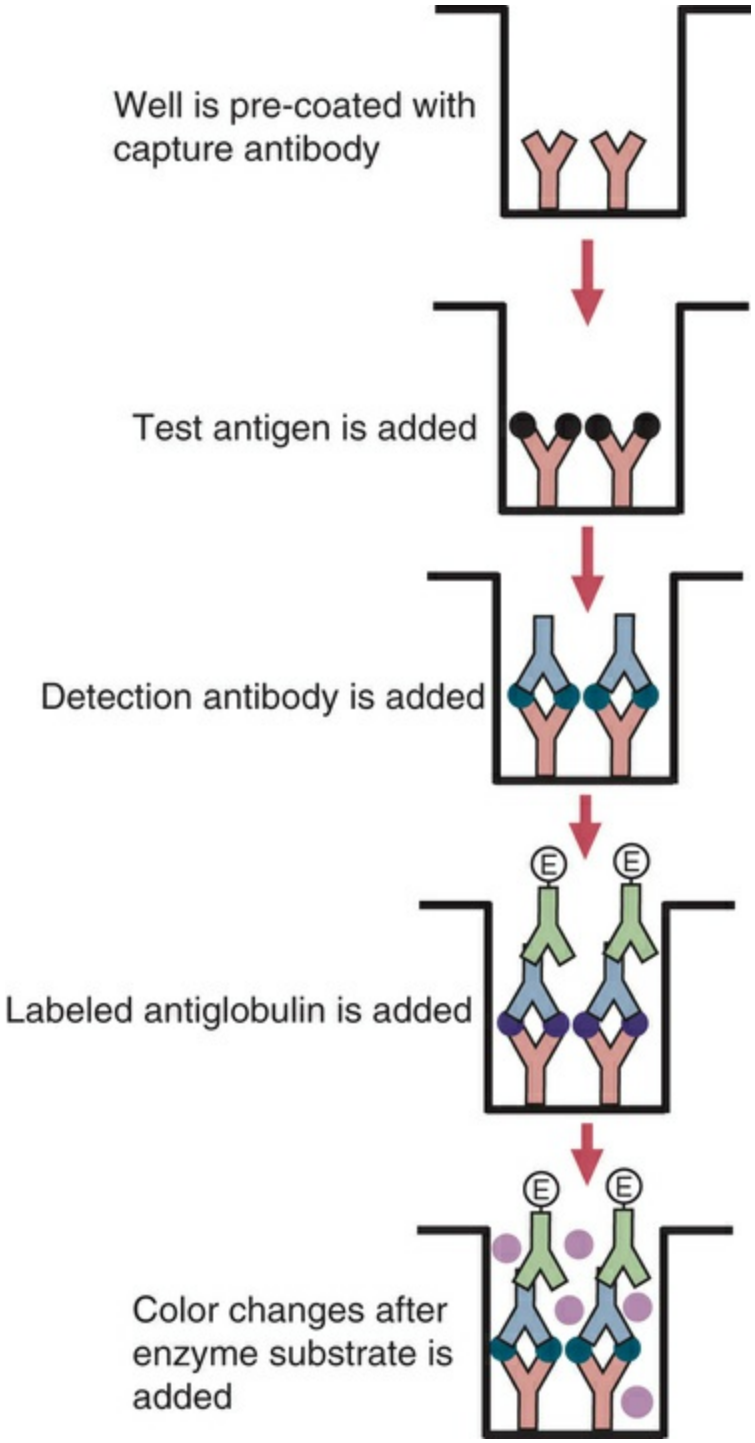


FIG. 42.7 The antibody sandwich ELISA. Antigen is bound to the plate by means of an antibody. The presence of that bound antigen is detected by sequential addition of a second antibody and an enzyme-labeled antiglobulin. Addition of the enzyme substrate leads to a color change proportional to the amount of bound antigen.

Another common modification of this technique is the labeled-antigen ELISA used to detect antibodies. This is favored in manufactured diagnostic kits. The antigen is bound to the

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microwells before testing (Fig. 42.8). The serum to be tested is added, followed, after washing, by labeled antigen. Any serum antibodies present will bind the labeled antigen to the microwell where it can be measured.

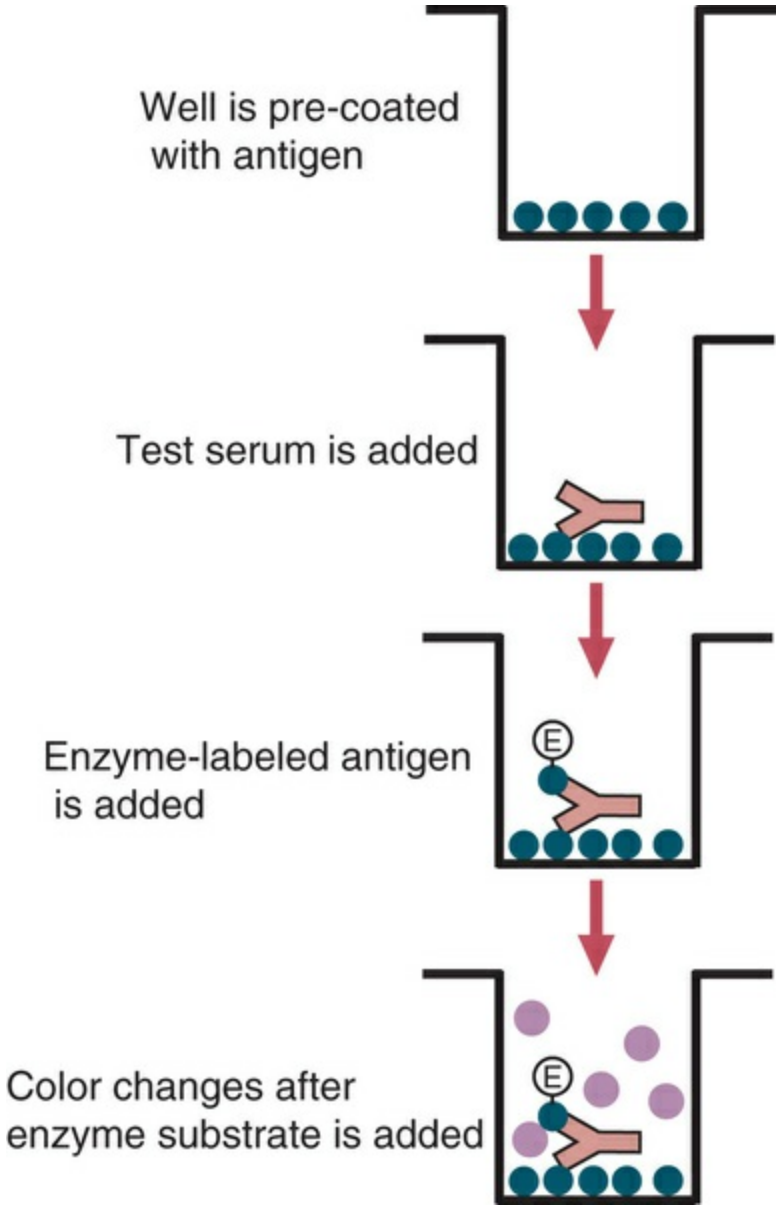


FIG. 42.8 The labeled antigen ELISA. The serum under test is added to an antigen-coated plate. Bound antibodies are then detected by an enzyme-labeled antigen.

A competitive ELISA can be used to measure hapten molecules or viral antigens (Fig. 42.9). In this technique, each microwell is coated with specific antibody before testing. In a single reaction, the

test sample and enzyme-labeled antigen are placed in the well where the antigens compete for the antibody-binding sites. The amount of labeled antigen bound to the microwell is inversely related to the concentration of antigen in the test sample. This technique is faster than other ELISA techniques. It can be made very sensitive if the sample antigen is permitted to react with the antibody before the labeled antigen is added.

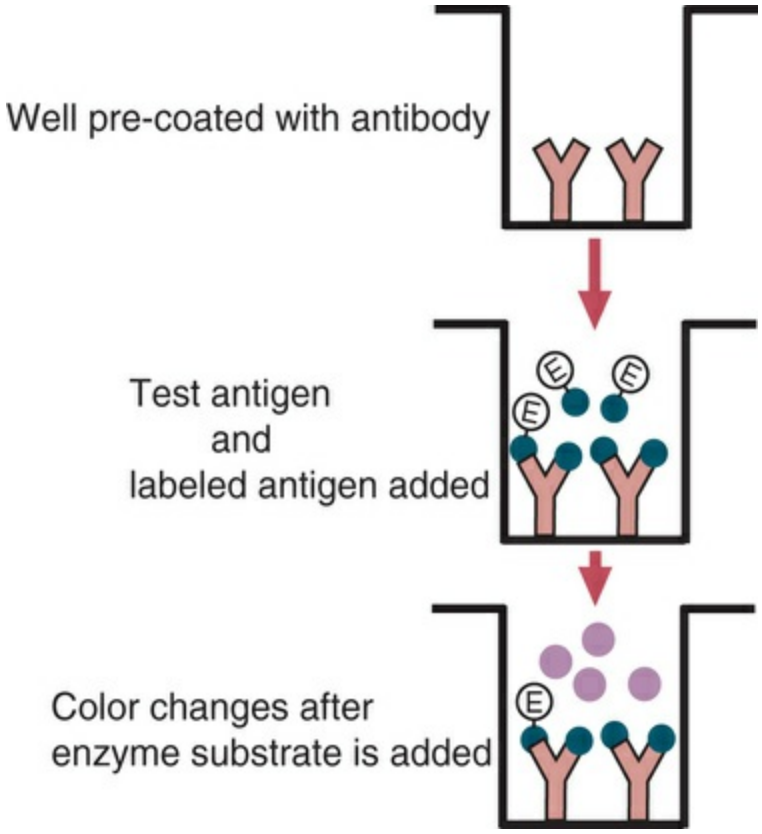


FIG. 42.9 The competitive ELISA. Labeled and unlabeled antigens compete for binding to antibody. Addition of the enzyme substrate leads to a color change inversely proportional to the amount of test antigen bound.

It is possible to conduct an ELISA on living cells growing in culture. This technique, called the enzyme-linked ImmunoSpot (ELISpot) (if a fluorescent label is used the test is known as a FluoroSpot) assay, uses exactly the same principle as antigen detection ELISAs; it simply detects the antigen on a cell surface rather than on plastic. This is commonly used to detect and measure cytokine production by cells since its limits may be as low as one cell in 100,000. Its sensitivity is similar to that of reverse-

transcriptase PCR analysis. Its use is discussed in Chapter 33.

Western Blotting

One solution to the problem of identifying protein antigens in a complex mixture is by use of a technique called Western blotting. This is a three-stage primary binding test (Fig. 42.10). Stage 1 involves electrophoresis of a protein mixture on gels so that each component is resolved into a single band. Stage 2 involves blotting or transfer of these protein bands to an immobilizing nitrocellulose membrane. This is accomplished by placing the membrane on top of the gel and sandwiching the two between sponges saturated with buffer. The membrane-gel sandwich is supported between rigid plastic sheets and placed in a buffer reservoir, and an electrical current is passed between the sponges. The protein bands are transferred from the gel to the membrane without loss of resolution.

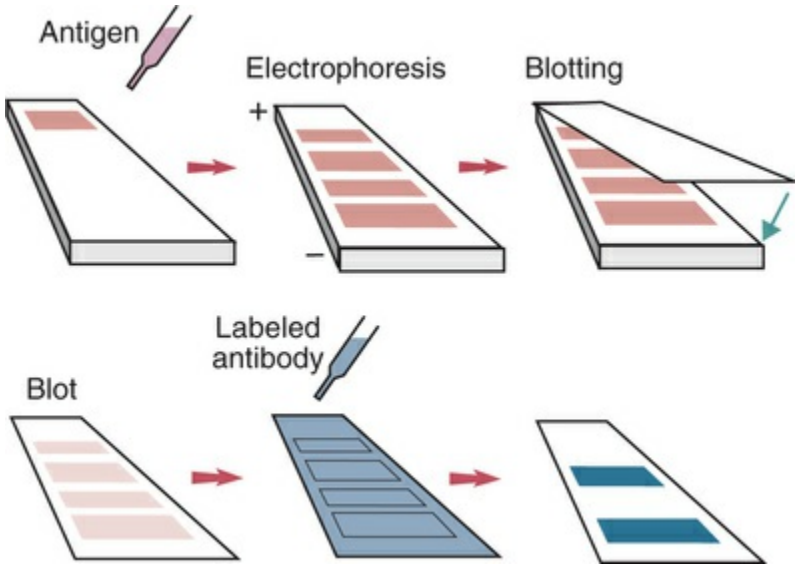


FIG. 42.10 The Western blotting technique. Serum is separated by electrophoresis and blotted onto nitrocellulose paper; the antigen bands are revealed by use of specific antibody and an enzyme- or isotope-labeled antiglobulin. The blotting stage may be a passive transfer or an electric potential may be used to accelerate the blotting process.

The third stage involves visualization of transferred antigens by means of an enzyme immunoassay or radioimmunoassay. When an enzyme immunoassay is employed, the membrane is first incubated

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in specific antiserum. After the membrane has been washed, an enzyme-labeled antiglobulin solution is added. When this is removed by washing, substrate is added, and a color develops in the bands where the antibody has bound to antigen. When isotope-labeled antiglobulin is used, an autoradiograph must be made and the labeled band identified by darkening of a photographic emulsion. Western blotting is used to identify the important antigens in complex microorganisms or parasites (Fig. 42.11). A variant form of the Western blot is the dot blot. Antigen solution is filtered through a nitrocellulose membrane so that any protein binds to the membrane. The presence of bound antigen can be determined using specific antiserum and enzyme-labeled antiglobulin in sequence. After exposure to enzyme substrate, the presence of a stained dot is a positive reaction. (Use of nasal washings as a source of the antigen, such as when trying to detect respiratory viruses, is called a snot-blot!)

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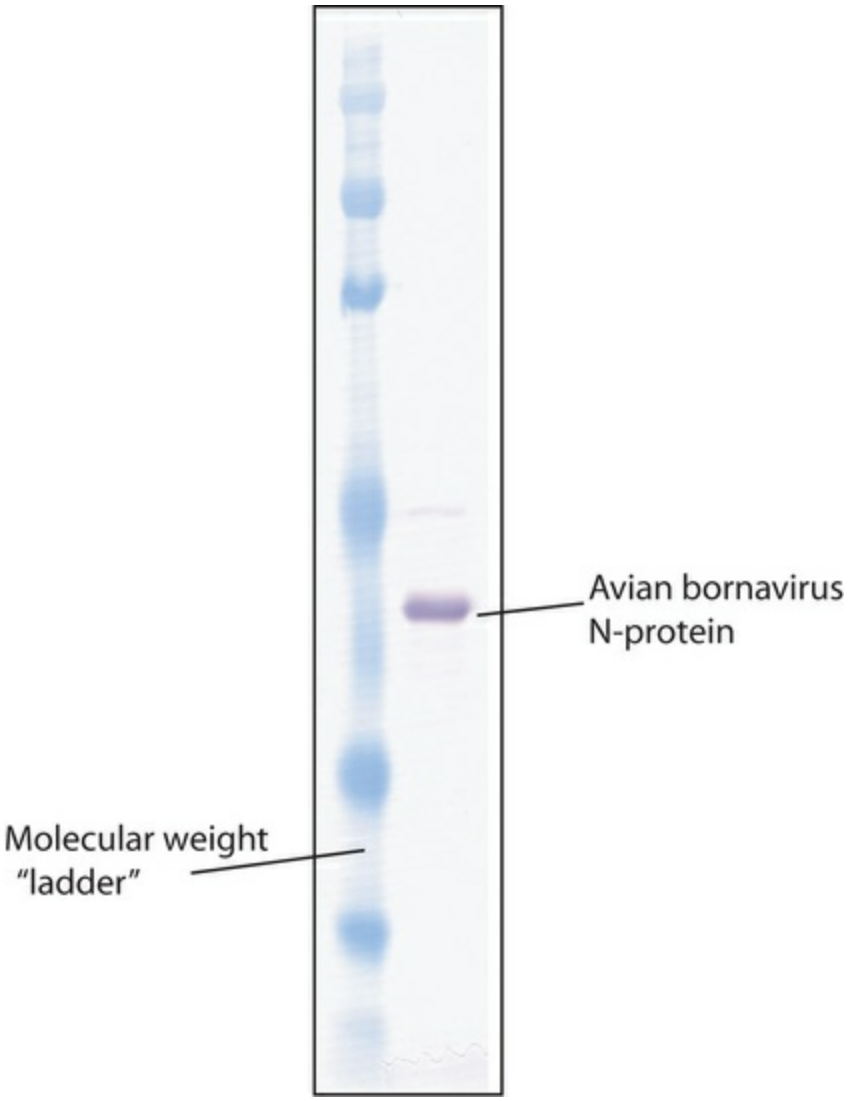


FIG. 42.11 A Western blot assay. In this example, the serum of a bird was tested for the presence of antibodies to avian bornavirus N-protein. Proteins from a culture of avian bornavirus were first separated by electrophoresis. The electrophoresed material was then blotted onto nitrocellulose paper. Serum from the bird to be tested was allowed to react with the viral proteins and unbound antibodies removed by washing. Finally, the presence of bound antibodies was revealed using an enzyme-labeled antiglobulin followed by enzyme substrate. The N-protein is revealed as a colored band of the correct size. The stained bands on the left are markers of defined molecular weights. (Courtesy Dr. I. Villanueva.)

It is possible to put "dots" of many different monoclonal antibodies on a single sheet of nitrocellulose. They may then be exposed to a complex labeled antigen mixture such as a cell protein extract, and after washing and development, the relative concentrations of many different antigens can be visualized. This is known as an antibody microarray.

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Alternatively, antigen dots, such as those from a virus, may be printed onto nitrocellulose membranes. Sequential addition and washing of the test serum, an enzyme labeled antiglobulin, and substrate will result in the development of a colored dot on the substrate. The intensity of the dot is proportional to the amount of antiviral antibody in the test sample. If multiple antigen dots plus control dots are printed in an array on a glass slide, many assays can be conducted simultaneously with small sample volumes. These can be measured using array scanners. This is called a multiantigen print immunoassay.

ELISAs can be used to test fluids other than blood. For example, saliva or tears can be tested for the presence of feline leukemia virus. In most cases, these are simply modified versions of the serum ELISA tests. However, in one such test, a hard plastic swab with antibody to feline leukemia virus bound to the tip is rubbed throughout the cat's mouth. The antibodies on the swab are protected by a sugar coating that is removed by soaking before the test. The antibody on the swab will bind any viral antigen in the saliva. The swab is then inserted into a tube containing enzyme-labeled monoclonal antibodies against feline leukemia virus antigens. After washing, the swab is placed in a solution of the enzyme substrate and the color change noted. This technique is much less sensitive than testing blood directly but is very convenient.

Immunohistochemistry

Enzymes conjugated to immunoglobulins or antiglobulins can be used to locate specific antigens in tissue sections. Horseradish peroxidase is the most widely employed label. The tests are performed in a manner similar to the immunofluorescence tests. In the direct immunoperoxidase test, the tissue section is treated with the enzyme-labeled antibody. After washing, the tissue is incubated in a solution of the appropriate enzyme substrate. Bound antibody is detected by the development of a brown color at the site of antibody binding (Fig. 42.12). In the indirect test, bound antibody is detected by means of a labeled antiglobulin. This technique has a significant advantage over immunofluorescence techniques in that the tissue can be examined by conventional light microscopy and

can be stained so that structural relationships are easier to see.

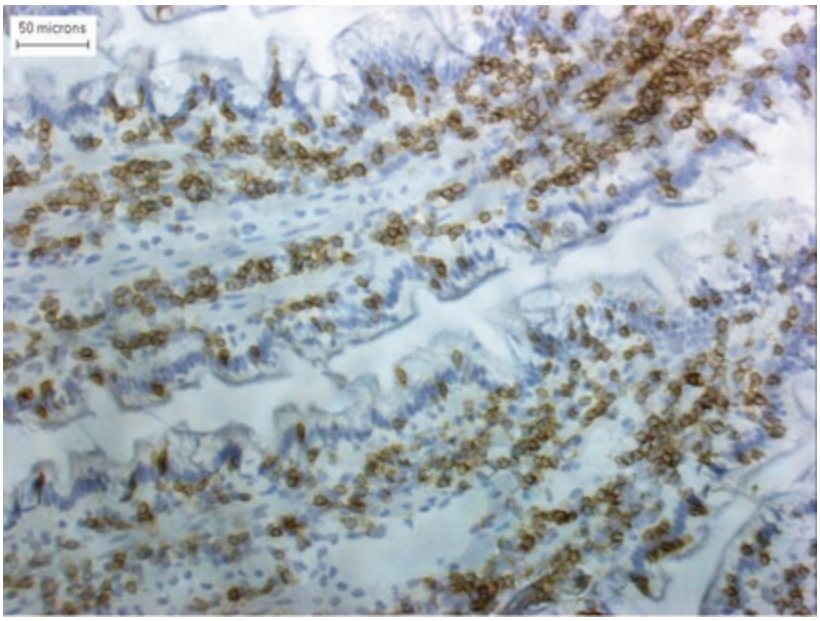


FIG. 42.12 The immunoperoxidase technique showing the presence of α/β T cells in the lamina propria and epithelium of canine duodenum. Cells binding the monoclonal antibody are exposed to peroxidase-labeled specific antiglobulin. The presence of the peroxidase is revealed as a brown deposit. (From German AJ, Hall EJ, Moore PF, et al: The distribution of lymphocytes expressing alphabeta and gammadelta T cell receptors, and the expression of mucosal addressin cell adhesion molecule-1 in the canine intestine, *J Comp Pathol* 121:249-263, 1999.)

Disposable Immunoassay Devices

Recent years have seen the development of simple immunoassays that can be employed in the clinic or office and will provide diagnostic results within a few minutes. These assays simply provide all necessary reagents in excess, and the sample under test becomes the limiting feature. Most disposable devices use this form of assay because the use of excess reagents makes the accurate metering of the sample unnecessary. Examples include flow ELISA techniques, and immunochromatography assays.

Bidirectional Flow ELISA Techniques

Bidirectional flow devices use a capture reagent (antibody or antigen) immobilized on a porous membrane to detect an analyte

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(antigen or antibody) in a test sample (Fig. 42.13). One simple method uses a polyethylene flow matrix containing capture reagents applied as sample and control spots. The flow matrix is set on a support base connected to reagent reservoirs and an absorbent bed. A test sample, such as blood containing an antigen, is mixed with a detection reagent, applied to the device, flows down the flow matrix, and interacts with deposited sample and control spots. The device is activated, which initiates the sequential application of defined volumes of wash solution and substrate reagent. The sample and detection reagent mixture is applied to one end of the flow matrix and flows in the forward direction, whereas wash and substrate are applied to the opposite end and flow in the reverse direction. The wash step and the bidirectional flow of sample and assay reagents are key features of the assay, which produce a clean, white background so that weak-positive results can be easily visualized. A positive result, where antigen or antibody has bound, is observed as a colored dot. Forms of this test can be single tests commonly employed for the detection of single analytes, or can be combination assays for detection of two or more antigen or antibody analytes. One such combination test is used for the detection feline leukemia virus antigen and feline immunodeficiency antibody, another combination test is used for detection of heartworm antigen and antibody to five tick-borne pathogens. When appropriately standardized, these tests can provide semiquantitative results by comparing the color intensity of the sample spot result to the intensity of a reference spot result. Semiquantitative in-clinic assays are available for pancreatic lipase and NT-proBNP (N-terminal pro-brain natriuretic peptide).

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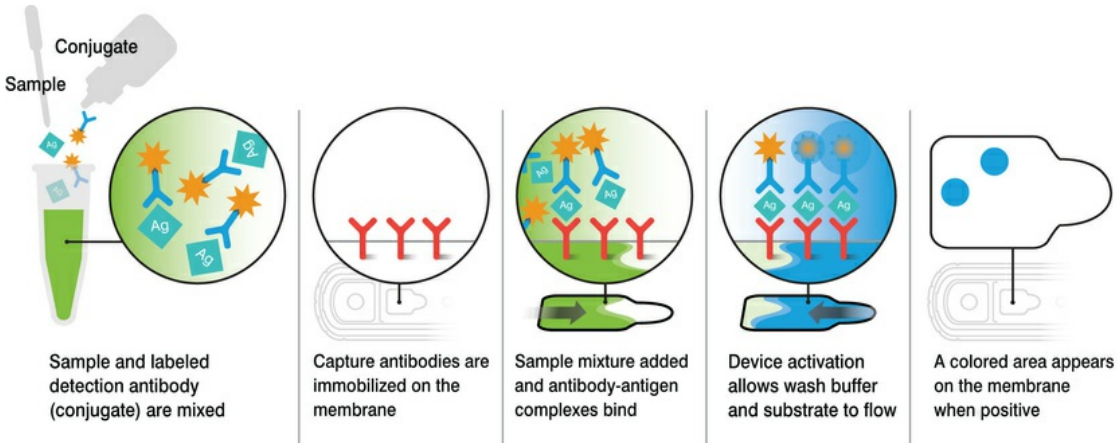


FIG. 42.13 An in-clinic ELISA technique. The SNAP® assay is an in-clinic device that performs each of the steps typically associated with an ELISA in a sequential fashion with minimal hands-on time. Sample and labeled detection antibody (conjugate) are applied to a flow matrix and interact with capture antibodies. The device is activated, which initiates sequential flow of wash and substrate, which removes unbound reagents, amplifies the signal, and generates the SNAP® test's distinctive blue color. In practice, this method is used in the form of a self-contained, plastic-mounted device containing reagent reservoirs. (Copyright IDEXX Laboratories, Inc.)

Immunochromatography

Immunochromatography assays are even faster and easier to read. In their simplest form, they involve allowing a solution containing antigen (such as infected blood) to flow laterally through a porous strip. As the solution passes through the strip, it first passes through a zone where it meets and solubilizes dried labeled antibody and forms immune complexes. This antibody may be labeled with either colloidal gold (pink color) or colloidal selenium (blue color). The fluid then flows through a detection zone containing immobilized antibody against the antigen where any immune complexes are captured. As a result, a pink or blue line or dot develops in the detection zone in a positive test (Fig. 42.14). This simple procedure permits multiple samples to be analyzed in a one-step procedure. A positive control band can be developed as well, and the use of an effective pre-filter can permit the use of whole blood. Lateral flow assays are used for the detection of heartworm or feline leukemia antigens. Similar assays may be used to detect many different viruses such as rota- or parvoviruses and bacteria such as salmonella or tuberculosis. Results are available within

minutes.

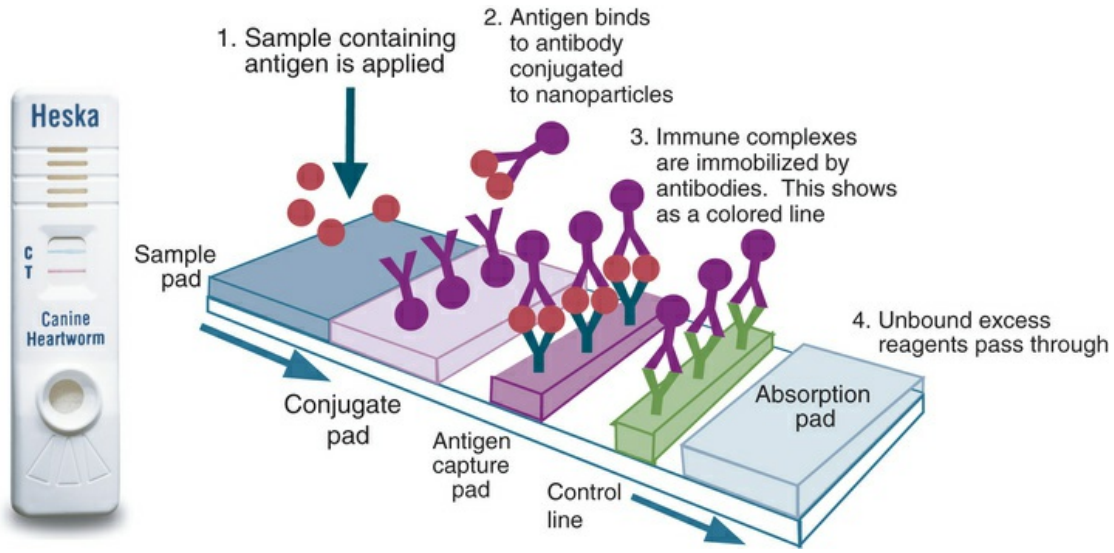


FIG. 42.14 Immunochromatography. A sample containing antigen flows through a porous strip. The antigen, if present, binds to labeled antibodies. If positive, the labeled antigen-antibody complexes are captured by antiglobulins so that positive reactions are shown by the appearance of a colored band. (Solo Step® photo courtesy Heska Corporation.)

Immunochromatography systems are made in several different formats. For example, the sample containing the antigen of interest can be applied to a porous membrane at one end of the strip. Then capillary action can draw the solution through a conjugate pad, a solid-phase detection zone, and into an absorption pad. Buffer may be added to speed the flow of antigen solution. In another form of this assay, the antigen solution is dropped onto a pad containing antibody. This is followed by wash buffer that drives the immune complexes through the pad to an area containing labeled antiglobulin. The immune complexes are captured at this point. Then buffer can be applied at the other end of the pad and used to flush labeled complexes back to the detection zone where they form a colored band or dot. These tests can also be employed for antibody detection using recombinant antigens bound to the substrate.

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Antibody Labels

Although radioisotopes and enzymes are commonly used as labels for primary binding tests, both have disadvantages. For example, radioactive isotopes may have a short half-life, are potentially hazardous, and may require expensive detection devices. Enzymes, though stable and relatively cheap, are large molecules that may inhibit antibody activity or lose enzymatic activity in the process of being bound to antiglobulin. One alternative is to use the small molecule biotin and its specific binding protein avidin. Biotin can bind to proteins without affecting their biological activity. Avidin binds very strongly and specifically to biotin and may be conjugated with enzymes.

The most popular enzymes used in ELISAs include alkaline phosphatase, horseradish peroxidase, and β-galactosidase. Enzyme assays involving the production of luminescent products, such as luciferase, may be many times more sensitive than conventional enzyme assays but require sophisticated instruments to measure the luminescence produced. Colored dyes linked to antibodies have been used in dipstick assays. Reagents linked to ferritin or colloidal gold may be used to identify the location of antigens in cells examined by electron microscopy because such labels are electron dense. As described previously, colloidal gold and colloidal selenium are colored and may be used as labels in simple immunochromatography tests.

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The Flow Cytometer

Because of the importance of identifying cell phenotypes, considerable effort has gone into developing rapid identification methods for cell surface antigens. Phenotypes can now be automatically analyzed in great detail and with high efficiency using a flow cytometer (Fig. 42.15). In this instrument a suspension of cells is pumped through a very narrow tube so that the cells pass through in single file. A laser beam is directed through the cell stream, and the effects of each cell on the light beam are measured. The scatter of the light beam in a forward direction can be used to give a measure of a cell's size. The light scattered to the side gives a measure of a cell's surface roughness and internal complexity. A combination of these two parameters can be used to identify all the leukocytes in a blood sample.

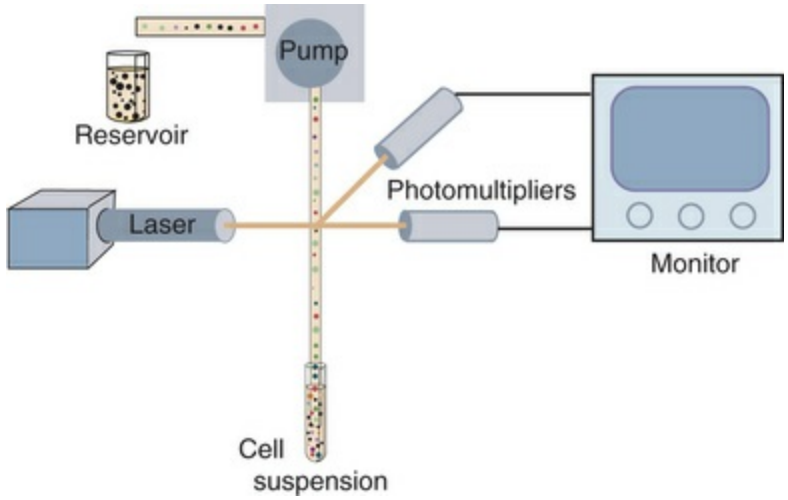


FIG. 42.15 A simplified view of the mechanism of action of the flow cytometer.

The flow cytometer can, however, be used to measure much more than this. If a cell suspension is mixed with a fluorescent monoclonal antibody, the labeled antibody will bind to cells carrying the appropriate antigen on its surface. This subpopulation can be characterized and counted (Figs. 42.16 and 42.17). By using antibodies labeled with different colors of fluorescent dyes, the expression of multiple cell surface antigens can be analyzed

simultaneously. It is possible to use the flow cytometer to follow sequential changes in the phenotype of mixed-cell populations (Fig. 42.18).

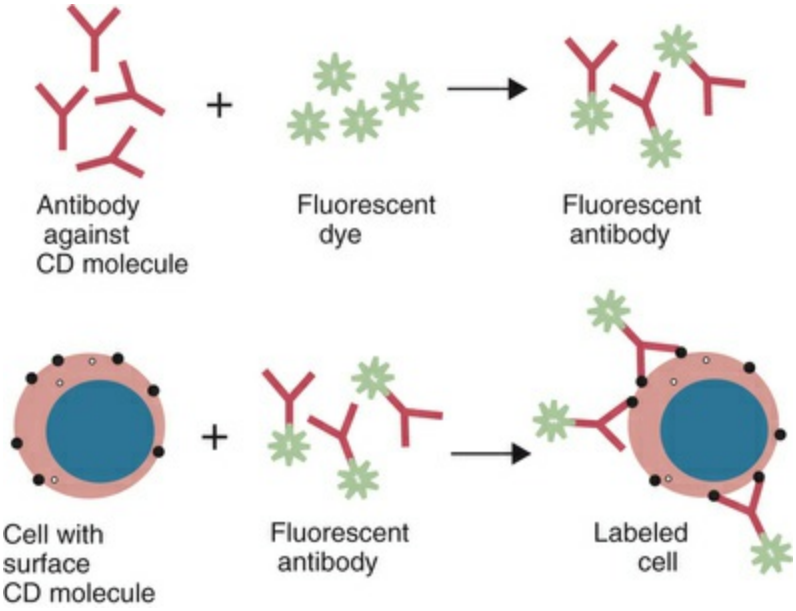


FIG. 42.16 How an immunoglobulin bound to a fluorescent dye can be used to identify cell surface CD molecules in a flow cytometer.

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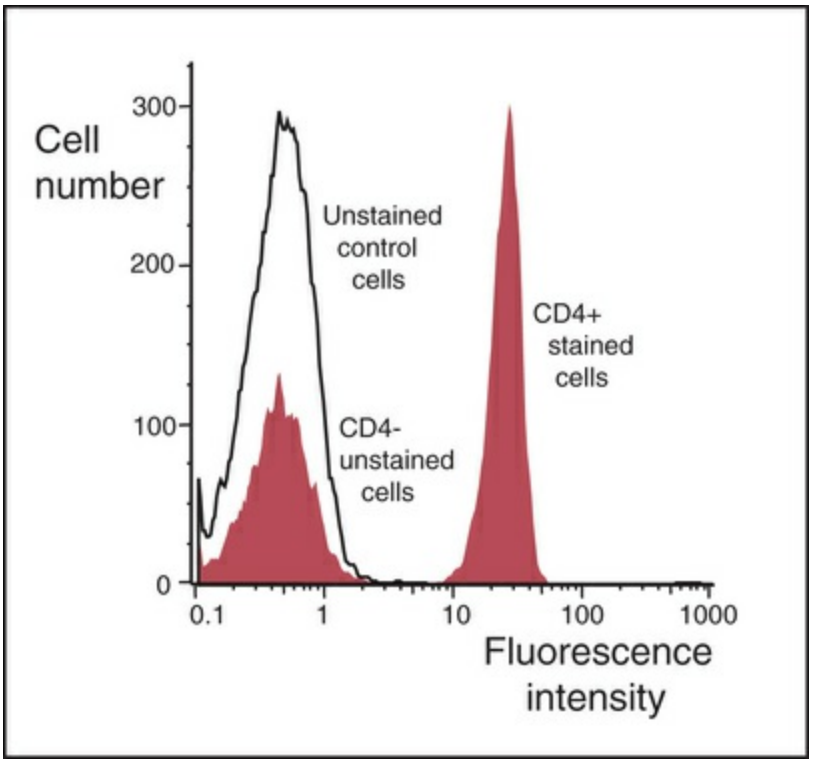


FIG. 42.17 A typical flow cytometer readout from labeling a cell population with antiequine CD4. The intensity of fluorescent labeling increases from left to right. Thus unlabeled control cells form the unshaded left peak. When a mixture of $CD4^+$ and $CD4^-$ cells is examined it forms two distinct peaks *(shaded area).* The left peak consists of unlabeled ($CD4^-$) cells. The right peak consists of labeled ($CD4^+$) cells. The area under each peak is a measure of the size of each cell subpopulation. (Courtesy Dr. R.R. Smith III.)

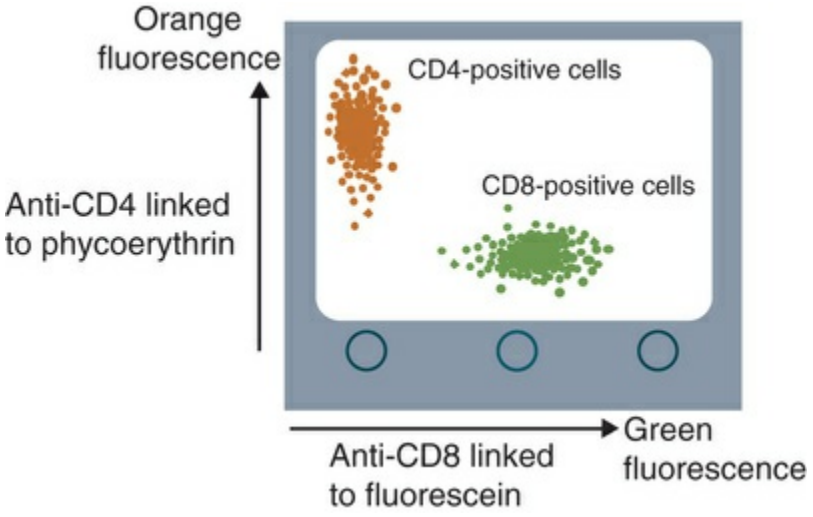


FIG. 42.18 The pattern seen on a flow cytometer screen when analyzing lymphocyte populations stained with two different fluorescence-conjugated antibodies. It is usual to label one

population with a green dye and the second population with a red or orange dye.

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Secondary Binding Tests

The reactions between antigens and antibodies are commonly followed by a secondary reaction. If antibodies combine with soluble antigens in solution, the resulting immune complexes may precipitate. Antibodies binding to particulate antigens (e.g., bacteria or red blood cells) may make them clump or agglutinate. If an antibody can activate the classical complement pathway and the antigen is on a cell surface, cell lysis may result. These reactions can be employed in many different serological assays.

Precipitation Tests

If a solution of soluble antigen is mixed with a strong antiserum, the mixture becomes cloudy within a few minutes, and then flocculent; finally, a precipitate settles to the bottom of the tube within an hour. The precipitate consists of antigen-antibody complexes. If increasing amounts of soluble antigen are mixed with a constant amount of antibody, the amount of precipitate that develops is determined by the relative proportions of the reactants. No obvious precipitate is formed at low antigen concentrations. As the amount of antigen increases, larger quantities of precipitate form until the amount is maximal. However, with the addition of yet more antigen, the amount of precipitate gradually diminishes, until none is present in tubes containing a large excess of antigen (Fig. 42.19). Equine IgG3 antibodies behave in a somewhat different fashion, producing a distinct flocculation over a very narrow range of antigen concentrations.

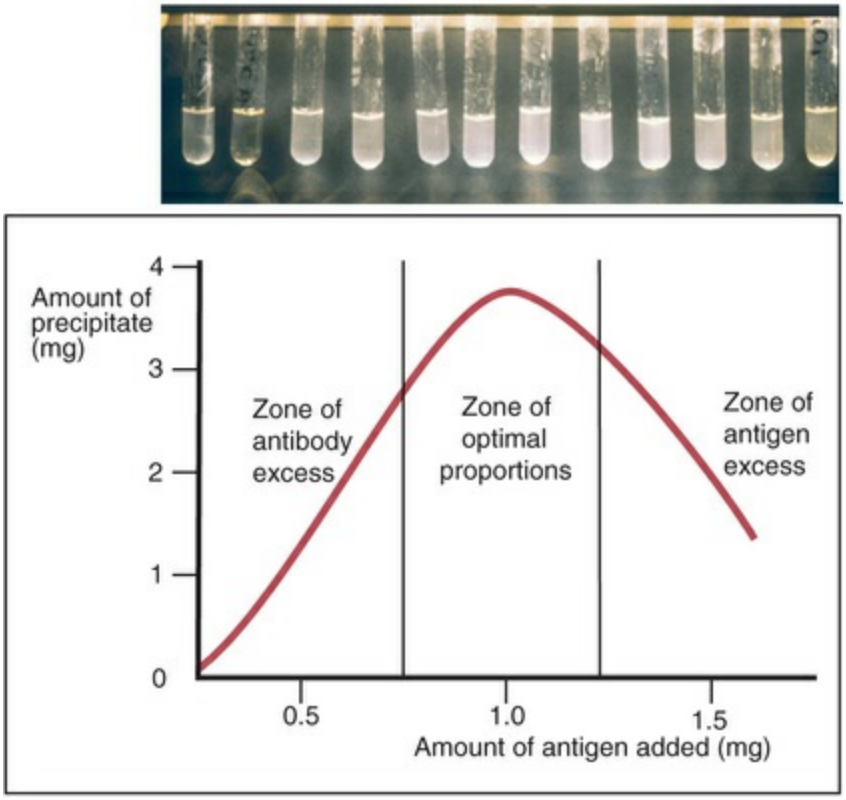


FIG. 42.19 The effect of mixing increasing amounts of antigen (bovine serum) with a constant amount of antibody (rabbit antiserum). The tube with the greatest amount of precipitate is the one in which the ratio of antigen to antibody is optimal. A quantitative precipitation curve of this test shows this effect graphically.

In the first stage of these reactions, only a little antigen is complexed to antibody, so little precipitate is deposited. In the tubes where most precipitation occurs, both antigen and antibody are completely complexed, and neither can be detected in the supernatant fluid. This is called the equivalence zone, and the ratio of antibody to antigen is optimal. When antigen is added to excess, a precipitate does not form, although soluble immune complexes are present, and free antigen may be detected in the supernatant fluid.

This pattern results from the fact that antibodies are bivalent and therefore can cross-link only two epitopes at a time, but complex antigens are generally multivalent, possessing many epitopes (Fig. 42.20). Where there is excess antibody, each antigen molecule is covered with antibody molecules, preventing cross-linkage and thus precipitation. When the reactants are in optimal proportions, the ratio of antigen to antibody is such that cross-linking and lattice

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formation are extensive. As this lattice grows, it becomes insoluble and eventually precipitates. In mixtures in which antigen is in excess, each antibody molecule binds two antigen molecules. Further cross-linkage is impossible, and since these complexes are small and soluble, no precipitation occurs. Mononuclear phagocytes are most efficient at binding and removing complexes formed at optimal proportions and in antibody excess. Small immune complexes formed in antigen excess are poorly removed by phagocytic cells but are deposited in vessel walls and in glomeruli, where they cause type III hypersensitivity (Chapter 32).

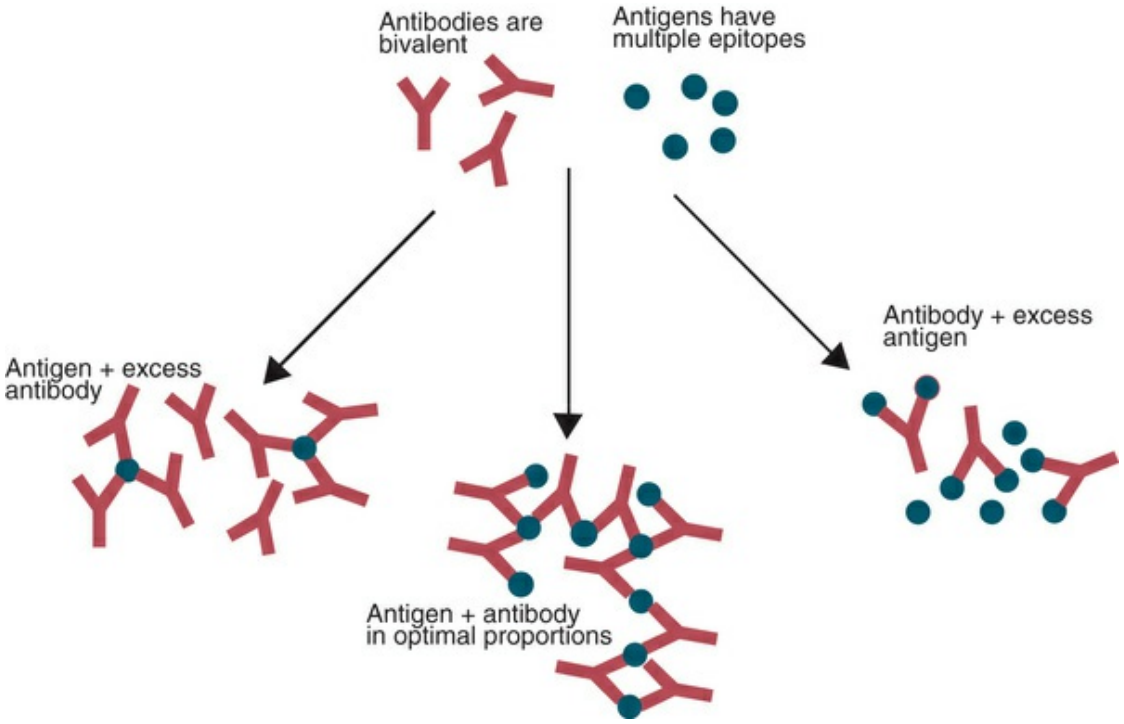


FIG. 42.20 The mechanism of immunoprecipitation. In both antigen and antibody, excess, small soluble immune complexes are produced. However, at optimal proportions, large insoluble complexes are generated.

Immunodiffusion

One simple method of demonstrating precipitation is immunodiffusion or gel diffusion. Round wells, about 5 mm in diameter and about 1 cm apart, are cut in a layer of clear agar. One well is then filled with soluble antigen and the other with antiserum; the reactants diffuse out radially. Where the reactants

meet in optimal proportions, an opaque white line of precipitated immune complexes forms (Fig. 42.21).

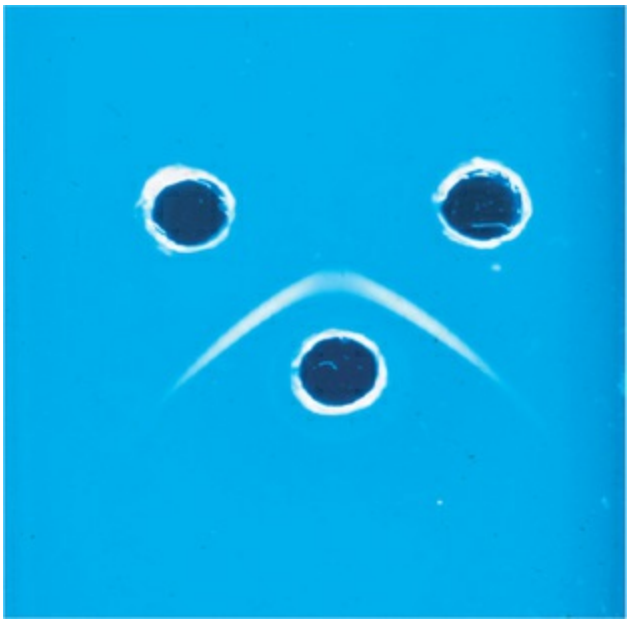


FIG. 42.21 Precipitation in agar gel. Antigen and antibody diffusing from their respective wells precipitate immune complexes in a region where optimal proportions are achieved. In this example, the antigen is identical in both top wells. As a result, the precipitation lines fuse to show complete identity.

If the solutions used contain multiple antigens and antibodies, the components are unlikely to reach optimal proportions in exactly the same position. Consequently, a separate line of precipitate is produced for each interacting set of antigens and antibodies. This test can be used to determine the relationship between antigens. If two antigen wells and one antibody well are set up, as in Figs. 42.22 and 42.23, lines will form between each antigen well and the antibody well. If these two lines join, the two antigens are probably identical. If the lines cross, the two antigens are completely different. If the lines merge with spur formation, a partial identity exists, indicating that one antigen possesses epitopes not present in the other. The Coggins test is a gel diffusion method used to detect antibodies against equine infectious anemia (EIA) virus in horse serum. In this test, an extract of infected horse spleen or a cell culture antigen reacts with the serum of the horse under test in agar gel, and the development of a line of precipitate constitutes a

positive reaction. A similar test is used to identify cattle infected with bovine leukemia virus. Immunodiffusion assays are, however, very insensitive and as a result are subject to numerous false-negative results. ELISA tests are significantly more sensitive. Thus the combination of ELISA plus immunodiffusion resulted in the detection of 17% more positive cases of EIA. These false-negative results would ensure that EIA cannot be eradicated if testing were based on the Coggins test alone.

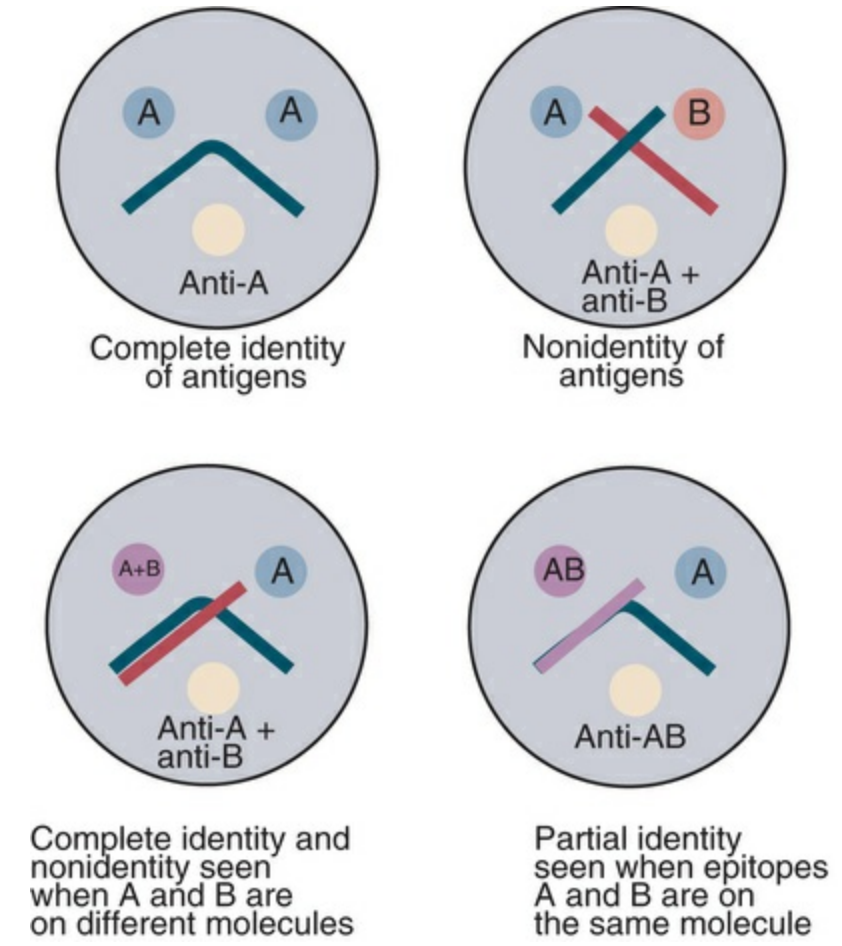


FIG. 42.22 The gel diffusion technique may be used to determine the relationship of two antigens.

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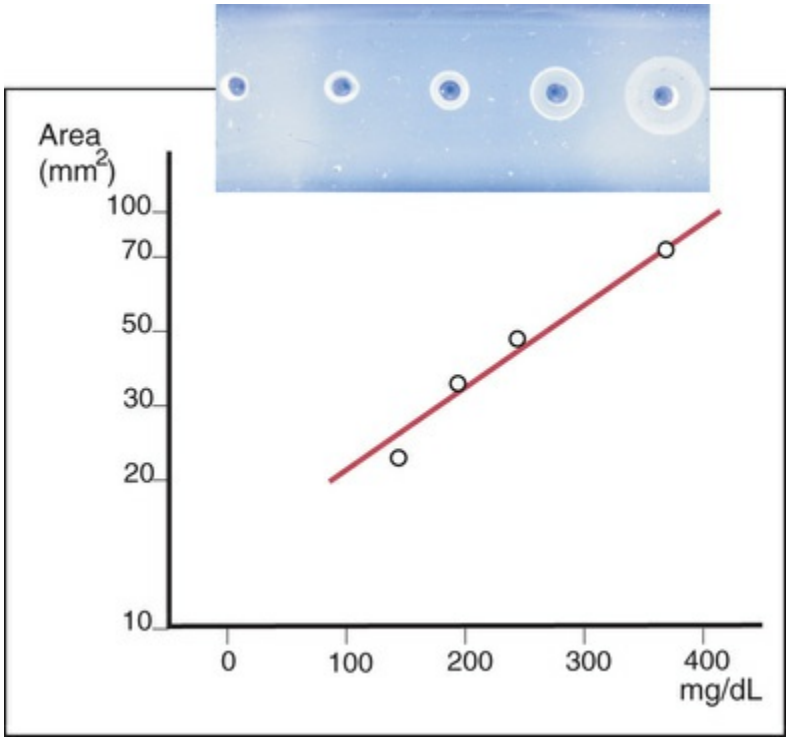


FIG. 42.23 A radial immunodiffusion assay. The area of precipitation is proportional to the concentration of antigen. In this case, antiserum to bovine IgA is incorporated in the agar and is used to measure bovine serum IgA levels.

Radial Immunodiffusion

If an antigen solution diffuses into agar in which specific antiserum has been incorporated, a ring of precipitate will form around the antigen well. The area of this ring is proportional to the amount of antigen in the well. A standard curve may therefore be constructed using known amounts of antigen (Fig. 42.23). Unknown solutions of antigen can then be accurately assayed by comparing the ring diameters from unknowns with the standard curve. This test is used to measure serum immunoglobulin levels in newborn foals (Chapter 23).

Immunoelectrophoresis and Related Techniques

Although conventional gel-diffusion techniques give a separate precipitation line for each antigen-antibody system in a mixture, it is often difficult to resolve all the components in a complex mixture. One way to improve the resolution of the system is to first separate the antigen mixture by electrophoresis before undertaking

immunodiffusion. This technique is called immunoelectrophoresis and is used to identify proteins in body fluids (Fig. 42.24).

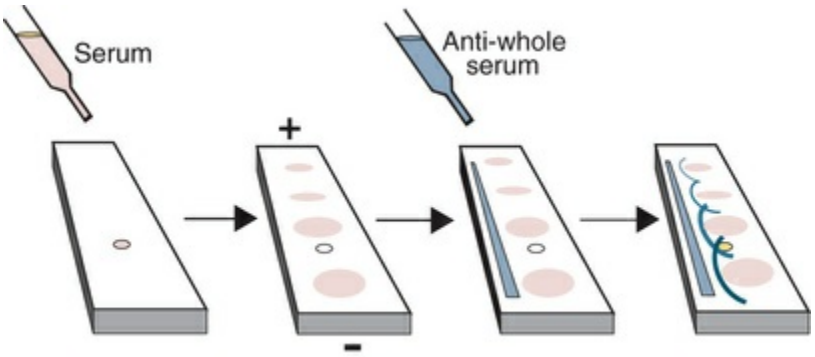


FIG. 42.24 The technique of immunoelectrophoresis (see text for details).

Immunoelectrophoresis involves the electrophoresis of the antigen mixture in agar gel in one direction. A trough is then cut in the agar parallel to this line of separated proteins. Antiserum against the whole serum is placed in this trough and allowed to diffuse laterally. When the diffusing antibodies encounter antigen, curved lines of precipitate are formed. One arc of precipitation forms for each of the constituents in the antigen mixture. This technique can resolve the proteins of normal serum into 25 to 40 distinct precipitation lines (Fig. 42.25). This technique has been used to identify the absence of a normal serum protein, as in animals with a congenital deficiency of some complement components. It is also used to detect the presence of excessive amounts of an individual component, as in animals with myeloma (see Fig. 15.23).



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FIG. 42.25 Immunoelectrophoresis of pig serum showing the lines of precipitation produced by some of the major serum proteins. (See also Fig. 15.23.)

If, instead of being permitted to passively diffuse into agar-containing antiserum as in the radial immunodiffusion technique, the antigen is driven into the antiserum agar by electrophoresis, the ring of precipitation around each well becomes deformed into a rocket shape. The length of the rocket is proportional to the amount of antigen placed in each well. This technique is called rocket electrophoresis.

Titration of Antibodies

Although the simple detection of antibodies or antigen is sufficient for many purposes, it is usually desirable to quantitate the reaction. One way of measuring specific antibody levels is by titration. The serum under test is diluted in a series of decreasing concentrations (Fig. 42.26). Each dilution is then tested for activity. The reciprocal of the highest dilution giving a positive reaction, called the titer, provides an estimate of the amount of antibody in that serum.

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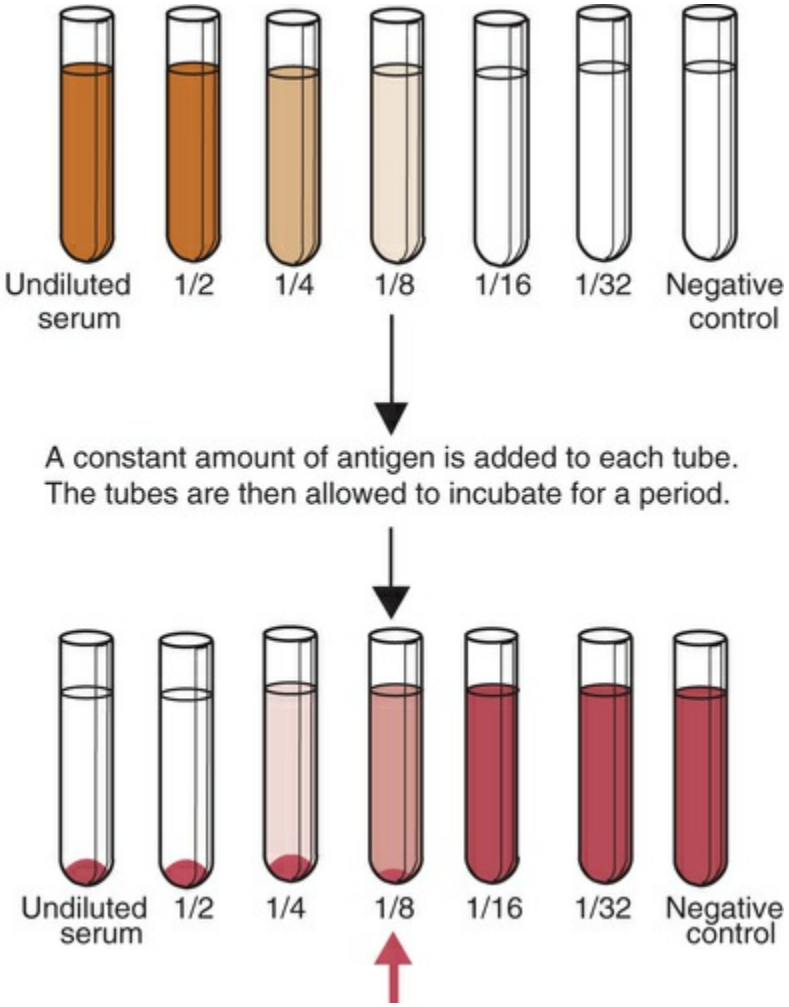


FIG. 42.26 The principle of antibody titration. Serum is first diluted in a series of tubes. A constant amount of antigen is then added to each tube, and the tubes are incubated. At the end of the incubation period, the last tube in which a reaction has occurred is identified. In this example, agglutination has occurred in all tubes up to a serum dilution of 1 : 8. The agglutination titer of the serum is said to be 8.

Agglutination

Because antibodies are bivalent, they can cross-link particulate antigens such as bacteria or foreign red cells, resulting in their clumping or agglutination. Antibodies differ in their ability to cause agglutination; for example, IgM antibodies are more efficient than IgG antibodies (Table 42.2). If excess antibody is added to a suspension of antigenic particles, then, just as in the precipitation reaction, each particle may be so coated by antibody that agglutination is inhibited. This lack of reactivity at high concentrations of antibody is termed a prozone. Another cause of

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prozone formation is the presence of antibodies that cannot cause agglutination. These nonagglutinating antibodies are also called "incomplete" antibodies. The reason for their lack of agglutinating activity is not completely understood; one possibility is that the epitopes with which they react lie deep within the surface coat of the particle, so deep that cross-linking cannot occur. An alternative suggestion is that they are capable of only restricted movement in their hinge region, causing them to be functionally monovalent (Chapter 16).

TABLE 42.2

Role of Specific Immunoglobulin Classes in Serological Assays

Property	IgG	IgM	IgA	Equine IgG3
Agglutination	+	+++	+	–
Complement activation	+	+++	–	–
Precipitation	+++	+	±	±
Time of appearance (days)	3-7	2-5	3-7	3-7
Time to peak titer (days)	7-21	5-14	7-21	7-21

Matrix gel agglutination is used for blood typing in dogs and cats. It relies on performing the agglutination test on top of a viscous gel layer containing specific antibody. Negative red cells will sink through the gel to the bottom of the tube, whereas agglutinated cells will clump and remain as a layer on top.

Antiglobulin Tests

If it is necessary to test for the presence of nonagglutinating antibodies on the surface of particles such as bacteria or erythrocytes, a direct antiglobulin test may be used. The washed particles may be mixed with an antiglobulin, and if antibodies are present on their surface, agglutination will occur (Fig. 42.27). This is sometimes called the Coombs test after its inventor.

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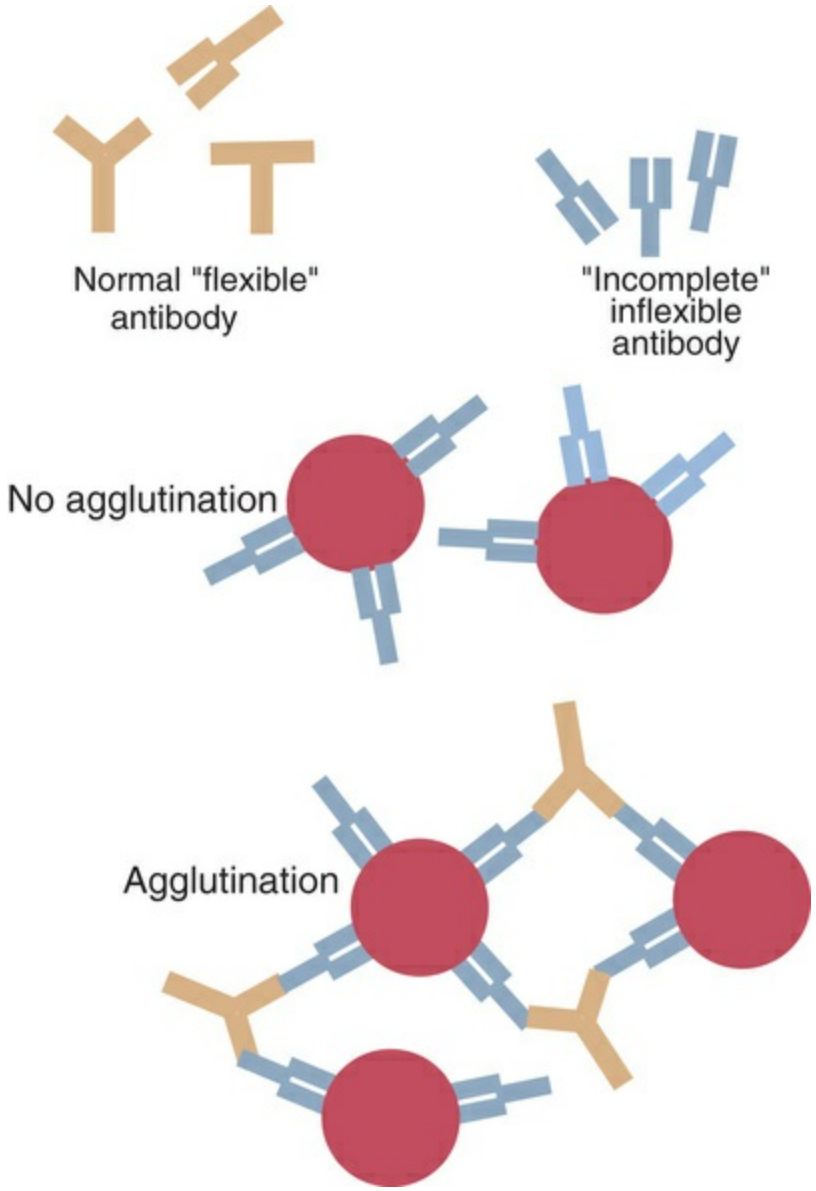


FIG. 42.27 The direct antiglobulin test. The presence of the antiglobulin is required to agglutinate particles coated with nonagglutinating antibody.

Passive Agglutination

Since agglutination is a much more sensitive technique than precipitation, it is sometimes useful to convert a precipitating system to an agglutinating one (Fig. 42.28). This may be done by chemically linking soluble antigen to inert particles such as erythrocytes, bacteria, or latex beads. Erythrocytes are among the best particles for this purpose, and tests that employ coated erythrocytes are called passive hemagglutination tests.

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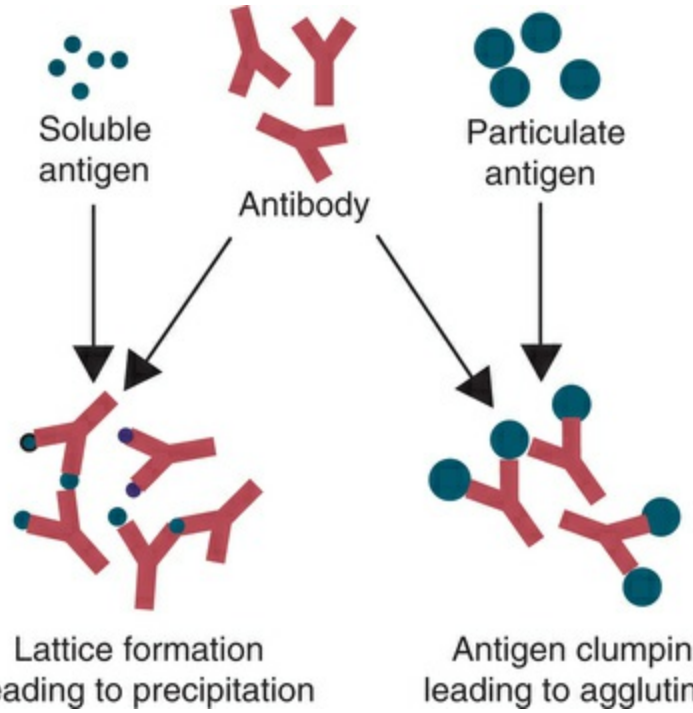


FIG. 42.28 The relationship between precipitation and agglutination. This is essentially a consequence of the size of the antigenic particle. Large particles agglutinate. Small particles and soluble molecules precipitate.

Viral Hemagglutination and Its Inhibition

Some viruses can bind and agglutinate red blood cells. This virus-induced hemagglutination may assist in characterizing an unknown virus. Inhibition of viral hemagglutination by antibody can then be used either as a method of identifying a specific virus or to measure antibody levels in serum. Hemagglutinating organisms include orthomyxoviruses and paramyxoviruses, alphaviruses, flaviviruses, and bunyaviruses, as well as some adenoviruses, reoviruses, parvoviruses, and coronaviruses. They also include some mycoplasma such as *Mycoplasma gallisepticum*.

Complement Fixation

The activation of the classical complement pathway by antibody bound to antigen results in the generation of terminal complement complexes that can disrupt cell membranes. If the antibody binds red cells, these will be ruptured, and hemolysis occurs. This phenomenon can be used to measure serum antibody levels in a

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test called the complement fixation test.

Complement is a normal constituent of all fresh serum, but the complement in fresh, unheated guinea pig serum is the most efficient in hemolytic tests. Serum used as a source of complement for serological applications should be stored frozen in small volumes. Once thawed, it should be used promptly. It should not be repeatedly frozen and thawed.

The complement fixation test is performed in two parts. First, antigen and antibodies (the serum under test deprived of its complement by heating at 56° C) are mixed and incubated in the presence of normal guinea pig serum as a source of complement. After the antigen-antibody-complement mixture reacts, the amount of free complement remaining in the mixture is then measured by adding an indicator system consisting of antibody-coated sheep red cells. Lysis of these cells (seen as the development of a transparent red solution) is a negative result because it indicates that antibody was absent from the serum under test and that complement was not consumed (or fixed) (Fig. 42.29). Absence of lysis (seen as a cloudy red cell suspension), indicating that complement was consumed, is a positive result. It is usual to titrate the serum being tested so that, if antibodies are present in that serum, as it is diluted the reaction in each tube will change from no lysis (positive) to lysis (negative). The titer is the highest dilution of serum in which no more than 50% of the red cells are lysed.

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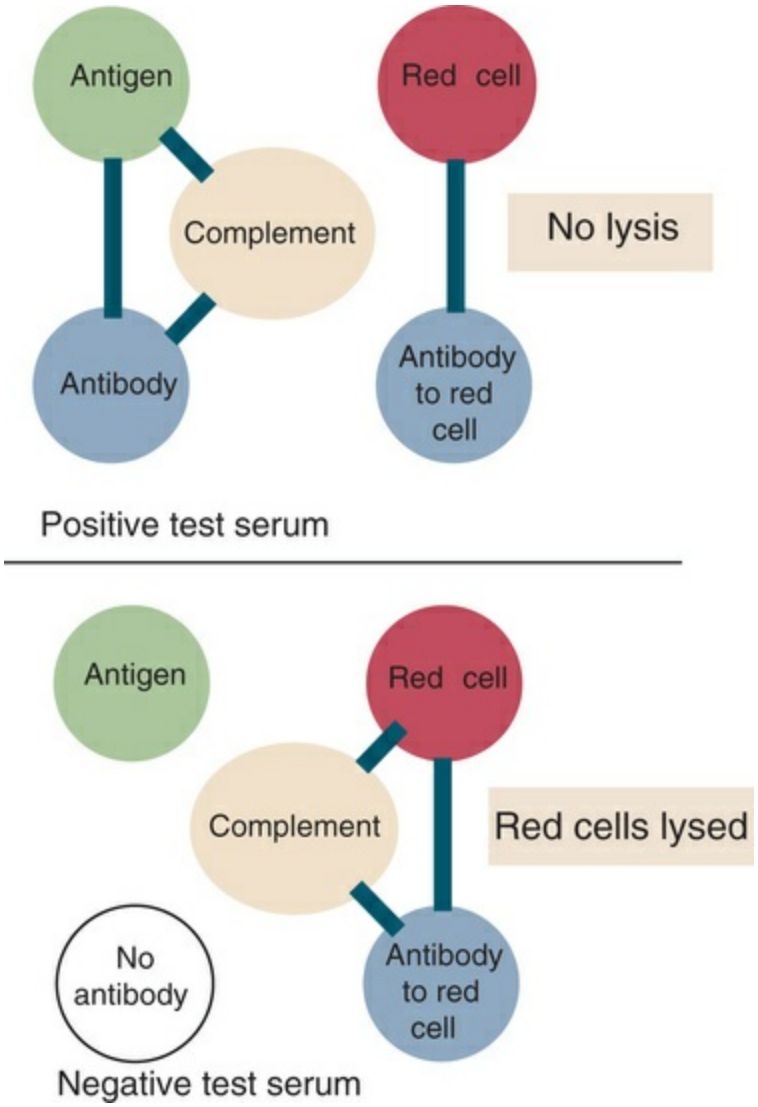


FIG. 42.29 The principle of the complement fixation test. Complement, if fixed by antigen and antibody, is unavailable to lyse the target cells in the indicator system. In the absence of antibody, the complement remains unfixed and is available to lyse the indicator system. (Modified from Roitt I: *Essential immunology,* Oxford, 1971, Blackwell Science.)

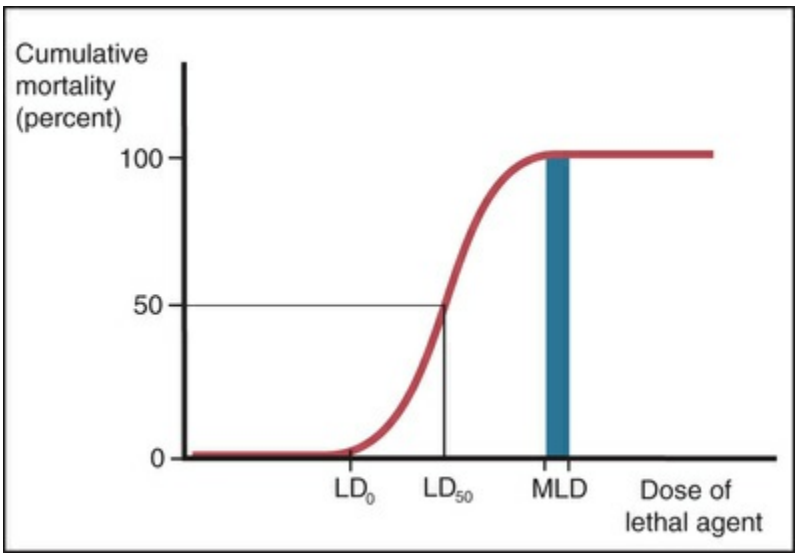
Cytotoxicity Tests

Complement may cause membrane damage, not only to erythrocytes but also to nucleated cells and to protozoa. Antibodies against cell surface antigens thus may be measured by reacting target cells with antibody and complement and estimating the resulting cell death. This form of assay has been employed to type cells by determining which major histocompatibility complex (MHC) class I molecules they are expressing.

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Assays in Living Systems

If an organism or antigen possesses biological activity, antibodies can be measured by their ability to neutralize this activity. The activities that may be neutralized include hemolysis of erythrocytes, lysis of nucleated cells, and disease or death in animals. Reactions such as these are subject to a high degree of variability because they tend to change gradually over a wide range of doses of organism or antigen. For this reason, results obtained from a single positive or negative neutralization test are usually of little use. For example, 0.003 mg of tetanus toxin may kill some mice in a test group, but about five times that dose is required to kill all mice in the same group. In addition, if an attempt is made to assess the lowest dose of tetanus toxin that will kill all the animals in a group (the minimal lethal dose), it is found to be highly variable. It is equally difficult to estimate with precision the highest dose of toxin that will just fail to kill all test animals. The most exact method of measuring the lethal effects of a toxin has been to estimate the dose that will just kill 50% of a group of test animals (Fig. 42.30). In practice, it is usually not possible to arrive at this 50% end point by direct experimentation. For this reason it is usually necessary to calculate it by plotting the results against the dose of toxin given and arriving at the 50% end point by calculation.



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FIG. 42.30 A cumulative mortality curve showing how the LD_{50} provides a more accurate estimate of the lethal effects of a toxin than either the LD_0 or the MLD.

In the example cited in the previous paragraph, the lethality of the toxin can be estimated by measuring the dose required to kill 50% of a group of experimental animals. This lethal dose is called the $LD_{50.}$ Similarly, the dose of complement that just hemolyses 50% of a red cell suspension is called the CH_{50}. The dose of organisms that infects 50% of animals is the ID_{50}, the dose that just infects 50% of tissue cultures is the $TCID_{50}$, and the dose of antiserum or vaccine that protects 50% of challenged animals is the PD_{50}.

Neutralization Tests

Neutralization tests estimate the ability of antibody to neutralize the biological activity of antigen when mixed with it in vitro. These tests may be used to identify bacterial toxins such as *Clostridium perfringens* α-toxin or staphylococcal α-toxin.

Viruses may be prevented from infecting cells after specific antibody has combined with and blocked their critical attachment sites. This reaction is the basis of the neutralization tests that are employed either for the identification of unknown viruses or for the measurement of specific antiviral antibody. Virus neutralization tests are highly specific and extremely sensitive. Thus antiserum to coliphage T4 will neutralize phage-induced lysis of *Escherichia coli* because antibodies can block the receptor on the phage tail, thus preventing its attachment to a bacterium. A single antibody molecule is sufficient to cause this, and a phage neutralization test may therefore detect as little as 0.00005 mg of antibody.

Protection Tests

A protection test is a form of neutralization test carried out entirely *in vivo.* The protective properties of a specific antiserum are measured by administering it in increasing dilutions to a group of test animals, which may then be challenged with a standard dose of pathogenic organisms or toxin. Although protection tests provide a

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direct measure of the therapeutic efficacy of an antiserum, they are also subject to great experimental variation because of differences among animals. Animals differ in their susceptibility to infection and in a number of other factors, such as the rate of absorption of antiserum, the level of activity of the mononuclear phagocyte system, and the half-life of the passively administered immunoglobulin. As in neutralization tests, meaningful results can be obtained only if large numbers of animals are employed and if the challenge dose is carefully standardized. It is usual to use a dose of organisms or toxin containing a known number of LD_{50} or ID_{50}. Similarly, the protective effect of an antiserum may be expressed in PD_{50}, the dose required to protect 50% of a group of animals.

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Molecular Methods

Although immunological assays have historically provided the most sensitive assays for diagnostic purposes, modern molecular techniques have proved to be even more sensitive and specific. The detection of the nucleic acid of an infectious agent through polymerase chain reactions (PCRs) is often superior to immunological methods (Fig. 42.31). This method is based on the ability of very small quantities of nucleic acid to be amplified in a highly specific manner so that it can be readily detected. Thus, for example, small amounts of viral DNA may be present in a tissue sample. If the sample is heated, the paired DNA strands will separate into two single strands. If the nucleotide sequence of this DNA is known, specific primers (oligonucleotides of single-stranded DNA) can be added to the sample, where they bind the viral DNA and act as a template for new DNA synthesis. These primers are selected so that they are complementary to the 3′ ends of the sequence to be amplified. Thus these primers will bind to the ends of the sample DNA, a process called annealing. By adding an enzyme called DNA polymerase, new complementary strands of DNA are then assembled on the primers. The cycle can then be repeated: heating → primer annealing → new DNA assembly. Each cycle doubles the amount of specific DNA present, so, in theory, 30 such cycles should result in the production of 2^{30} copies of the original DNA sample. Once cycling is completed, the products can be examined by gel electrophoresis and the characteristic DNA bands identified. If necessary, the bands may be sequenced to provide assurance that the correct DNA has been amplified.

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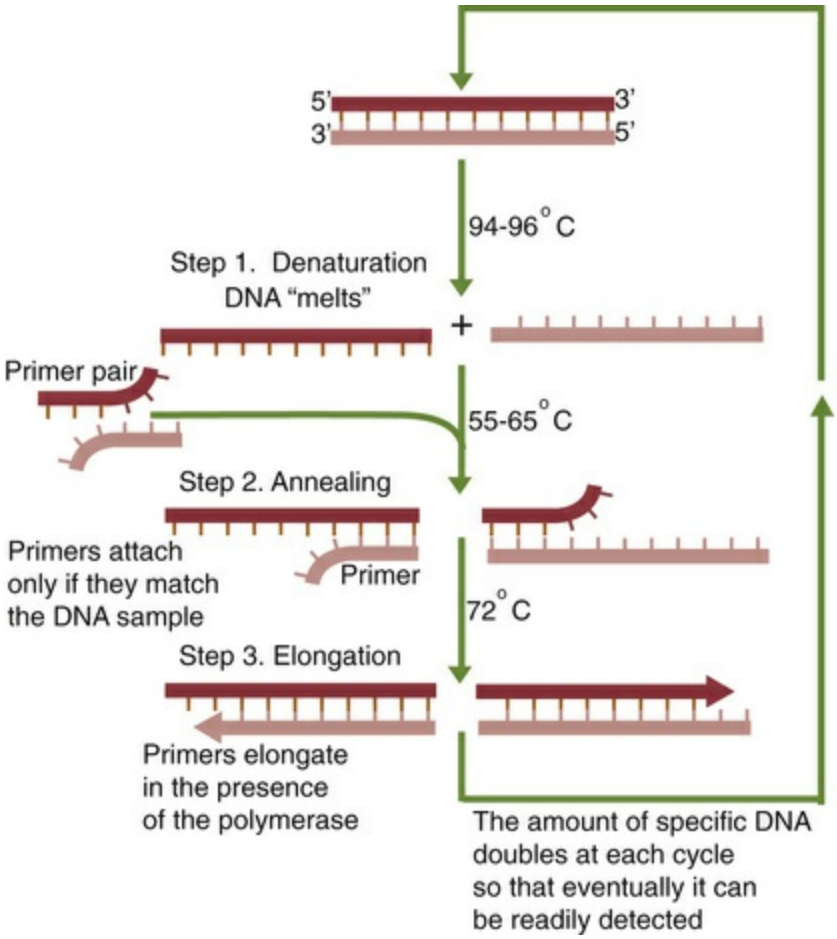


FIG. 42.31 The principle of the polymerase chain reaction (PCR) test. Essentially, by performing a cycle of reactions repeatedly, it is possible to produce large amounts of DNA coding for the gene of interest. Once produced in sufficient amounts, this DNA can be detected by electrophoresis.

As might be anticipated, many different variations of the basic PCR process have been developed. For example, if the nucleic acid of interest is RNA rather than DNA (e.g., detecting an RNA virus), a reverse-transcriptase PCR may be performed. This simply involves the initial conversion of the viral RNA to DNA using a reverse transcriptase, before beginning the PCR cycle.

Real-time PCR in effect simply measures the amplification of the DNA of interest as it is amplified (in real time). The current methods measure fluorescence-resonance energy transfer to quantitate the amplification of a specific DNA of interest. As the cycles proceed, the amount of fluorescence gradually increases and can be plotted as a curve. With this test, it is possible to measure the initial copy number, and it is less susceptible to contamination errors.

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PCR assays are not only useful in detecting the presence of trace amounts of viral nucleic acid in tissues. They can also be used to amplify specific genes from within an animal's own DNA. For example, if the primers are correctly selected, they can be used to amplify normal or abnormal genes. Thus PCR can identify SCID foals or cattle with leukocyte adherence deficiency (Chapter 39).

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Diagnostic Applications of Immunological Tests

Obviously the presence of antibodies to a specific organism in an animal's serum indicates previous exposure to an epitope present on that organism. It does not, however, prove that infection exists or that any concurrent disease is actually caused by the organism in question. For example, the fact that the sera of most healthy horses contain antibodies to *Salmonella typhimurium* does not prove that most horses are suffering from salmonellosis. The presence of antibodies to an organism in a single serum sample is rarely of diagnostic significance. Only if at least two samples are taken 1 to 3 weeks apart and show at least a four-fold rise in titer can a diagnosis be made. This should be done only in conjunction with careful clinical assessment.

A second feature that must be considered in the interpretation of serological tests is the possibility of errors. Technical errors are usually prevented by incorporation of appropriate controls into the test system. Other errors, however, are largely unavoidable. For example, if test results are obtained from a known diseased population and from a known disease-free population, it will be rare to find that the results obtained separate perfectly. Much more commonly, the test results overlap, and the test cannot distinguish normal from diseased with 100% accuracy (Fig. 42.32). As a result, irrespective of the selected cut-off point, there will be some correct results and some incorrect ones. There will be four types of result: true-positive and true-negative results and false-positive and false-negative results. A test in which a large proportion of the positive results are true is considered to be specific, whereas one that correctly identifies the true-negative responses is considered sensitive. A perfect test would be highly sensitive and highly specific. In ideal tests, it would be desirable for the criteria used in interpreting the test results to be so obvious and absolute that each test would be absolutely sensitive and specific. Unfortunately, such ideal tests are uncommon. In general, the level of test errors can be adjusted by the point used to differentiate positive from negative

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reactions. If this cut-off point is adjusted downward, so that the criterion for a positive test is made less strict, the number of false-positive results will increase, but there will also be a decrease in the number of false-negative results. In practice, therefore, highly sensitive tests tend to be relatively nonspecific, and highly specific tests are generally insensitive. The establishment of the cut-off point in reading test results and, from this, the sensitivity and specificity of a test are determined both by the requirements of the test procedure and by the significance of false-positive and false-negative reactions.

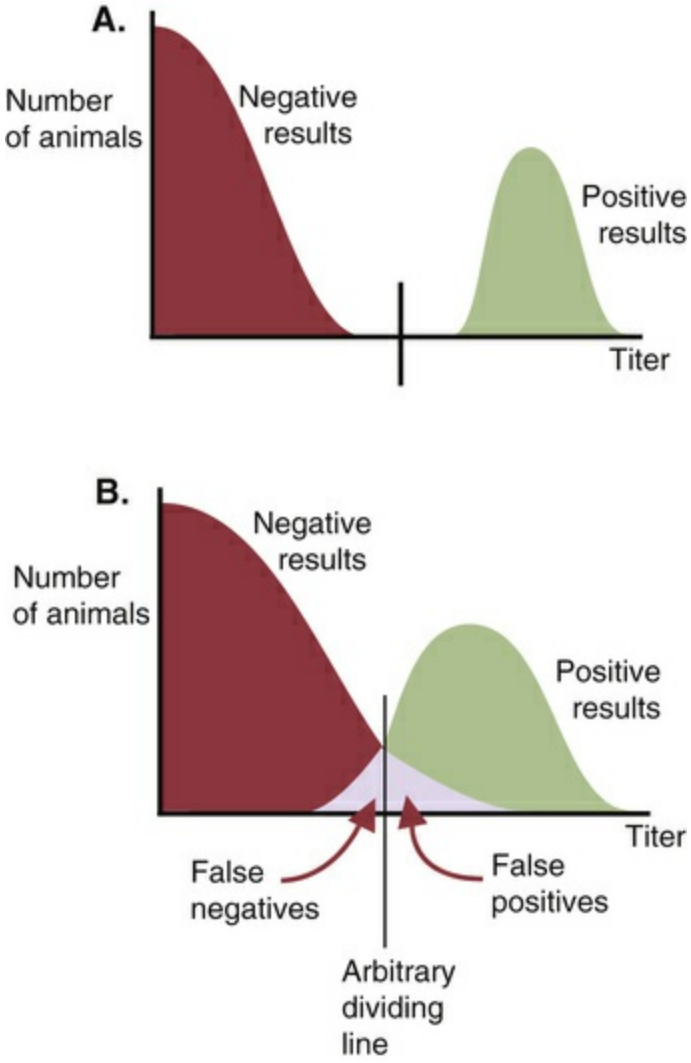


FIG. 42.32 Schematic diagrams depicting the errors associated with immunological tests. The top diagram **(A)** depicts an ideal test in which there is no ambiguity in interpreting test results. The bottom diagram **(B)** depicts a more typical test in which an arbitrary line must be used to separate positive from negative results. By moving

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this dividing line, the relative proportions of false-positive and false-negative results may be changed.

The sensitivity and specificity of any test can be calculated using the number of true-positive (a), false-positive (c), true-negative (d), and false-negative (b) results. The sensitivity of a test is the probability that a test result will be positive when the disease is present (true-positive rate) will be a/(a + b). The specificity of a test is the probability that a test will be negative when the disease is absent (the negative rate) will be d/(c + d). Because of the reciprocal nature of sensitivity and specificity (one goes up as the other goes down), it is possible to plot this graphically using a receiver operating characteristic (ROC) curve (Fig. 42.33). In this procedure, the test sensitivity is plotted as a function of 100 minus the specificity for multiple different cut-off points. Each point on the ROC curve thus represents sensitivity/specificity for a given cut-off point. A test with perfect discrimination will thus have an ROC plot that passes through the upper left corner (100% sensitivity and 100% specificity). In less than perfect tests, an investigator can determine the optimal cut-off point by selecting the point on the curve closest to the upper left corner. The area under the curve also provides a measure of how well the test separates the two populations being tested. An area of 1 represents a perfect test, whereas an area of 0.5 represents a test whose results do not differ from random, and hence is useless. ROC curve analysis is very useful in determining the best way to interpret a serological test, especially assays such as ELISAs, in which quantitative data is obtained, but their significance is not immediately apparent. Tests with a high sensitivity are needed if it is essential that no positive cases be missed, as in disease eradication programs. Tests with a high specificity are needed if the false positive results would have inappropriate consequences such as requiring unnecessary animal euthanasia.

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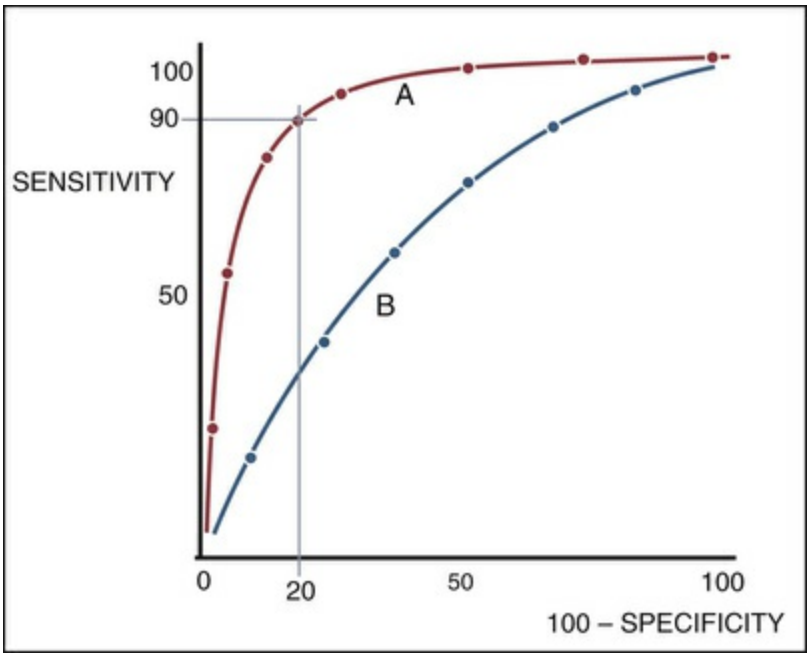


FIG. 42.33 An ROC curve plots a test sensitivity against 100-specificity for an entire range of cut-off points (e.g., different optical density values in an ELISA test). In a good test, specificity and sensitivity approach 100%, and the appropriate cut-off value may be very obvious. Thus in test *A*, the best cut-off point would give a sensitivity of 90% and a specificity of 80%. Unfortunately, this is not always the case *(B)*.

As has been evident from the discussions earlier in this chapter, the advantages and disadvantages of each immunodiagnostic test vary according to the requirements of the investigator, the nature of the antigen employed, and the complexity, sensitivity, and specificity of each method. In general, the selection of a diagnostic test represents a compromise among its sensitivity, its specificity, and its complexity. The latter includes the number of steps involved, the time involved, the degree of technical expertise required, its cost, and the nature of the equipment needed to conduct the test. Although precise guidelines cannot be drawn, it is usually most appropriate to use the most sensitive and specific test that can be satisfactorily performed with the available technical assistance and equipment at the lowest cost in the shortest possible time.

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43

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Evolution of the Immune System

CHAPTER OUTLINE

Immunity in Invertebrates, 491
Physical Barriers, 491
Innate Immunity, 491
Phagocytosis, 491
Prophenoloxidase (proPO) System, 491
Antimicrobial Peptides, 492
RNA Interference, 492
Adaptive Immunity, 492
Graft Rejection, 492
Immunity in Vertebrates, 493
Immunity in Cyclostomes, 493
Immunological "Big Bang," 493
Immunity in Jawed Fish, 494
Innate Immunity, 494
Adaptive Immunity, 494
Immunoglobulins, 495
Cell-Mediated Immunity, 496

Immunity in Amphibians, 496
Urodele Amphibians, 496
Anuran Amphibians, 497
Immunity in Reptiles, 498
Immunity in Birds, 499
Major Histocompatibility Complex Molecules, 500
Immunoglobulin Classes, 501
Immunoglobulin Y, 501
Immunoglobulin M, 501
Immunoglobulin A, 501
Generation of Antibody Diversity, *502*
Maternal Immunity, 502
Immunity in Monotremes and Marsupials, 502
Mammalian Phylogeny, 503
Fever, 503

LEARNING OBJECTIVES

After reading this chapter, you should be able to:

- Understand that invertebrates rely exclusively on the use of innate immune mechanisms to protect themselves against infectious agents.
- Explain how the jawless fish mainly rely on innate immunity, although they also possess a remarkably complex and diverse adaptive antigen-binding receptor system.
- Understand that cartilaginous fish were the first vertebrates to utilize an adaptive immune system.
- Define the immunological “big bang.”

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- Describe how jawed fish and all the more evolved vertebrates possess both antibody and cell-mediated immune systems.
- Understand that the major chicken immunoglobulin is called IgY.
- Describe the structure of the chicken MHC.
- Define the prophenoloxidase system, RNA interference, variable lymphocyte receptor, translocon pattern, and behavioral fever.
- Describe how maternal immunity is transferred to newly hatched birds.

All multicellular animals, regardless of their complexity or evolutionary history, must be able to defend themselves against invading microorganisms. Innate immune defenses evolved very early in animal evolution and are found in both invertebrates and vertebrates. The much more complex, lymphocyte-based, adaptive immune system, however, evolved only after the emergence of the vertebrates.

The subsystems of the innate immune system evolved in response to the threats imposed by many different invading microbes. The relative contribution of these innate subsystems in any individual species reflects the optimal mixture that evolved to ensure survival. Specific components of the innate immune system may therefore vary greatly even within the same class of organism. Natural killer (NK) cells, the use of type I interferons (IFNs), and certain specialized leukocytes such as eosinophils and basophils occur only in vertebrates. Likewise, subsystems that depend on an intact vasculature do not work in invertebrates that have an open circulatory system.

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Immunity in Invertebrates

Invertebrates are classified based on the presence of a body cavity or coelom (Fig. 43.1). The acoelomates include the sponges and coelenterates (jellyfish and sea anemones). The coelomates evolved into two major branches. One branch includes the annelids, mollusks, and arthropods, collectively called the protostomes. The other branch, including the echinoderms, protochordates, and chordates, is called the deuterostomes. It is from deuterostome-like ancestors that the vertebrates evolved.

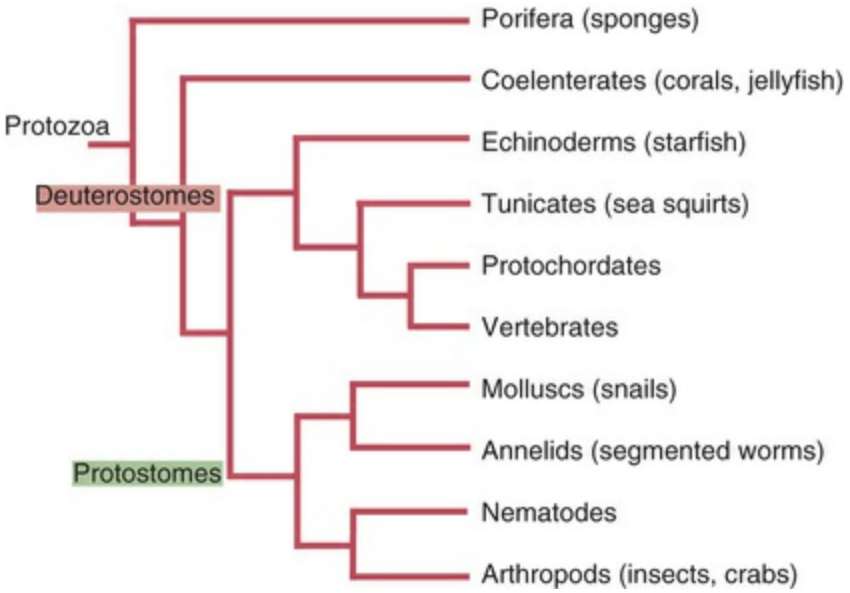


FIG. 43.1 A phylogenetic tree showing the major divisions of the invertebrates.

Physical Barriers

Physical barriers are most obvious in the arthropods. Tough chitinous exoskeletons protect arthropods against many attackers. The horseshoe crab *(Limulus polyphemus)* not only has a hard exoskeleton but also can protect itself against bacteria in polluted water by secreting a specialized glycoprotein through pores in the carapace. On contact with endotoxins, this glycoprotein coagulates, sealing the pores and immobilizing any invading bacteria.

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Likewise, if bacteria enter horseshoe crab hemolymph, clotting factors are activated by lipopolysaccharides and generate clots that trap the invaders. Other invertebrates such as the coelenterates, annelids, mollusks, and echinoderms secrete masses of sticky mucus containing antimicrobial peptides when attacked, thus immobilizing and killing invaders.

Innate Immunity

Invertebrates use three major innate defense subsystems: phagocytosis by blood or body cavity cells; protease cascades that lead to fluid clotting, melanin formation, and opsonization; and the production of many different antimicrobial peptides. Their initial defensive response is to use phagocytic cells. They can be highly efficient, killing most of the invading bacteria. Any persistent invaders will induce the production of antibacterial peptides.

Phagocytosis

In 1884, the Russian biologist Elie Mechnikov discovered phagocytosis when examining starfish larvae. He showed that mobile macrophage-like cells attacked rose thorns introduced into the coelom of these larvae. Since then, phagocytosis has been shown to be a universal defense mechanism within the animal kingdom. Several types of phagocytic cells are recognized in coelomate invertebrates. They occur in blood (hemocytes) and in the body cavity (coelomocytes). These cells undertake chemotaxis, adherence, ingestion, and digestion. They contain proteases and potent oxidants. Some phagocytes aggregate and plug wounds to prevent bleeding. Where phagocytic cells cannot control the invaders, they may be walled off in cellular nodules similar to vertebrate granulomas.

Invertebrates also produce cytokine-like molecules. One of these, an interleukin-1 (IL-1)–like molecule, may activate phagocytic cells. LPS stimulation of mollusk hemocytes may stimulate the release of TNF-like, IL-6–like, or IL-1–like proteins.

Prophenoloxidase (proPO) System

This enzyme system, found in arthropod hemolymph, generates a

cascade of proteases and other enzymes leading to the production of the enzyme phenoloxidase (Fig. 43.2). The system is triggered by the interaction of bacterial and fungal lipopolysaccharides, peptidoglycans, and glucans with hemocytes. This activates a serine protease cascade that eventually generates phenoloxidase. Phenoloxidase acts on tyrosine to generate a series of intermediates that eventually form the black molecule eumelanin. Melanin polymer is deposited in the tissues surrounding invaders to form an impermeable barrier that blocks their nutrient uptake. Oxidizing agents and other antimicrobial molecules are also generated during melanin synthesis.

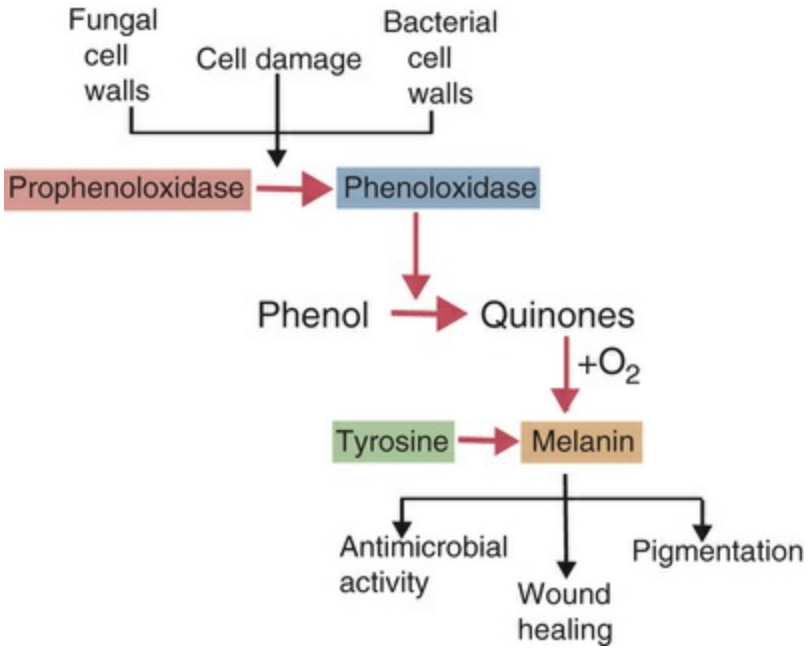


FIG. 43.2 The prophenoloxidase pathway is an enzyme cascade system found in many invertebrates, in which it serves a key defensive role.

Antimicrobial Peptides

When bacteria infect insects, their pathogen-associated molecular patterns (PAMPs) are recognized by toll-like receptors (TLRs) and other pattern-recognition receptors (PRRs). Because of their dependence on innate immunity, invertebrates have evolved many different PRRs. For example, in the sea urchin *(Strongylocentrotus purpuratus),* there are 222 *TLR* genes, about 300 *NOD*-like genes,

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and about 180 scavenger receptors. TLRs have been identified in even the least evolved invertebrates such as the sponges. In contrast to mammals, in which TLRs directly recognize pathogens, drosophila toll is activated by a protein ligand (called spätzle) that is generated after pathogen recognition.

Activation of these PRR pathways causes arthropod cells to produce antimicrobial peptides. These peptides are mainly produced in the fat body (the functional equivalent of the mammalian liver), although some may be produced locally on body surfaces. The peptides appear within 2 hours after bacterial invasion and reach peak levels at 24 hours. About 400 antimicrobial peptides, including defensins, have been identified in invertebrates. Invertebrates also generate C-type lectins and pentraxins. These invertebrate lectins act as opsonins and enhance activation of the prophenoloxidase system.

The complement system is ancient, with two complement-like proteins, C3 and factor B (FB), having been traced back to the coelenterates. Proteins homologous to mammalian MBL, ficolins, MASPs, C3, C2/FB, and a C3 receptor have been identified in all deuterostomes analyzed so far. Thus invertebrates have both alternative and lectin pathways.

RNA Interference

The intracellular RNA interference pathway (RNAi) is a gene-silencing system that prevents virus replication. It is especially important in invertebrates (Fig. 43.3). RNA normally occurs only in a single-stranded (ss) form. Long segments of double-stranded RNA (dsRNA) only occur in cells infected by RNA viruses. Any dsRNA formed is therefore recognized and rapidly degraded into many short fragments by host endonucleases called dicers. The small fragments, 21 to 23 nucleotides in length, are called small-interfering RNAs (siRNA). siRNAs are bound and stabilized by a protein complex called the RNA-induced silencing complex (RISC). Half of these siRNAs are complementary to the original viral messenger RNAs (mRNAs) and as a result will bind them tightly and "silence" them. Once these viral mRNAs have been bound by a RISC, they are rapidly degraded so that viral replication is effectively blocked.

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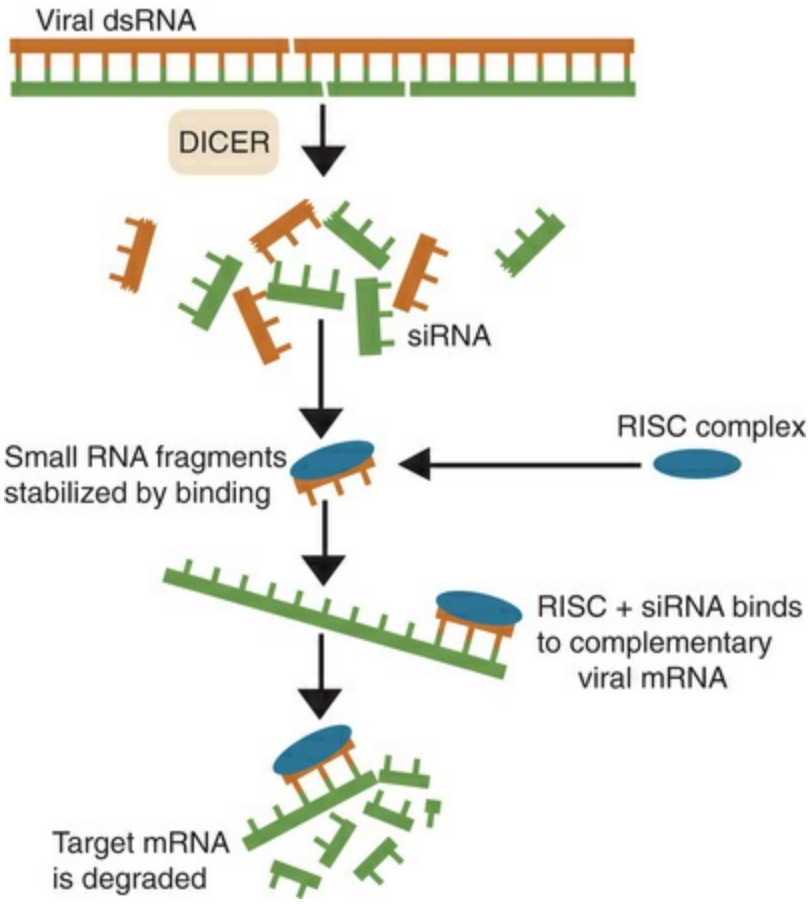


FIG. 43.3 The mechanism of RNA interference, an important defensive mechanism in invertebrates (and plants). Double-stranded RNA should not be present in the cytoplasm of normal healthy cells. Its presence indicates that an RNA virus is infecting a cell. This dsRNA is degraded by an enzyme called dicer into short fragments (short-interfering RNA). The short fragments are then stabilized by a set of proteins called the RISC complex. Half of these siRNA fragments will be complementary to viral messenger RNAs within the cell. As a result, they will bind specifically to the mRNA. Once this happens the mRNA will be degraded.

Adaptive Immunity

Proteins belonging to the immunoglobulin superfamily have been detected in arthropods, echinoderms, mollusks, and protochordates. In insects, for example, a member of the immunoglobulin superfamily called Dscam can be diversified by alternate splicing. Isoforms of Dscam are expressed in immune tissues and secreted as soluble proteins into the hemolymph. *Drosophila* species have the potential to express more than 18,000 isoforms of Dscam. Individual hemocytes may express 14 to 50

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forms of Dscam that can bind bacteria and enhance their phagocytosis.

Graft Rejection

Invertebrates can reject allografts and xenografts. For example, cell-mediated allograft rejection occurs in sponges, coelenterates, annelids, and echinoderms. When two identical sponge colonies are placed side by side and made to grow in contact with each other, no reaction occurs. If, however, sponges from two unrelated colonies come into contact, tissue destruction occurs along the area of contact as each sponge attempts to destroy the other.

Annelids such as earthworms can reject both allografts and xenografts. The rejection of xenografts (from other species of earthworms) takes about 20 days. Cells invade the graft, and the grafted tissue turns white, swells, becomes edematous, and eventually dies. If the recipient worms are grafted with a second piece of skin from the same donor, the second graft is rejected faster than the first. This ability to reject second grafts rapidly may be adoptively transferred by coelomocytes from sensitized animals. Thus invertebrates have some form of immunological memory.

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Immunity in Vertebrates

There are seven classes of living vertebrates: jawless fish, cartilaginous fish, bony fish, amphibians, reptiles, birds, and mammals (Fig. 43.4).

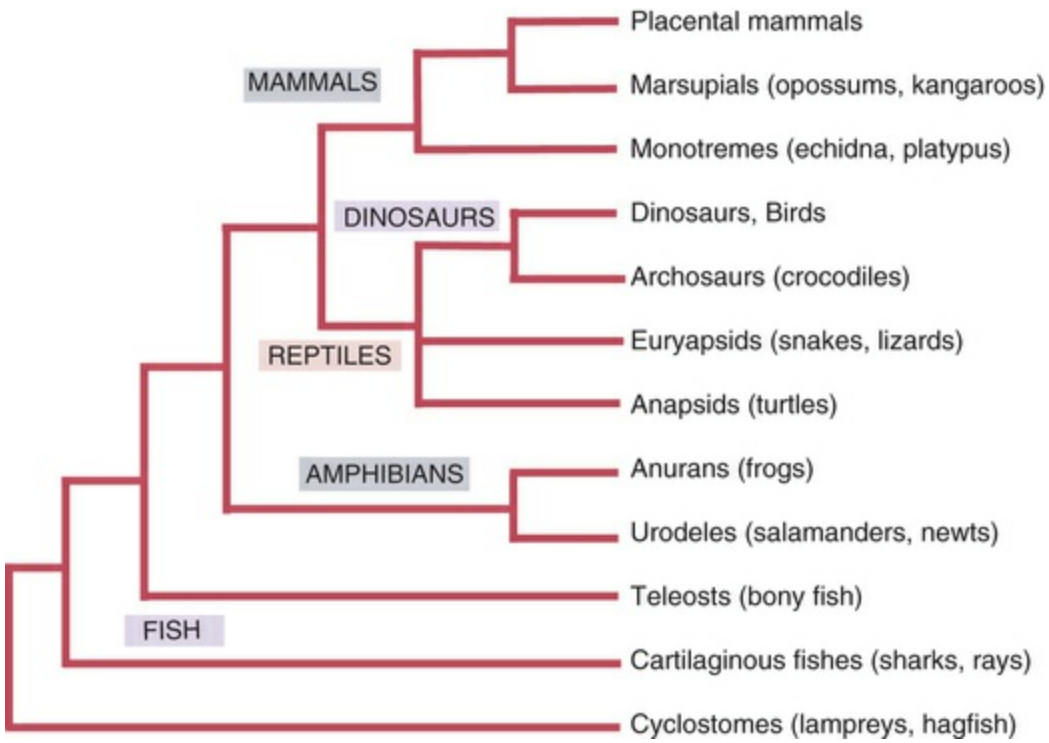


FIG. 43.4 A simplified phylogenic tree showing the major relationships among the vertebrates.

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Immunity in Cyclostomes

The least evolved of living vertebrates are the cyclostomes, the fish without jaws. These include the lampreys and the hagfish. They have both innate and adaptive defenses. For example, they make proteins that can bind to bacteria and enhance their phagocytosis by leukocytes. Cyclostomes possess both the alternate and lectin complement pathways but lack the lytic components of the complement system. The lamprey complement system thus promotes phagocytosis rather than lysis.

Cyclostomes have two types of blood leukocyte. One type resembles monocytes. The other resembles lymphocytes. Lacking the appropriate recombinases, cyclostomes cannot make antibodies or T cell receptors (TCRs). Instead, they mount a form of adaptive humoral response that employs variable lymphocyte receptors (VLRs) unrelated to immunoglobulins. There are three types of VLR: VLRA is only found on cells that resemble α/β T cells, VLRB is found on and secreted by cells resembling B cells, and VLRC is found on cells resembling γ/δ T cells.

Cyclostomes generate enormous diversity in their VLRs by rearranging their DNA by gene conversion. This involves inserting several variable leucine-rich repeat cassettes into an incomplete VLR germline gene. These cassettes are obtained from a large library of such cassettes located at each end of the *VLR* gene. As a result, the protein-binding site of the VLR proteins is lined by hypervariable positively selected amino acids. It is calculated that they may be able to assemble as many as 10^{14} unique receptors in this way. It also appears that each lamprey lymphocyte expresses a specific VLR, suggesting that clonal selection operates in this system. All the VLRs are anchored to the lymphocyte membrane, but $VLRB^{+}$ cells bind native antigens and differentiate into cells that secrete soluble VLRB.

Thus VLRA and VLRB are expressed on cells that resemble T cells and B cells, respectively. VLRC is expressed on a cell type that resembles γ/δ T cells. VLRA- and VLRC-positive cells share donor LRR cassettes. (VLRB receptors use a completely different set of cassettes.) The production of VLRs therefore uses a very different

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mechanism from that involving immunoglobulin or TLR diversity, which emerged around the same time (about 500 million years ago). Most advanced vertebrates developed α/βT, γ/δT, and B cells. The jawless fish developed VLRA, VLRB, and VLRC. Thus the emergence of three cell lineages occurred in the last common ancestor of these vertebrates.

Discrete thymus-like lymphoepithelial structures called thymoids are found in the gill filaments of lamprey larvae. This is where VLRA and VLRC cells develop. They express the transcription factor FoxN1 that also is a marker of thymic function in the jawed vertebrates.

Immunological "Big Bang"

The adaptive immune system uses two key antigen-receptor systems, TCRs and BCRs. Both require the rearrangement of *V*, *D*, and *J* gene segments to form antigen-binding receptors. Sometime during the 100 million years between the divergence of jawless and jawed vertebrates and the emergence of cartilaginous and bony fish, about 450 Mya, the enzymes needed for the recombination of *V* gene segments emerged. The mechanism of this sudden appearance is unknown. It has been suggested, however, that a transposon carrying the precursors of the recombinase-activating genes *RAG1* and *RAG2* (most likely a bacterial integrase) was successfully inserted into an immunoglobulin superfamily *V*-like gene within the germline of the early jawed vertebrates (Fig. 43.5). As a result, the *V*-like gene could be expressed only after splicing by the RAG enzymes. Thus emerged the ability to generate antigen-binding sites and functional immunoglobulins. The advantages of this new "improved" system were such that it is now a feature of all jawed vertebrates. This did not, of course, result in discarding of the innate immune defenses. Lectins, the complement system, and the NK cell system remain essential components of vertebrate immunity. It is also important to point out that adaptive immunity did not confer invincibility to infectious agents. It simply made life more difficult for them and conferred an incremental selective advantage on animals with such defenses.

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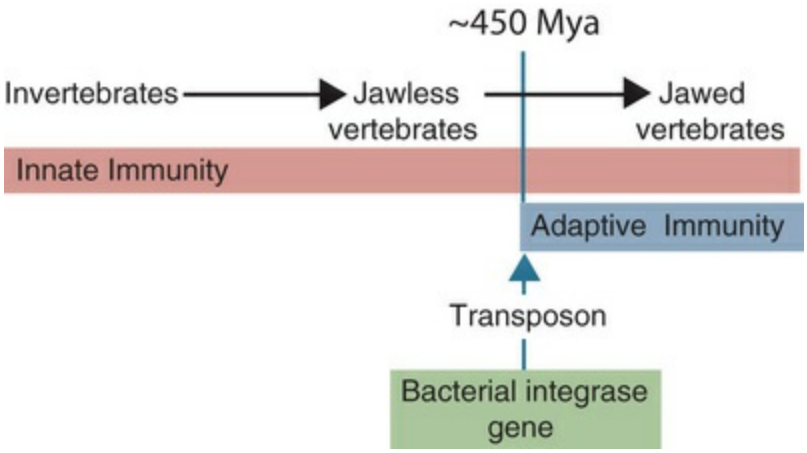


FIG. 43.5 Innate immunity is a feature of all animals, both vertebrates and invertebrates. Adaptive immunity, in contrast, is found only in the jawed vertebrates, that is, animals more evolved than the jawless fish. It has been suggested that the ability to mount an adaptive immune response depends on the transfer of a bacterial integrase gene to a vertebrate germ cell through a transposon.

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Immunity in Jawed Fish

The fish emerged about 500 to 420 Mya, long before the appearance of the mammals. The Chondrichthyes, the fish with cartilaginous skeletons that include the rays and sharks (the elasmobranchs) emerged first. The most complex fish are the bony fish of the class Osteichthyes, which include the overwhelming majority of modern fish, the teleosts.

Innate Immunity

Fish employ PRRs similar to those found in mammals. These include TLRs, RIG-1–like receptors, and NOD-like receptors. There are 17 fish TLRs. (TLR14, 20, 21, 22, and 23 are not found in mammals.) Fish are constantly exposed to aquatic viruses and must defend themselves accordingly. They thus possess a type I IFN system activated through TLRs. There are multiple subgroups of fish type I interferons. Most fish have one or two subgroups but salmonids have six, IFN-a thru IFN-f. Depending on the stimulus, different organs produce different interferons. Thus poly-IC induces IFN-a, -b and -c in the head kidney. Hemorrhagic septicemia virus, in contrast, induces IFN-d, -e, and -f in the kidney and spleen.

In fish inflammation, granulocytes arrive first, and their numbers peak by 12 to 24 hours. This is followed by a wave of macrophages and possibly lymphocytes. The response tends to be prolonged, and macrophage numbers peak after 2 to 7 days. In fish, granulocytes originate from the head kidney, whereas their macrophages develop from blood monocytes. Fish macrophages are found in many organs, especially the mesentery, splenic ellipsoids, kidney, and atrium of the heart.

Teleost neutrophils are similar in morphology and probably function to mammalian neutrophils. They are phagocytic, and their numbers increase in response to infections. The release of oxidants from neutrophils at inflammatory sites may cause severe tissue damage since the fat of fish is highly unsaturated as an adaptation to low temperatures. Polyunsaturated fats are prone to oxidation,

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and ROS may therefore oxidize tissue lipids. Fish use melanin to moderate this response. Melanin can quench free radicals, and melanin-containing cells are common in their inflammatory lesions. They probably protect tissues against oxidants.

Both bony and cartilaginous fish produce lysozyme, lectins, defensins, complement, and acute-phase proteins. Lysozyme is present in their eggs and may protect the developing embryo. Fish acute-phase proteins include the pentraxins, C-reactive protein, serum amyloid A, serum amyloid P, C3, transferrin, and cathelicidins. However, their rise is much less pronounced than in mammals. Natural cytotoxic cells resembling mammalian NK cells have been described in bony fish. They are produced in the anterior kidney. Antimicrobial peptides called piscidins are found in the mast cells and phagocytic cells of bony fish. These peptides can kill both extracellular and intracellular invaders.

Cartilaginous and bony fish possess all three complement pathways. Their lytic pathway generates a terminal complement complex similar to that formed in mammals, although it works at a lower temperature (~25° C). Unlike other vertebrates, where C3 is coded for by a single copy gene, bony fish produce multiple functional isoforms of C3. For example, rainbow trout have four C3 isoforms, carp have eight, and sea bream have five. They differ in their ability to bind to activating surfaces.

Adaptive Immunity

Both cartilaginous and bony fish have a complete set of lymphoid organs except for bone marrow and they can mount adaptive immune responses (Fig. 43.6). Their thymus is located just above the pharynx and arises from the first gill arches. In immature fish, small pores lead from the pharynx to the thymus, thus exposing it to antigens in the surrounding water. Thymectomy leads to prolonged allograft survival and reduced antibody responses (Box 43.1). Antibodies or antigen-binding cells may be detected in the thymus, suggesting that it contains both T-like and B-like cells. Although the thymus may involute in response to hormones or season, age involution is inconsistent, and a thymus may be found in old fish.

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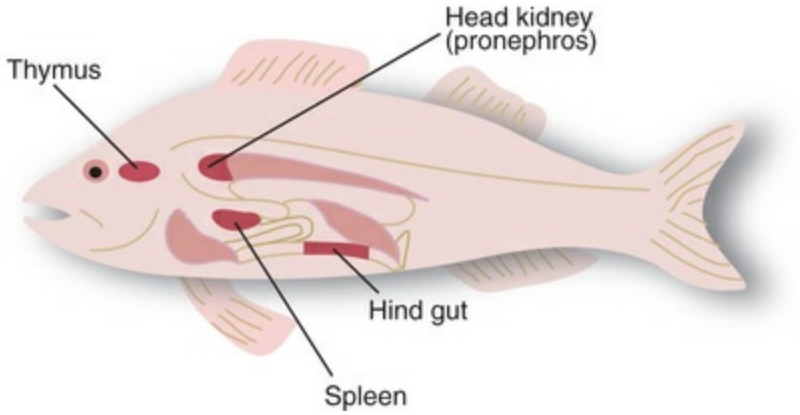


FIG. 43.6 The lymphoid organs of a bony fish.

Box 43.1

Vaccine-Induced Autoimmunity in Fish

Oil-adjuvanted vaccines are widely employed in aquaculture for the prevention of infections. Under some circumstances administration of these vaccines to farmed Atlantic salmon can result in polyclonal B cell activation, the production of multiple autoantibodies, and the development of a membranoproliferative glomerulopathy and spondylitis. The autoantibodies are directed against nuclear and cytoplasmic antigens, immunoglobulins (rheumatoid factors), ssDNA, chromatin, thyroglobulin, erythrocytes, and ferritin. These are not non-specific reactions; rather, each fish develops its own pattern of autoreactivity with different levels of response against each autoantigen. The kidney lesions that develop are consistent with immune complex–mediated reactions. These vaccine-induced reactions may well have an impact on the continuing use of oil-based adjuvants in salmon vaccines.

Koppang EO, Bjerkås I, Haugarvoll E, et al: Vaccination-induced systemic autoimmunity in farmed Atlantic salmon, *J Immunol* 181:4807-4814, 2008.

The fish kidneys differentiate into two sections. The opisthonephros or posterior kidney is an excretory organ similar to the mammalian kidney. In contrast, the pronephros or anterior kidney is a lymphoid organ containing antibody-forming cells and phagocytes. Its function is analogous to mammalian bone marrow

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and lymph nodes. Fish have a spleen with a structure and location similar to that in mammals.

Aggregates of lymphocytes are prominent in the fish intestinal tract. In addition, lymphomyeloid structures that produce granulocytes are found in the submucosa of the esophagus (Leydig organ) and in the gonads (epigonal organ) of sharks. Some species possess both, but others may have only one. The epigonal organ and the Leydig organ in cartilaginous fish appear to be primary lymphoid organs. The epigonal organ appears to be a source of B cells.

Fish have clusters of macrophages containing melanin and hemosiderin. These melanomacrophage centers are found in the spleen, liver, and kidney. Antigens may persist in these centers for long periods, and they appear to be precursors of the germinal centers found in more evolved vertebrates. Teleosts also have dendritic-like cells that can present antigens to T cells.

Fish lymphocytes resemble those of mammals. B cells can be found in the thymus, anterior kidney, spleen, Leydig organ, and blood, and their surface immunoglobulins act as antigen receptors. These B cells can mature into plasma cells. Unlike mammalian B cells, however, teleost B cells can phagocytose particles, generate phagolysosomes, and kill ingested microbes. This supports the idea that B cells may have evolved from an ancestral phagocytic cell and may account for the similarities between macrophages and mammalian B-1 cells. Both helper and cytotoxic T cells can be detected in fish.

Immunoglobulins

The cartilaginous fish such as the sharks are the least evolved vertebrates known to produce antibodies. They produce diverse immunoglobulin isotypes, not all of which are directly related (orthologous) to mammalian Igs.

Fish differ from mammals in the organization of immunoglobulin gene segments within the genome. For example, elasmobranch fish have clustered immunoglobulin genes, where *V*, *D*, *J*, and *C* segments form clusters that are duplicated many times; thus:

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-*VDJC* — *VDJC* — *VDJC* — *VDJC* — *VDJC* —

There are 100 to 500 of these *VDJC* clusters in sharks; each about 16 kilobases (kb) in size. About half of these clusters appear to be functional. Teleost fish, in contrast, have an immunoglobulin heavy chain gene arrangement similar to that of mammals (the translocon pattern), with multiple *VH* genes arranged thus:

-*V*-*V*-*V*-*V*-*V*-*V*-*V*-*D*-*D*-*J*-*J*-*J*-*J*-*C* —

In catfish, heavy chains are arranged in the translocon pattern, and light chain genes are in the clustered pattern. Shark immunoglobulins show evidence of somatic hypermutation.

IgM: IgM is found in both bony and cartilaginous fish (Fig. 43.7) (but not in the Coelacanth!). It is mainly produced by plasma-like cells in the anterior kidney. Cartilaginous fish usually have both pentameric and monomeric serum IgM. Bony fish have tetrameric and monomeric IgM. Fish antibody responses are characterized by the predominance of IgM. Fish antibodies in the presence of complement can lyse target cells and they are effective at agglutination, but there is no evidence that they can act as opsonins, nor have Fc receptors been detected on their phagocytic cells. The blood vessel walls of fish are permeable to IgM. As a result, antibodies are found in most tissue fluids (plasma, lymph, skin mucus).

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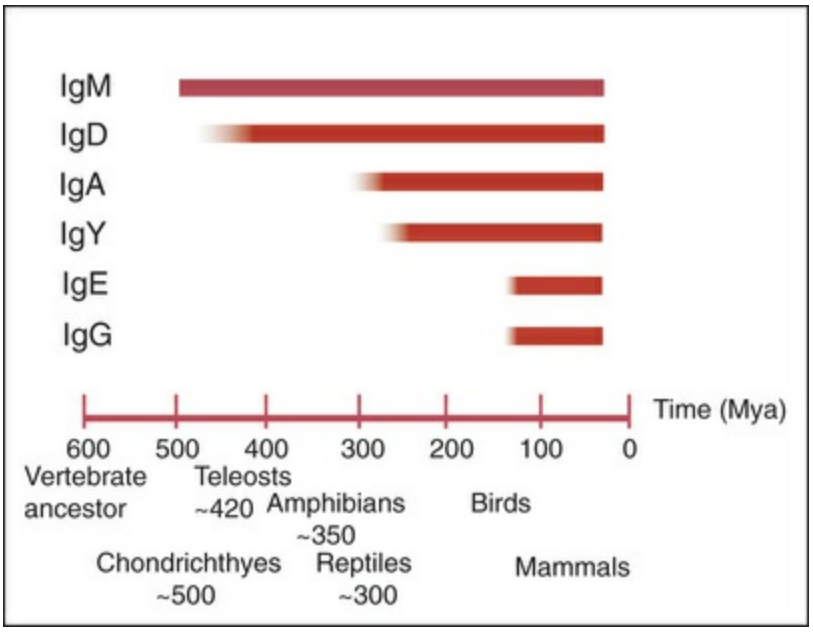


FIG. 43.7 The evolution of the major immunoglobulin classes.

IgD: IgD has been identified in catfish, halibut, salmon, and cod and secretory IgD has been described in catfish and trout. As in other vertebrates, its gene organization is very variable. It has some similarities to mammalian IgD, including co-expression with IgM on B cell surfaces. Fish do not produce IgA but they secrete a special mucosal IgM together with IgD. This secreted IgD may act as a PRR since it lacks a variable region (in catfish but not in trout). It occurs in two forms: a conventional form and a short, truncated form similar to the truncated form of avian IgY.

IgT: IgT is the major mucosal and skin immunoglobulin in teleosts. In serum it is monomeric; in the gut and skin mucus it is multimeric. Mucosal IgT and IgM both associate with a polymeric Ig receptor for transport into the gut lumen. The skin of teleosts consists of live epidermal cells in contact with a water medium. The skin-associated lymphoid tissues (SALT) of these fish resemble the gut-associated lymphoid tissues and in addition are populated by a diverse microbiota. B cells in teleost skin resemble those in the gut. Thus they produce polymeric IgT in the skin mucus layer.

Other immunoglobulins: Three additional immunoglobulin isotypes have been identified in elasmobranchs. These are IgW in the sandbar shark, IgNAR in the nurse shark, and IgR in the skate. IgNAR is a homodimeric heavy chain antibody with no associated light chains. Three additional immunoglobulin isotypes have been

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identified in teleosts. These include IgZ in zebrafish, IgH in pufferfish, and a chimeric IgM-IgZ in the common carp.

Not all antigens are effective immunogens in fish. Soluble protein antigens are poorly immunogenic, in contrast to particulate antigens such as bacteria or foreign erythrocytes that are highly immunogenic. Many cartilaginous fish show seasonal effects on antibody production; that is, under constant conditions of light and temperature, immune responses are poorer in winter than in summer. Social interactions can also influence their immune response; fish kept at high population density are immunosuppressed.

Cell-Mediated Immunity

Fish make rearranged TCRs and TCR homologs whose overall structure is similar to that in mammals. Their germline *TCR* genes are not rearranged and are organized in the cluster pattern.

Fish have both MHC class I and II genes, but agnathans do not. The basic structure of each MHC molecule is conserved, as is the organization of the MHC class I and class II loci, although in teleosts, class I and II loci are on at least three different chromosomes (Box 43.2).

Box 43.2

The Curious Case of the Cod!

The Atlantic cod, *(Gadus morhua)* has an unusual immune system. It totally lacks MHC class II molecules as well as the chaperone invariant chain (Ii)! In addition, the gene for CD4, the MHC II binding protein on T cells, is a truncated pseudogene. As a result, the cod is unable to process and present processed bacterial and parasitic antigens to its T cells in the conventional manner. Despite this, the cod does not appear to be unusually susceptible to infectious diseases in its natural cold water habitat. It must therefore compensate somehow for the lack of an MHC class II pathway. It has accomplished this by greatly expanding the number and complexity of its MHC class I genes. It has, in addition, a uniquely large population of TLR families. The cod thus relies heavily on TLRs, especially on TLR9, to detect bacterial

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DNA. Atlantic cod also have unusually high levels of serum IgM and abundant neutrophils in their bloodstream.

Star B, et al. The genome sequence of Atlantic cod reveals a unique immune system, *Nature* 407:207-210, 2011.

Many homologs of mammalian cytokines have been identified in fish including proinflammatory interleukins such as IL-1β, IL-18, IL-6, and IL-11; interleukin-2 subfamily members, IL-2, -4, -7, -15, and -21; interleukin-10 subfamily members, IL-10, -20–like, and -22/26; five interleukin-17 members; plus the heterodimeric interleukin chains, p19, p35, p40, and EB13 (IL-27β). TNF-α, TGF-β, IFN-β, and IFN-γ are also found in fish.

Cartilaginous fish reject scale allografts slowly, whereas bony fish reject them much more rapidly. Repeated grafting leads to accelerated rejection. The rejected allografts are infiltrated by lymphocytes and show destruction of blood vessels and pigment cells. As in all ectotherms, graft rejection is slower at lower temperatures.

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Immunity in Amphibians

There are two major orders of amphibians: the less evolved Urodela, which includes the long-bodied, tailed amphibians such as the salamanders and newts; and the Anura, an advanced, tailless order that includes the frogs and toads.

As vertebrates evolved, their immune systems changed (Fig. 43.8). This is well seen in amphibians, where there are marked differences between the urodeles and the anurans. In addition, amphibians go through a complex metamorphosis as they change from the tadpole into an adult form. This affects the development of the immune system. A notable feature of amphibian innate immunity is the presence of very potent antimicrobial peptides in their skin. Amphibians also possess a complement system that, although similar to that of mammals, is more effective at 16° C.

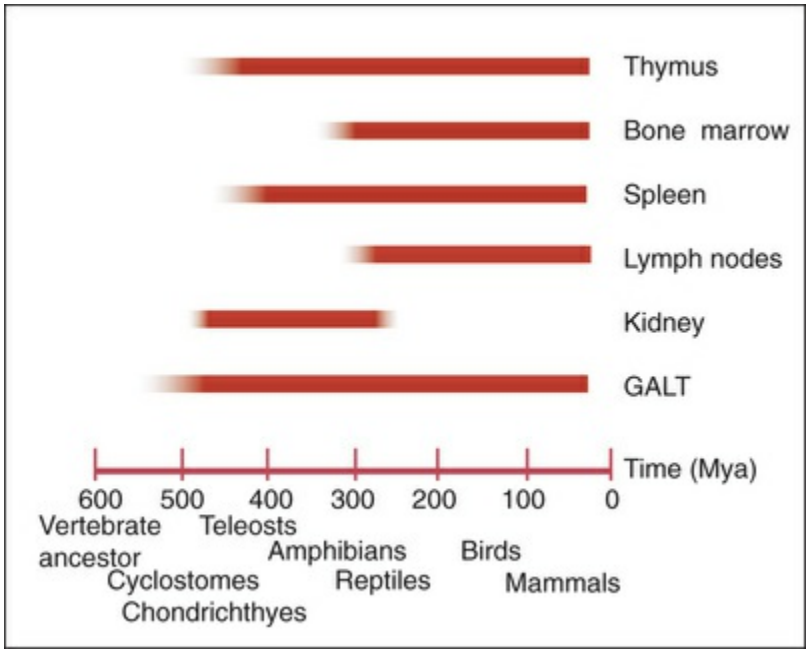


FIG. 43.8 The evolution of the major lymphoid organs in vertebrates.

Urodele Amphibians

Urodeles generally lack a bone marrow, although some

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salamanders may have a small amount of lymphoid tissue within their long bones. They have a thymus that develops slowly, only appearing at the seventh week of life. Thymectomy delays or blocks rejection of skin allografts. The kidney retains its lymphoid function, as in fish. In the spleen, the red and white pulps are not separate.

Urodeles produce a monomeric IgM and can mount a good but slow antibody response against bacterial antigens. They do not respond to soluble protein antigens such as serum albumin or ferritin. Other immunoglobulin isotypes found in urodeles are IgY and IgD.

It takes about 28 to 42 days for a skin allograft to be rejected in urodeles. The allograft looks healthy for about 3 weeks, and it is then slowly rejected. Destruction of the pigment cells makes rejection of the graft readily visible because it turns white. Second-set rejection takes about 8 to 20 days in the newt, and the memory of this lasts for at least 90 days.

Anuran Amphibians

In contrast to urodeles, a fully functional bone marrow is present in anurans (frogs and toads). Their thymus arises from the second pharyngeal pouches and involutes by about 1 year of age. It also involutes during metamorphosis from the tadpole to the adult stage and then rapidly regenerates. The thymus lies just below the skin posterior to the middle ear. It shows a distinct separation between the outer cortex and central medulla. The cortex is full of proliferating lymphocytes. The medulla contains fewer lymphocytes, but thymic corpuscles are present. Immunoglobulins can be found on about 80% of these thymocytes. Larval thymectomy reduces their response to foreign red blood cells, but the response to bacterial LPS is unaffected, suggesting this is a T-independent response. Thymectomy slows but does not completely prevent allograft rejection in toads. In frogs and toads, for the first time, boundary layer cells separate the red pulp and the periarteriolar white pulp of the spleen. Structures that resemble lymph nodes are seen in some anuran amphibians. These proto-lymph nodes consist of a mass of lymphocytes surrounding blood

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sinusoids. As a result, they filter blood rather than lymph. Lymphoid aggregates do not seem to be present in the intestine of urodeles but are seen in anurans.

Larval anurans such as the bullfrog tadpole have lymphomyeloid organs in their branchial region called ventral cavity bodies. Sinusoids in these organs are lined with macrophages that effectively remove particulate antigens from the blood. Removal of these organs renders tadpoles incapable of making antibodies to soluble antigens. They disappear at metamorphosis. During metamorphosis from larval stage to adult, there is a temporary immunosuppression as shown by slowing of allograft rejection. Some allografts may even be tolerated at this time. As tadpoles change into frogs or toads, the thymus shrinks, and there is a drop in the numbers of B cells and antibody levels (Box 43.3).

Box 43.3

Chytridiomycosis

The massive recent decline in global amphibian numbers is in large part the result of chytridiomycosis, a disease caused by the fungus *Batrachochytrium dendrobatidis*. More than 250 amphibian species are susceptible to this chytrid fungus found on all the temperate continents. It affects adult amphibians by causing hyperplasia of skin keratinocytes. The resulting increase in skin thickness probably affects osmoregulation and ion balance across the skin. Frog mortality can reach 100% in some systems and have little effect in others. In tadpoles, it causes loss of keratinized mouthparts and hence reduces food intake and survival. It appears that amphibians have difficulty in clearing this infection. *B. dendrobatidis* cells and its culture supernatants impair lymphocyte proliferation and induce their apoptosis, although fungal recognition and phagocytosis by macrophages and neutrophils are not affected. The suppressive factor(s) is present in the fungal cell wall. The occurrence of this immunosuppression may explain in part why this organism has had such a devastating effect.

Fites JS, et al. The invasive Chytrid fungus of amphibians paralyzes lymphocyte responses, *Science* 342:366-369, 2013.

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Both adult and larval amphibians have circulating B and T cells that probably originate in the ventral cavity bodies or the liver. About 80% of circulating lymphocytes carry surface IgM. Frogs also possess NK-like and T cytotoxic–like killer cells.

Anuran amphibians have up to five immunoglobulin isotypes: IgM, IgD, IgX, IgY, and IgF. The two most important are IgM and IgY. They are the least evolved vertebrates to show isotype switching.

IgM and IgY: Frogs immunized with bacteria or foreign erythrocytes will produce only IgM. Bacteriophages or soluble foreign proteins induce both IgM and IgY. The IgY takes up to 1 month to appear, and its level is very low. Amphibians do not mount a secondary response to erythrocytes and bacteria, but memory develops in response to the antigens that stimulate an IgY response. Studies of immunological memory are complicated by the fact that antigens may persist in the circulation for several months after injection.

IgX: IgX is orthologous to avian IgA. It is produced in response to oral immunization. (Fig. 43.9). Anurans have secretory immunoglobulins in bile and the intestine (but not in skin mucus). These consist of IgM and IgX but not IgY.

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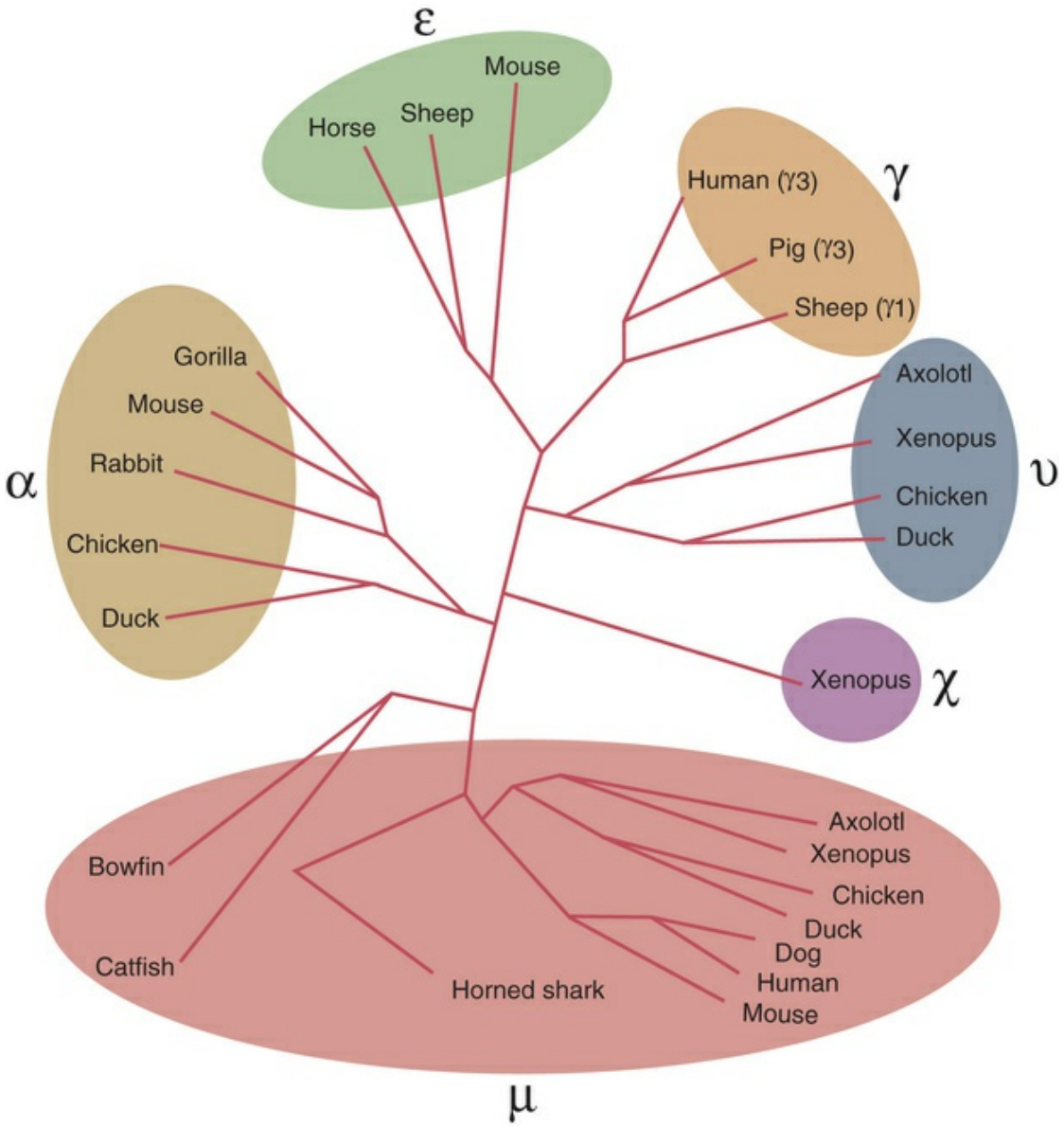


FIG. 43.9 The evolutionary relationships among the major vertebrate immunoglobulin heavy chains. This is a distance tree constructed by aligning the amino acid sequences of representative vertebrate IgH-chain constant regions. (From Warr GW, Magor KE, Higgins DA: IgY: clues to the origins of modern antibodies, *Immunol Today* 16:392-398, 1995.)

IgD: The gene for the IgD heavy chain (δ) has been identified in the toad *Xenopus tropicalis.* It is expressed on mature B cells. As in mammals, the *Cδ* heavy chain gene is located immediately 3′ to the *IgM* gene. Xenopus IgD is orthologous to IgW found in cartilaginous fish and lungfish. This implies that IgD/W was present in these ancestors of all living jawed vertebrates. This IgD is structurally variable. Its gene shows many duplications, deletions of domains, the presence of multiple splice forms, or even the loss

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of the entire gene.

IgF: An additional isotype, IgF with a Φ heavy chain and only two constant domains, has been identified in *Xenopus.* It is unique in having a hinge region, the earliest example of such a structure. The IgH locus of *X. tropicalis,* has the following order: *5′-V_H-D_H-J_H-Cμ-Cδ-Cχ-Cυ-CΦ-3′.* Amphibian antibody diversity is generated in a fashion similar to that in mammals.

Amphibians have T cells with functional TCRs, and anurans such as bullfrogs and toads can reject allografts. A first-set reaction takes about 14 days at 25° C. Second allografts do not even become vascularized and are destroyed within a few days. If these amphibians are kept in the cold, skin allograft rejection may take as long as 200 days. Delayed hypersensitivity reactions have been described in the axolotl *(Ambystoma)* and *Xenopus* in response to mycobacterial sensitization.

The African clawed frog, *Xenopus* has a well-characterized MHC with class I, II, and III regions called XLA. The class II region contains genes for both α and β chains that code for 30- to 35-kDa transmembrane glycoproteins. About 20 class I and 30 class II alleles are believed to exist. The class III region contains a gene for C4. It is interesting to note that although MHC class II molecules are expressed early in larval development on B cells and tadpole epithelia, MHC class I molecules are not expressed before larval metamorphosis.

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Immunity in Reptiles

Three subclasses of reptiles currently exist: the Anapsida, which includes the turtles; the Lepidosaura, which consists of the lizards and snakes; and the Archosauria, which includes the crocodiles and alligators.

The reptilian thymus develops from the pharyngeal pouches and is structurally similar to that seen in other classes of vertebrates. Both age and seasonal involution have been reported. Thus the thymus shrinks in winter and enlarges in summer. The reptilian spleen usually shows a clear separation between red and white pulps.

Lizards often shed their tails in an attempt to escape from predators. The resulting stump is covered by a blood clot that develops into a scab. Phagocytic cells destroy invading bacteria while activated keratinocytes cover the stump surface. Studies on the wounds created by tail loss have shown the release of β-defensins from the granulocytes attracted to the tail stump wound. By protecting the wounded tissues from microbial invasion, this defensin (and likely many others), initiates tissue regeneration.

Lymphomyeloid nodes that resemble lymph nodes are seen in reptiles. They have a simple structure consisting of a lymphoid parenchyma with phagocytes and intervening sinusoids. Primitive lymph nodules surround the aorta, vena cava, and jugular veins. Lymphocytes and plasma cells are found in nodules in the intestinal wall of the more evolved reptiles. Some turtles and snakes have lymphoid aggregations that project into the cloacal lumen, called the cloacal complex. These aggregates are larger in adults than in young turtles and cannot be regarded as a primitive bursa. A few lymphocytes are found in the kidney of reptiles. Basophils are abundant in reptiles. For example, more than half the blood leukocytes in snapping turtles *(Chelydra serpentina)* are basophils. They express FcR on their surface and release histamine in response to treatment with anti-turtle immunoglobulin serum.

The reptiles that have been studied possess IgM, IgD, and IgY. The IgM of turtles is comparable to mammalian IgM in size, chain structure, and carbohydrate content. They produce several isoforms

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similar to IgD2, as well as a mixed IgM/IgD molecule. IgY is found in both the full-sized and truncated isoforms (although some turtles may have only the truncated isoform). Some turtles may have multiple IgY isoforms. Some reptiles such as the Leopard gecko *(Eublepharis macularius)* produce IgA but anoles (*Anolis* sp.), turtles, and snakes do not. Sequence analysis shows that while its C_H1 and C_H2 domains are homologous to *Xenopus* IgY, its C_H3 and C_H4 domains are homologous to *Xenopus* IgM! It appears therefore that recombination between *IgY* and *IgM* genes gave rise to this IgA.

Turtles in general seem to have one IgM gene, one IgD gene, several IgY genes, and several IgD2 genes. (IgD2 is a variant of IgD with its first four CH domains similar to IgD and the last two C_H domains similar to the C_H3 and C_H4 domains of IgA.) Green turtles *(Chelonia mydas)* have novel asymmetrical antibodies. Green anole lizards *(Anolis carolensis)* and Chinese-soft shelled turtles *(Pelodiscus sinensis)* also express three immunoglobulin heavy chain isotypes: IgM, IgD, and IgY. As in some birds, two forms of IgY are produced: a full-length form and a truncated form. These lizards completely lack an *IgA* gene in their IgH locus. On the other hand, alligators *(Alligator mississippiensis)* and crocodiles *(Crocodylus porosus)* have four *IgM* genes, one *IgD,* three *IgA,* three *IgY,* and two *IgD2* genes.

Genes coding for IgM, IgD, and two classes of IgY (IgYa and IgYb) have been identified in snakes. Their IgD structure is similar to that seen in other reptiles. In some species, (the coast garter snake *[Thamnophis elegans]* and the Burmese python *[Python molurus]*) there is a third *IgY* gene *(IgYc).* Its constant region lacks a C_H2 domain and hence contains only three C domains.

Turtles and lizards immunized with bovine serum albumin, pig serum, or red blood cells can mount primary and secondary antibody responses. IgM is produced in the primary response; IgY predominates in the secondary response. All reptile antibody responses appear to be T-dependent. Secondary responses and IgY antibody production do not occur in response to bacterial antigens such as *Salmonella enterica Adelaide* or *Typhimurium,* or *Brucella abortus.* The reader may recollect that a similar situation occurs in mammals, in that thymus-independent antigens such as *Escherichia*

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coli LPS induce a prolonged IgM response that is distinctly different from that induced by soluble protein antigens (Chapter 15).

As in other ectotherms, reptile allograft rejection is temperature dependent. Turtles, snakes, and lizards reject allogeneic skin grafts in about 40 days at 25° C. Other evidence of cell-mediated immune responses such as delayed hypersensitivity reactions have been demonstrated in reptiles.

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Immunity in Birds

The dinosaurs were sufficiently different from true reptiles to be put in a class of their own, the Dinosaura. Although most dinosaurs disappeared 65 Mya at the end of the cretaceous period, their modern descendants are the birds, the members of the class Aves. Unlike the reptiles, birds are (and dinosaurs were) endothermic, or warm blooded. As a result of this, birds share with mammals all the benefits that come from greatly increased physiological and biochemical efficiency.

Most studies on the avian immune system have focused on chickens. Thus the statements to follow, although generally true of chickens, may not necessarily apply to the other approximately 10,000 bird species. The dinosaurs diverged from the mammalian line about 300 Mya, which has provided ample opportunity for the immune systems of mammals and birds to evolve major differences.

Analysis of the complete chicken genome provides some interesting insights into the evolution of the immune system in this species. For example, it has proved possible to identify chicken orthologs of several immune-related genes that were previously believed to be confined to mammals. These include cathelicidin, colony-stimulating factors, and IL-3, IL-4, IL-7, IL-9, IL-13, and IL-26. Chickens have TLR1 to TLR5, and TLR7 but not TLR6, 8, 9, or 10. TLR 1 and 2 have duplicated genes. Conversely, TLR15 (also found in reptiles) is found in chicken spleen, bursa, and bone marrow, but not mammals. Its does not bind specific ligands but is activated by fungal and bacterial proteases. TLR 21 (also found in fish) is the chicken ortholog of mammalian TLR9 and is activated by CpG oligodeoxynucleotides. Chickens lack RIG-1.

Birds have many different host defense peptides including defensins and cathelicidins. Chickens have 14 β defensin genes (but zebra finches have 22). Some defensins (ovodefensins) have been identified in egg white. Chickens have four cathelicidin genes including fowlicidin-1.

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Major Histocompatibility Complex Molecules

Studies on chickens have suggested that the their MHC is small and simple. It lacks the framework genes present in mammals. The core of the chicken MHC occupies only 92 kb and contains only 19 genes as compared to the human whose MHC occupies 3.6 mb and contains 224 genes. It is divided into two independent regions designated MHC-B and MHC-Y. Both are located on microchromosome 16, but they are separated by the nucleolar organizer region (Fig. 43.10). The class III region contains the *C4*, steroid 21-hydroxylase gene *(CYP21)* and tenascin genes *(TNXB).* The class I region contains two class I α-chain genes (*BF1* and *BF2*), and the tapasin genes (*TAP1* and *TAP2*). The class II region contains two class II genes *DMA* and *DMB* and two class II β-chain genes (*BLB1* and *BLB2*).

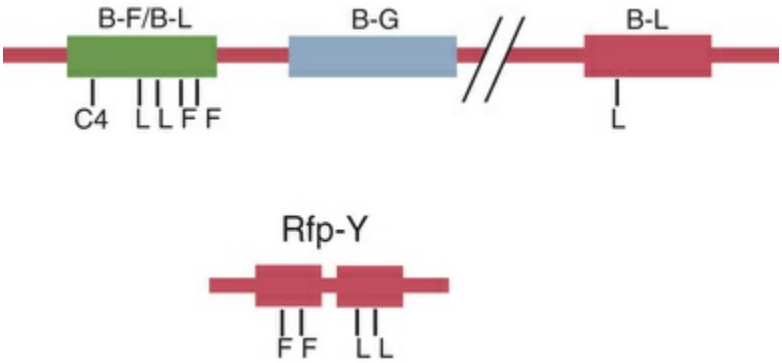


FIG. 43.10 Structure of the chicken B region *(top)* and the Rfp-Y region *(bottom). F* genes are class II genes, and *L* genes are class I genes.

The Y region consists of two gene clusters containing two class I and two class II genes as well as a C-type lectin gene. They differ from the B region genes in that their products are not expressed on red cells. Genes within the Y region also regulate NK cell recognition. The B locus of the turkey is very similar to that of the chicken. It is a small, constricted locus with 34 genes in near-perfect synteny with that of the chicken B locus. The quail B locus is 191 kb in size with a similar structure. However, the quail has seven class I and seven class II β-chain genes, while the chicken has only two of each.

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In the common chicken haplotypes, only one class I and one class II molecule are dominantly expressed. Since viruses contain relatively few proteins, MHC-dependent disease susceptibility depends on virus antigens binding to the dominant MHC class I molecule. As a result, possession of specific haplotypes determines viral disease susceptibility. For example, the haplotype B^{21} is associated with resistance to Marek's disease, whereas the B^{19} haplotype is associated with susceptibility. Chickens homozygous for B^{1} generally have high adult mortality, are highly susceptible to Marek's disease, and respond poorly to *Salmonella pullorum* or to human serum albumin. Birds homozygous for B^{5} are able to mount a better antibody response and develop less severe lesions in response to infection with *Eimeria tenella* than B^{2} homozygous birds. Certain MHC genotypes ($B^{A4/A4}$ and $B^{A12/A12}$) are significantly over-represented in birds suffering from bacterial arthritis and osteomyelitis caused by *Staphylococcus aureus.*

Neither chickens nor turkeys are typical of birds as a whole. Passerine birds, for example, have a complex MHC structure with multiple duplicated copies of each class of gene, pseudogenes, and longer introns and intergenic regions. Even the kiwi, a very primitive bird, has at least five MHC class II genes.

The thymus in birds and in primitive mammals is similar to that in eutherian mammals. The germinal centers of bird lymphoid organs are large and well defined. Although birds are commonly considered not to possess lymph nodes, they do possess structures that can be considered to be their functional equivalent. These avian lymph nodes consist of a central sinus that is the main lumen of a lymphatic vessel. The sinus is surrounded by a sheath of lymphoid tissue that contains germinal centers (Fig. 43.11). Avian lymph nodes have no external capsule.

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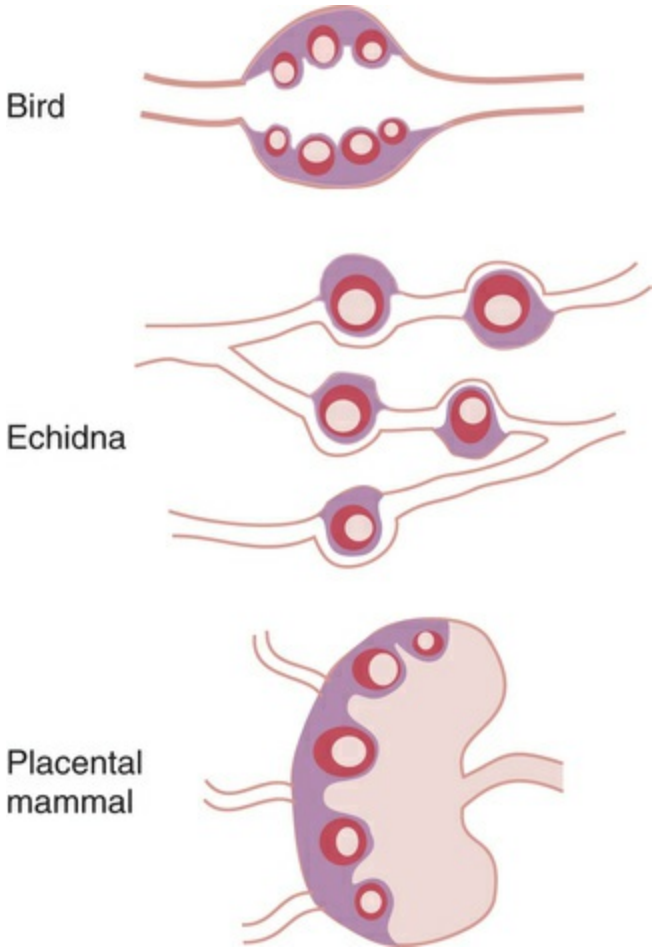


FIG. 43.11 The structure of lymph nodes in birds, echidna, and placental mammals.

The bursa of Fabricius was described in Chapter 12. Bursectomy results in a loss of antibody production, although bursectomized birds can still reject skin allografts. These results have been interpreted to suggest that the bursa is a primary lymphoid organ whose function is to serve as a maturation and differentiation site for the cells of the antibody-forming system. The bursa, however, contains some T cells; it can trap antigens and undertake some antibody synthesis. Birds also have large numbers of lymphocytes in the cecal tonsils and in the skin.

Bird lymphocytes originate in the yoke sac and migrate either to the bursa or the thymus. Immature lymphocytes that enter the thymus mature under the influence of molecules produced by thymic epithelial cells, and cells with recognizable T cell markers emigrate from the thymus. T cells constitute between 60% and 70% of blood lymphocytes.

Chicken NK cells are $CD8^+$ lymphoid cells that are asialo-GM_1

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positive and share surface antigens with T cells. They are probably large granular lymphocytes. Some NK-like cells are $CD4^+$, CD3- and generate IFN-γ. NK activity is found in the blood, thymus, bursa, spleen, and intestinal epithelium. They use chicken Ig-like receptors (CHIR), a highly diverse receptor family with over 100 genes that consists of activating, inhibiting, and bifunctional receptors. They show homology to mammalian KIR and LILR receptors (Chapter 19). Chicken NK cells can attack human cancer cells, lymphoid leukosis, leukemia virus, and Marek's disease virus-infected cells. Chickens possess conventional dendritic cells in their spleen that resemble the cells found in mammals.

Immunoglobulin Classes

The four principal immunoglobulin classes are found in birds: IgY, IgM, IgD, and IgA. Immunoglobulin D is widely distributed in birds. Thus δ genes are found in species such as eagles, shorebirds, flamingos, storks, pelicans, emu, and ostrich. They are absent from chickens, ducks, and doves.

Immunoglobulin Y

The principal immunoglobulin in chicken serum is IgY. Like the immunoglobulins of mammals, IgY consists of two heavy and two light chains (Fig. 43.12). The heavy chains, with upsilon (υ) chains, usually consist of one variable and four constant domains, and the complete molecule is about 180 kDa. However, some birds have a truncated isoform that has only two constant domains (it lacks the third and fourth constant domains). This isoform is about 120 kDa. Some birds such as ducks and geese have both full-sized and truncated IgY. Others, such as chickens, have only full-sized molecules.

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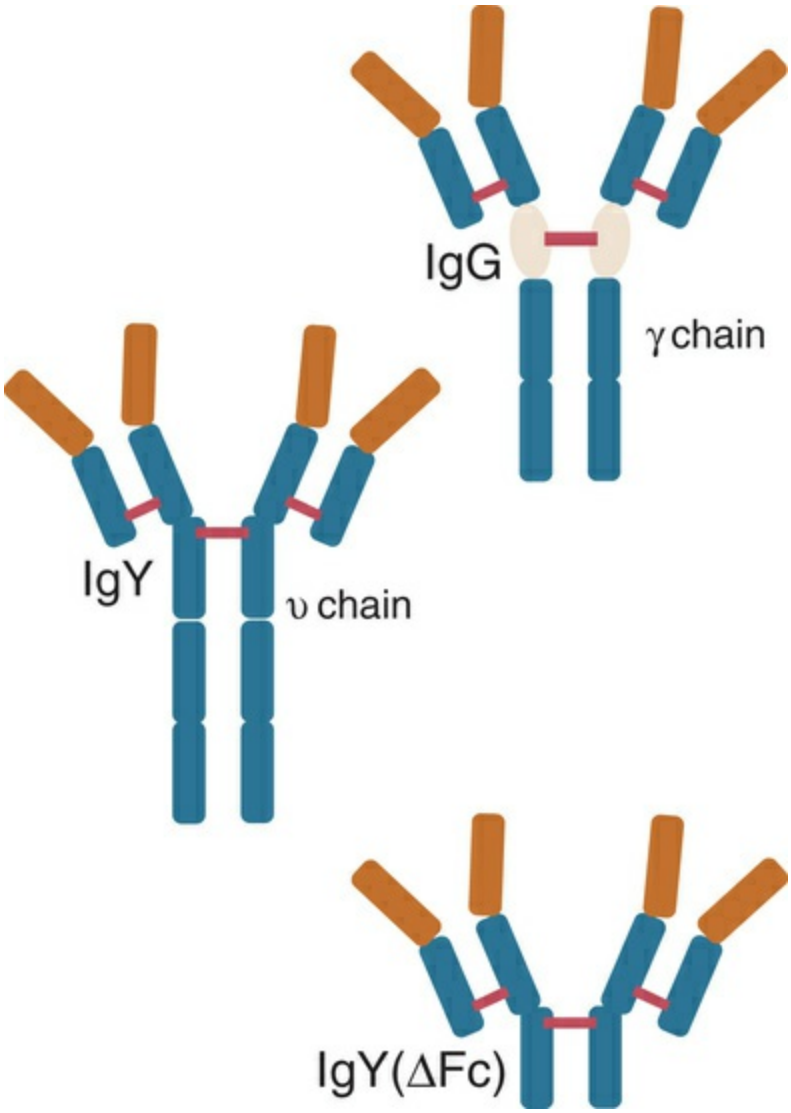


FIG. 43.12 The structure of IgY and IgY(ΔFc) compared with mammalian IgG.

The truncated isoform of IgY is produced as a result of alternative splicing of heavy chain mRNA. Its correct name is therefore IgY(ΔFc). Because it lacks an Fc region, it cannot activate complement nor bind to Fc receptors. Its function is unclear. There has, however, been a tendency during evolution to make low-molecular-weight immunoglobulins. Similar truncated immunoglobulins have also been described in some fish and turtles. Evidence from mallards *(Anas platyrhynchos)* suggests that the ratio of IgY(ΔFc) to intact IgY affects the efficiency of phagocytosis and determines whether immune complexes are phagocytosed in the spleen or liver.

Both isoforms of IgY lack a hinge. As a result, these molecules are somewhat inflexible and only cause precipitation or agglutination

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in the presence of high salt concentrations. They tend to show somewhat restricted diversity and limited affinity maturation. Studies clearly show that IgY is related to both IgG and IgE in mammals (see Fig. 43.9). In fact, it may have been an evolutionary precursor of these two classes.

It is of interest to note that chickens can develop anaphylaxis. The signs of acute anaphylaxis in chickens and other birds are similar to those in mammals, although it is likely mediated by IgY. They show increased salivation, defecation, ruffling of feathers, dyspnea, convulsions, cyanosis, collapse, and death. The major target organ is probably the lung, and death is due to pulmonary arterial hypotension, right-sided heart dilation, and cardiac arrest. The pharmacological agents involved include histamine, serotonin, the kinins, and leukotrienes.

Immunoglobulin M

The predominance of IgM in the avian primary immune response and of IgY in the secondary response is less marked than in mammals. A monomeric IgM can be detected in chicken eggs and in 1-day-old chicks. It is thought to be derived from oviduct secretions in the hen.

Immunoglobulin A

Chicken IgA is similar to IgA in mammals. The only significant difference is that chicken IgA has four heavy chain C domains, whereas mammalian IgA has only three. Chicken serum IgA exists in both dimeric (340 kDa) and monomeric (170 kDa) forms. Intestinal IgA is associated with secretory component.

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Generation of Antibody Diversity

Chickens generate antibody diversity in a manner unlike that seen in mammals. Chickens have only one functional *V* gene and one *J* gene for both light and heavy chains, although they do have 16 *D* genes. Although they have only one functional *V* gene, chickens have a large number of *V* pseudogenes that serve as sequence donors. They therefore diversify their *V* genes by gene conversion. During recombination of the *V* and *J* genes, single bases are also added to each gene (N region addition), and joining occurs at random. Chicken immunoglobulins are further diversified by somatic hypermutation and imprecise *V-J* joining.

A second major difference involves the timing of the process. In mammals, rearrangement of immunoglobulin genes occurs throughout life. Chickens, in contrast, rearrange their immunoglobulin genes as a single wave between 10 and 15 days of embryogenesis, at a period when there is clonal expansion of B cells in the bursa of Fabricius. During that 5-day period, birds generate all the antibody specificities they will need for the rest of their lives. After the bursa degenerates at puberty, the chicken must largely make do with the B cell diversity generated in early life. However, once a mature chicken B cell is stimulated by exposure to an antigen, it can generate additional V region diversity by further gene conversion. If gene conversion is blocked, somatic mutation can occur. Indeed, species that undertake gene conversion also show limited somatic mutation, although the reverse is not true. The chicken can generate about 10^6 different immunoglobulin molecules. This is approximately one order of magnitude less than the mouse.

Chicken T cells participate in delayed hypersensitivity reactions, graft-versus-host disease, and allograft rejection. Avian homologs of mammalian γ/δ TCR (TCR-1) and α/β TCR (TCR-2 and TCR-3) have been identified. TCR-2 and TCR-3 are subsets of α/β TCRs that use distinctly different Vβ gene segments. TCR-2 cells undergo *V-DJ* joining by gene deletion, whereas TCR-3 cells undergo *V-DJ* joining by chromosome inversion. Chickens possess both Th1 and Th2 cells. For example, chicken IL-18 stimulates IFN-γ release from

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$CD4^+$ T cells.

Chickens also possess a second TCRδ locus that differs from the conventional TCR α/β locus in that its *V* genes are more related to IgH *V* genes than to TCR *V* genes. In this it resembles the marsupial/monotreme TCRμ locus.

Birds reject skin allografts in about 7 to 14 days. Histological examination shows massive infiltration of the grafted tissue with lymphocytes. These cells are believed to be T cells since neonatal thymectomy results in a failure to reject grafts. If chicken T cells are dropped onto the chorioallantoic membrane of 13- to 14-day-old chick embryos, the cells will attack the chick tissues. This will result in pock formation on the membrane and splenic enlargement. The grafted cells attack the hematopoietic cells of the recipient. A few days after hatching, chicks become resistant to this form of graft-versus-host attack.

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Maternal Immunity

Newly hatched birds emerge from the sterile environment of the egg and, like mammals, require temporary immunological assistance. Serum immunoglobulins are actively transported from the hen's serum to the yolk while the egg is still in the ovary. (The yolk sac has an IgY receptor called FcRY that is distinctly different from mammalian Fc receptors.) IgY in the fluid phase of egg yolk is therefore found at levels equal to or greater than those in hen serum. In addition, as the fertilized ovum passes down the oviduct, IgM and IgA from oviduct secretions are acquired with the albumin (Fig. 43.13). As the chick embryo develops in ovo, it absorbs the yolk IgY, which then appears in its circulation. At the same time, the IgM and IgA from the albumin diffuse into the amniotic fluid and are swallowed by the embryo. Thus when a chick hatches, it possesses IgY in its serum and IgM and IgA in its intestine. The newly hatched chick does not absorb all its yolk sac antibodies until about 24 hours after hatching. These maternal antibodies effectively prevent successful vaccination until they disappear between 10 and 20 days after hatching. Newly hatched chicks begin to make their own IgA at day 3 in the bursa and day 7 in the gut and lung. Interestingly, maternal IgA persists for at least 7 days since it appears to be retained by the intestinal mucus.

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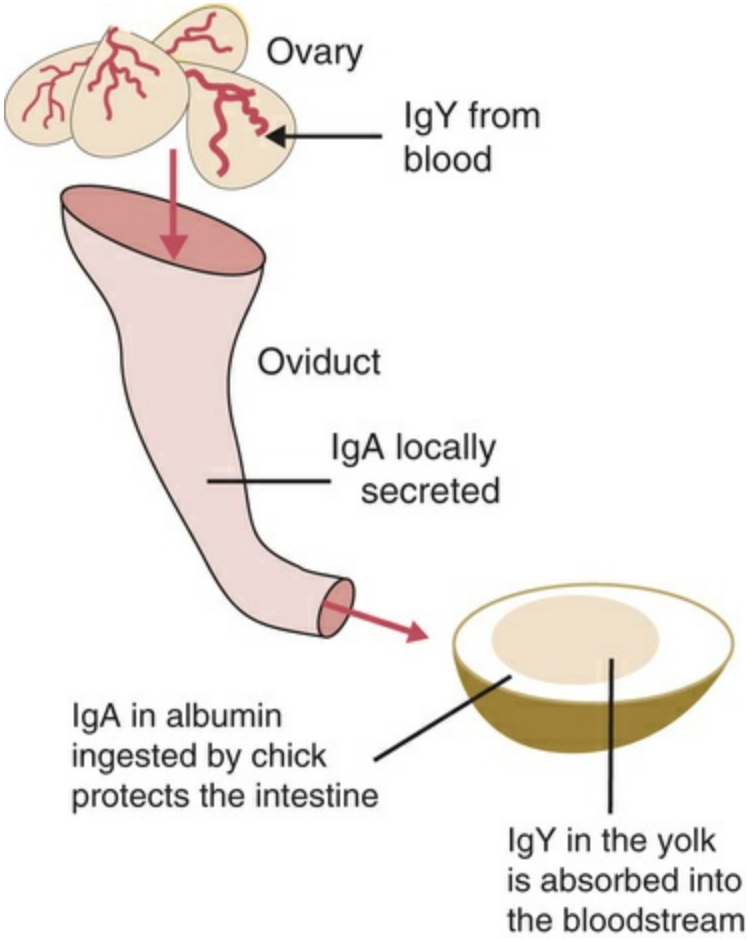


FIG. 43.13 Passive transfer of immunity from the hen to the chick.

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Immunity in Monotremes and Marsupials

The least evolved mammals, the egg-laying monotremes, such as the duck-billed platypus *(Ornithorhynchus anatinus)* and the echidna *(Tachyglossus aculeatus)*, branched from the other mammals about 166 Mya. They have a spleen, thymus, and gut-associated lymphoid tissues that are as well developed as those in marsupials and eutherian mammals. However, instead of typical mammalian lymph nodes, they have structures consisting of several lymphoid nodules, each containing a germinal center suspended by its blood vessels within the lumen of a lymphatic plexus. Thus each nodule is bathed in lymph. There is usually just one germinal center per nodule. Monotremes express the typical mammalian immunoglobulins. They have eight heavy chain isotypes, including two IgG isotypes, two divergent IgA isotypes, one IgD, IgE, IgM, and a unique isotype called IgO arranged in order -μ–δ–ο–γ2–γ1–α1–ε–α2-.

The *o* gene contains four C_H domains, and a hinge, and appears to be structurally intermediate between IgY and IgG. The δ gene contains 10 C_H domains and thus resembles the δ gene in reptiles, amphibians, and fish. All the major structural changes that gave rise to the immunoglobulin classes of modern mammals evolved before the separation of the monotremes from the marsupials and placental mammals and probably soon after the split from reptile lineages 300 Mya. Monotremes, like other mammals, produce predominantly IgM in the primary immune response and IgG in secondary immune responses.

Marsupials split from the placental mammals about 147 Mya. The complete sequencing of the genome of the marsupial opossum *Monodelphis domestica* has allowed its immune system genes (its immunome) to be examined in detail. Thus it contains genes for all the key immune gene families. There has been substantial duplication or gene conversion involving leukocyte receptors, NK complexes, immunoglobulins, type I interferons, and defensins. The opossum, like the platypus and wallaby, produces a TCR chain

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expressed early in development before conventional TCRs and which may provide protection during the first few days of life before their immune system is functional. This receptor chain is called TCRμ. (μ or M for marsupial.) Its C regions are related to TCRδ, while its V regions are related to immunoglobulin V regions. The V regions of TCRμ are constructed from *V, D,* and *J* genes, some of which have been recombined as in eutherian mammals while others are prejoined in the germline DNA.

Marsupials produce immunoglobulins in a manner similar to eutherian mammals. They possess four immunoglobulin isotypes: IgM, IgG, IgE, and IgA, but not IgD. The marsupial opossum *(Didelphis)* resembles more primitive vertebrates in that it responds well to particulate antigens, such as bacteria, but responds poorly to soluble antigens.

Poly-Ig receptors, FcR-like and the common FcR γ chain first appear in bony fish. Fc receptors for IgM first appear in monotremes and receptors for IgA are only present in placental mammals. Likewise, IgG and IgE receptors are found only in marsupials and placental mammals.

Mammals collectively possess a very large number of *VH* genes. When the sequences of their conserved framework regions are analyzed, they can be shown to cluster into three major "clans" (I, II, and III). Comparative studies have shown that these three clans have probably existed for more than 400 million years. Fish *VH* genes are most closely related to mammalian clan III, although they also possess two additional clans not found in mammals. The *VH* genes of birds (chickens), monotremes, marsupials, and some eutherians (rabbits and pigs) also belong to clan III. This has led to the suggestion that clan III is the most ancient of the mammalian clans. However, cattle and sheep also use only a single *VH* gene family, and this belongs to clan II. Humans and mice possess *VH* genes belonging to all three families.

Marsupials, like eutherians, develop within the uterus and use a placenta. Their pregnancy is short and their placenta is much less invasive. For much of their development their embryos are protected by a maternally secreted protein layer called the "shell coat." As a result, placental invasion of the maternal endometrium does not occur until late in pregnancy. As in eutherians, the

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marsupial placenta expresses nonclassical MHC class I antigens. Marsupials have relatively undeveloped young that migrate immediately to the mother's pouch where they complete the development process. Newborn marsupials do not have a functioning immune system but are exposed to a diverse array of microorganisms while developing in the pouch. Protection of the developing young comes from passive immunity through milk, prenatal immunoglobulin transfer, the presence of antimicrobial compounds secreted in the pouch, and repeated cleaning via maternal licking. Young marsupials also possess a diverse array of innate defenses, especially antimicrobial peptides.

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Mammalian Phylogeny

The mammals consist of three orders: the prototherians, composed of the monotremes, or egg-laying mammals such as the platypus and the echidna; the metatherians, composed of the marsupials or pouched mammals, such as the opossum and the kangaroos; and the eutherians, or placental mammals. The marsupials and eutherians are each other's closest relatives.

This book has focused on immunity in a small group of eutherian domestic mammals. These mammals have been selected not as representatives of mammalian diversity but for the behavioral traits that lend them to domestication or for the ease with which they are maintained in captivity. If we examine their place in mammalian phylogeny, we can see that most domestic animal species are relatively closely related (Fig. 43.14). Even domestic pets such as dogs and cats are closer to farm animal species than to primates. Likewise, laboratory animals tend to cluster in a separate group. It is unsurprising, therefore, that significant differences exist among the immune systems of species of interest to veterinarians. It is also clear that if we are to understand the significance of these differences and how they evolved, we must examine the immune systems of other, unrelated mammals. Even within the major domestic herbivores, their phylogeny demonstrates why there are significant differences between their immune systems (Fig. 43.15).

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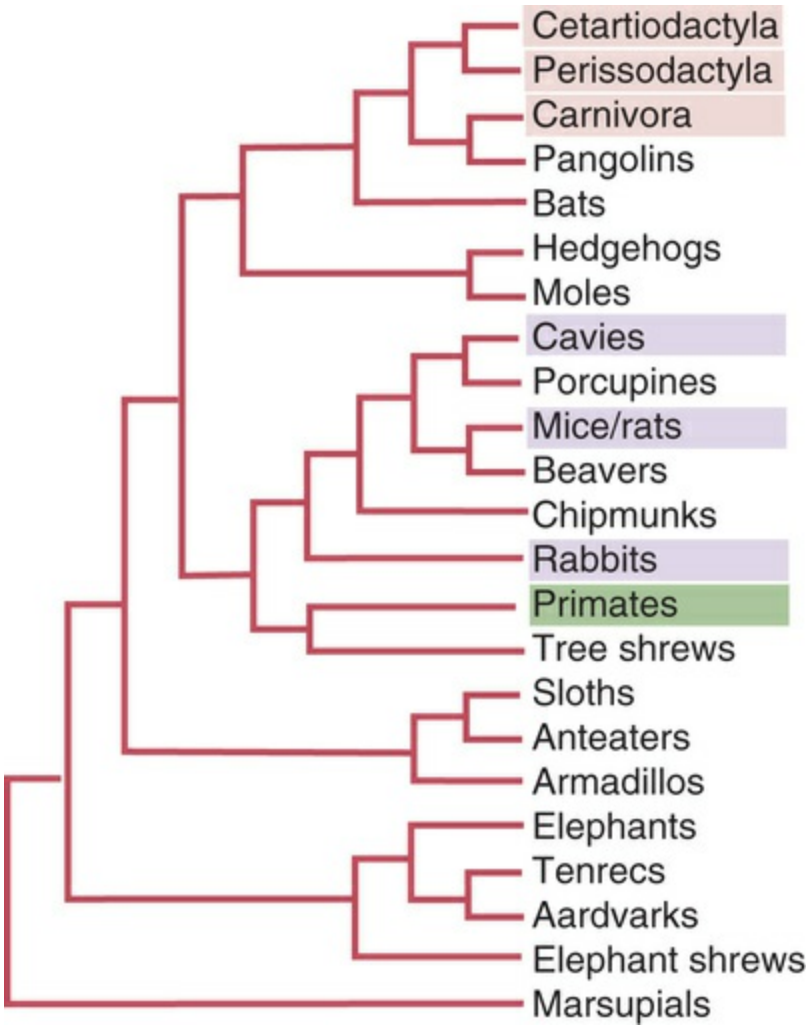


FIG. 43.14 The currently accepted phylogeny of mammals as based on analysis of gene sequences. Note that none of the domestic animal species can be considered representative of mammals as a whole.

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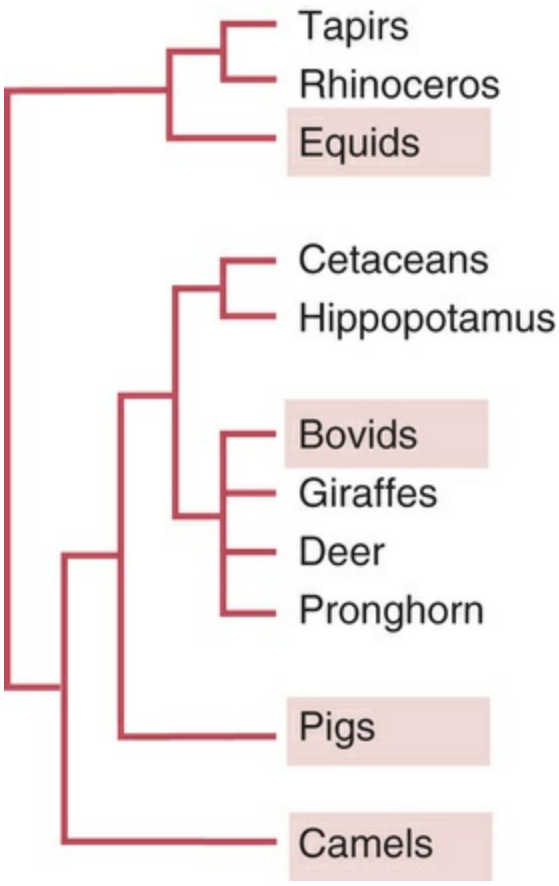


FIG. 43.15 The molecular phylogeny of the domestic herbivores. Many gaps remain in our knowledge of the immunology of these species.

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Fever

Innate and adaptive immune responses are enhanced at higher body temperatures (Chapter 7). Conversely, low temperatures in ectotherms may be significantly immunosuppressive. In chilled fish, the lag period following vaccination may be long, or there may be a complete absence of a detectable antibody response. Only certain phases of the antibody response are temperature dependent. For example, secondary immune responses can be elicited at low temperatures provided primary immunization is carried out at a high temperature. The cells that are sensitive to low temperature in fish are helper T cells, as a result of a loss of T cell membrane fluidity and reactivity to interleukins. Acclimatization to low temperatures can also occur. For example, goldfish that are acclimatized at a low temperature may be able to produce a similar number of antibody-forming cells to those that have remained at a warmer temperature. The nature of the antigen is also critical in that certain T cell–dependent mitogens are ineffective at low temperatures, again implying that the target cell is a helper T cell.

Although endotherms such as mammals develop a fever when infected, ectotherms are unable to change their body temperature by physiological mechanisms. As a result, they cannot develop a fever if maintained in a constant temperature environment. If, however, they are maintained in an environment with cool and warm areas, they cycle between these areas and maintain their body temperature within well-defined limits. For example, it has been observed that normal iguanas *(Dipsosaurus dorsalis)* maintain their temperature between 37° and 41° C. However, iguanas infected with the bacterium *Aeromonas hydrophila* modify their behavior so that they spend more time in the warm environment (Fig. 43.16). As a result, their body temperatures cycle between 40° and 43° C. Once the bacterial infection is cured, the iguanas resume their normal behavior. Thus the iguanas effectively induce a fever by their behavior. A similar behavioral fever is seen in goldfish and zebrafish kept in two interconnected tanks maintained at different temperatures. In response to microbial infection, the fish will choose to spend more time in the warmer water, effectively raising

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their body temperature. The benefits of this are obvious because, as pointed out earlier, their immune systems function more efficiently at higher temperatures. Many insects also respond to fungal or bacterial infections by developing a behavioral fever. That is, they raise their mean body temperature by spending more time in a warmer environment. It is interesting to note, however, that not all insect pathogens can stimulate such a response and that not all insects respond in the same way. For example, the bacterium *Serratia marcescens* can elicit a fever in the desert locust but not in the domestic cricket. Even plants do it! The temperature of the leaves of the bean plant increases by as much as 2° C in response to fungal infection.

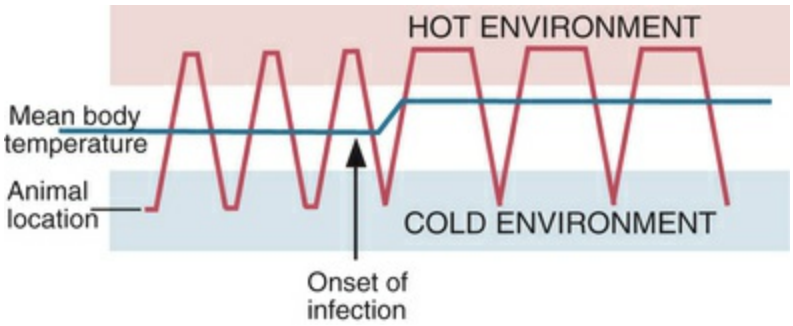


FIG. 43.16 A fever can be induced in ectotherms by modifications in behavior. Simply spending more time in a warm environment will effectively raise average body temperature. This behavioral response occurs in response to microbial infection.

Some mammals hibernate, most notably bears, bats, and some rodents. In the prior summer, their gut microbiota is highly efficient and thus promotes adiposity. As a result, bears are obese when they enter hibernation. During hibernation, periods of metabolic depression known as torpor are interspersed by transient activity called arousal. During torpor, the body temperature may fall to less than 10° C. This affects both the innate and adaptive immune systems. Torpor drastically reduces blood leukocyte numbers, complement levels, phagocytosis, TNF-α and IFN-γ production, T cell proliferation, and antibody synthesis. For example, if bats are cooled to about 8° C, they cease antibody production, but rewarming permits rapid resumption of antibody synthesis. This cessation of the antibody response in hibernating bats may allow

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them to act as persistent carriers of viruses such as rabies and increases their infection risk, as shown by the development of white-nose syndrome, a fungal infection in hibernating bats (see Box 7.2).

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APPENDIX 1

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Annotated List of Selected CD Molecules

Note: Of the 371 currently recognized cell surface CD molecules, many have no known function or do not play a significant role in immunity. Most are glycoproteins. This list summarizes the key features of only the most important.

CD1 A family of class Id MHC-like molecules that are antigen-presenting molecules for lipids and glycolipids. Found on thymocytes, macrophages, dendritic cells, NKT cells, and some B cells.

CD2 Also called LFA-2, this is a cell adherence molecule whose ligands are CD58 (non-rodents) and CD48 (rodents only). It is found on T cells and some B cells.

CD3 A collective designation for the signal transducing molecules of the TCR. They are found only on T cells.

CD4 A receptor for MHC class II molecules that plays a key role in the recognition of processed antigens by helper T cells. It is expressed on helper T cells, thymocytes, and monocytes.

CD5 A receptor for CD72. It is found on T cells and a subpopulation of B cells (B-1a cells) in most species, including mice and humans, but not on B cells in rats or dogs.

CD8 This dimeric glycoprotein is a receptor for MHC class I molecules that plays a key role in the recognition of endogenous antigen. It is expressed on cytotoxic T cells.

CD9 A glycoprotein expressed on platelets, immature B cells, eosinophils, basophils, and activated T cells.

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CD10 An endopeptidase expressed on T and B cell precursors.

CD11 Also called LFA-1, this is an integrin α chain found on leukocytes. Three forms are known, 11a, 11b, and 11c. They bind leukocytes to vascular endothelium.

CD14 This is the receptor for lipopolysaccharide-binding protein and therefore regulates the biological activities of this molecule. It is found on macrophages and granulocytes.

CD15 A complex carbohydrate called Lewis-X. Its sialylated form, sialyl Lewis^x, is expressed on NK cells. Its ligand is the selectin CD62. It is found on many cells, especially granulocytes.

CD16 Also called FcγRIII, this is a low-affinity receptor for IgG and for CD4. It is found on NK cells, granulocytes, and macrophages.

CD18 This is the integrin β1 chain found on all leukocytes. It associates with the various forms of CD11. A mutation in the *CD18* gene is responsible for leukocyte adherence deficiency in calves.

CD19 A protein that associates with CD21 and plays a key role in regulating the B cell response to antigen. It is expressed on B cells and their precursors but not on plasma cells. It is also expressed on dendritic cells.

CD21 A complement receptor also called CR2. Its several ligands include CD23 and C3d. It regulates B cell responses in association with CD19. CD21 is found on B cells, some T cells, and dendritic cells.

CD22 Also called siglec-2; a B cell inhibitory receptor and a receptor for soluble IgM.

CD23 A receptor for IgE, also called FcεRII. In its soluble form it regulates the production of IgE. It can also regulate B cell responses by binding to CD21. It is found mainly on mature B cells.

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CD25 The α chain of the IL-2 receptor. CD25 associates with the IL-2Rβ chain (CD122). It is expressed on activated T cells, B cells, and monocytes. CD25 expression is a feature of regulatory T cells.

CD27 A member of the tumor necrosis factor receptor family and the ligand of CD70. It is homologous to SWC2.

CD28 The ligand for both CD80 and CD86 that plays a key role in T cell co-stimulation. It is expressed on activated B cells and other antigen-presenting cells. It delivers a stimulatory signal to T cells as opposed to CD152, which delivers a suppressive signal.

CD29 A β_1 integrin expressed on leukocytes and platelets. In conjunction with its α chain (one of the forms of CD49), it binds these cells to extracellular matrix proteins.

CD31 Mediates adhesion between cells that express CD31 (e.g., leukocytes to endothelial cells) in a homophilic manner (CD31 binds CD31 on the apposing cell). It regulates the phagocytosis of dead and dying cells.

CD32 A medium-affinity IgG receptor also called FcγRII, different forms of which are expressed on macrophages, granulocytes, and B cells.

CD33 Siglec-3, a lectin expressed on immature hematopoietic stem cells.

CD34 Also called sialomucin, this glycoprotein is a ligand for certain integrins. It is expressed on endothelial cells and some dendritic cells.

CD35 This is the receptor for the complement components C3b and C4b, so it is also called CR1. It is expressed on granulocytes, monocytes, B cells, NK cells, and primate erythrocytes.

CD36 A pattern-recognition receptor that binds many different ligands, especially lipids. CD36 on intestinal γ/δ T cells binds bacterial lipoteichoic acids and helps trigger innate responses. Found on many different cell types.

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CD40 A member of the tumor necrosis factor receptor superfamily. Binding to its ligand CD40L (CD154) on activated helper T cells is essential for a successful antibody response and class switching. It is expressed on all antigen-presenting cells.

CD41 An integrin α chain expressed on platelets and macrophages. It associates with its β chain (CD61) and binds fibrinogen.

CD43 Also called sialophorin or leukosialin, this glycoprotein serves as an anti-adhesive molecule on leukocytes. It is expressed on T cells as well as granulocytes, macrophages, NK cells, platelets, and activated B cells.

CD44 A receptor for hyaluronic acid that mediates binding of cells to high endothelial venules. It is expressed in large amounts on T and B cells, monocytes and granulocytes, and many other cells.

CD45 A family of tyrosine phosphatases, some of which are required for signaling through the TCR. Multiple isoforms of CD45 are generated by alternative splicing of three exons. They are found on all cells of hematopoietic origin except red cells.

CD46 Also called membrane cofactor protein. CD46 is a receptor for C3b and C4b. Once bound, these complement components are destroyed by factor I. It is expressed on T cells, B cells, monocytes, granulocytes, NK cells, platelets, fibroblasts, endothelial cells, and epithelial cells, but not on red cells.

CD48 A GPI-linked glycoprotein that is a ligand for CD2 and CD247 in rodents. It is expressed on all blood lymphocytes.

CD49 A family of integrin α chains associated with the CD29 β chain. They are expressed in various forms on leukocytes, platelets, and epithelial cells. Their ligands are extracellular matrix proteins.

CD51 An integrin α chain found on platelets and endothelial cells. Its β chain is CD61, and its ligand is vitronectin.

CD52 The homolog of SWC1. It is believed to be an anti-adhesion

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protein,

CD54 Also called ICAM-1, this glycoprotein is the ligand for the CD11a/CD18 and CD11b/CD18 integrins. It is expressed on a wide variety of cells, most notably vascular endothelial cells.

CD55 Also called decay accelerating factor, this glycoprotein blocks the assembly of C3 convertase and accelerates its disassembly. It thus protects normal cells against attack by complement. It is broadly distributed on many cell types.

CD56 An adhesion molecule expressed on NK cells and nerve cells.

CD58 Also called LFA-3, this is a glycoprotein found on most cells where it is a ligand for CD2.

CD59 A glycoprotein also called protectin, it acts as an inhibitor of the terminal complement pathway by binding to C8 and C9 and blocking the assembly of the terminal complement complex. It is expressed on leukocytes, vascular endothelium, and epithelial cells.

CD61 A β_3-integrin that associates with CD41 to bind to extracellular matrix proteins. It is expressed on platelets and macrophages.

CD62 The selectins (S-lectins) bind to carbohydrate structures such as CD15s (sialyl Lewis^x) on neutrophils. CD62E is E-selectin, CD62L is L-selectin, and CD62P is P-selectin. They are expressed on platelets, lymphocytes, and endothelial cells.

CD64 Also called FcγRI, this high-affinity IgG receptor plays a key role in antibody-dependent cellular cytotoxicity. It is expressed on monocytes and interferon-γ–stimulated granulocytes.

CD66e Also called carcinoembryonic antigen, this glycoprotein is expressed in large quantities by malignant intestinal cells. Its detection is therefore diagnostic of intestinal malignancy in humans.

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CD71 A transferrin receptor expressed on activated leukocytes. It is required by dividing cells to import iron. It may also act as a selective IgA receptor.

CD72 Found on B cells (but not plasma cells), CD72 is a ligand for CD5. It may participate in an alternative pathway of B cell and T cell activation.

CD74 Also called the γ or invariant chain, this protein associates with intracellular MHC class II molecules. Found in all MHC class II–positive cells. It is believed to prevent the premature binding of endogenous peptides.

CD79 CD79a is an alternative name for the BCR signal-transducing peptide, Ig-α, and CD79b is another name for Ig-β.

CD80 Also called B7-1, this is high-affinity receptor for CD28 and CD152 (CTLA-4). The interaction of CD80 with its ligands is crucial to T cell communication with antigen-presenting cells.

CD81 Also called TAPA-1, CD81 is a widely expressed cell surface protein that regulates both B cell and T cell responses. On B cells it forms a complex with CD19 and CD21 and is involved with co-stimulation of T cells.

CD83 A member of the immunoglobulin superfamily used as a marker for mature dendritic cells. Its function is unknown.

CD85 A family of leukocyte Ig-like receptors (LIRs) that act as receptors for MHC class I molecules. They are expressed on macrophages, dendritic cells, and B cells.

CD86 Related to CD80 and also called B7-2, this co-stimulatory receptor is expressed on antigen-presenting macrophages, activated B cells, and dendritic cells. Its ligands are CD28 and CD152 (CTLA-4).

CD88 The C5a receptor found on granulocytes, macrophages, and mast cells.

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CD89 This IgA receptor (FcαRI) is expressed on granulocytes, monocytes, and some subpopulations of T and B cells.

CD90 Otherwise known as Thy-1, this glycoprotein is expressed on thymocytes and T cells in some species. It is also expressed on some brain cells.

CD91 The heat-shock protein receptor. This protein, expressed on macrophages and dendritic cells, is important in the intracellular processing of these molecules.

CD93 The C1q receptor. Found on monocytes and neutrophils, but not lymphocytes. It modulates phagocytosis of apoptotic cells.

CD94 An NK cell receptor that associates with NKG2D and binds target cell MHC class I molecules.

CD95 Otherwise known as Fas, this is a receptor for CD95-ligand (FasL or CD178) and a signaling component of an important cell death pathway. It is found on myeloid and T cells and plays a key role in the negative selection of self-reactive T cells.

CD102 Also called ICAM-2, a glycoprotein expressed on vascular endothelial cells, resting lymphocytes, and monocytes, but not neutrophils. It is the ligand for the integrin CD11a/CD18.

CD105 The TGF-β receptor expressed on endothelial cells.

CD106 Also called VCAM-1, a glycoprotein expressed on endothelial cells. It is the ligand for CD49d/CD29 (VLA-4).

CD115 The M-CSF receptor expressed on macrophages and their precursors.

CD116 The α chain of the GM-CSF receptor is found on granulocytes, monocytes, and eosinophils. It shares a common β chain with IL-3R and IL-5R.

CD117 Also called c-kit, this is the receptor for stem cell factor. It is an immunoglobulin superfamily tyrosine kinase found on

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hematopoietic stem cells.

CD119 The IFN-γ receptor is found on B cells, macrophages and monocytes, fibroblasts, and endothelial cells.

CD120 There are two TNF receptors (TNFR-I [CD120a], and TNFR-II [CD120b]). TNFR-I is found at higher levels on epithelial cells, whereas TNFR-II is more highly expressed on myeloid cells.

CD121 These are the two IL-1 receptors, IL-1RI and IL-1RII, expressed on thymocytes, fibroblasts, keratinocytes, endothelial cells (type I), and macrophages and B cells (type II).

CD122 The IL-2 receptor β chain expressed on T cells, activated B cells, NK cells, and monocytes.

CD123 The IL-3 receptor α chain.

CD124 The IL-4 receptor expressed on T and B cells, fibroblasts, endothelial cells, and stem cells.

CD125 The IL-5 receptor α chain.

CD126 The α chain of the IL-6 receptor expressed on B cells, plasma cells, epithelial cells, and hepatocytes.

CD127 The IL-7 receptor expressed on stem cells, T cells, and monocytes.

CD128 The IL-8 receptors expressed on leukocytes and keratinocytes. Also called CXCR1 and -2.

CD130 The β chain of the IL-6 (with CD126) and IL-11 receptors is found mainly on B cells but expressed at lower levels on most leukocytes, epithelial cells, hepatocytes, and fibroblasts.

CD131 The common β chain of the IL-3 (with CD123), IL-5 (with CD125), and GM-CSF (with CD123) receptors.

CD132 The common γ chain of IL-2 (with CD25 and CD122), IL-4

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(with CD124), IL-7 (with CD127), IL-9 (with CD129), and IL-15 receptors.

CD134 A member of the TNF receptor family that serves as the cell-binding receptor of feline immunodeficiency virus.

CD140 Platelet-derived growth factor (PDGF) receptor.

CD150 Also called SLAM (signaling lymphocyte activation molecule). The receptor for canine distemper virus.

CD152 Also known as CTLA-4, this is the ligand for CD80 and CD86 and a suppressor of T cell activation. It is expressed on antigen-presenting cells.

CD154 A member of the TNF family. Since it serves as the ligand for CD40, it is also called CD40L. Found on activated Th cells, it plays a key role in T cell activation by cross-linking with CD40 on antigen-presenting cells.

CD158 The KIR family of MHC class I receptors expressed on NK cells. They play a key role in NK cell activation in primates and cattle.

CD159 Members of the NKG2 family that serve as inhibitory receptors on NK cells.

CD166 A leukocyte adhesion molecule that acts as a receptor for IL-6.

CD169 Siglec-1 or sialoadhesin, a macrophage lectin-like adhesion molecule.

CD172a A signal regulatory protein expressed on monocytes and a subset of dendritic cells.

CD178 Also called CD95-L (Fas-ligand). A member of the tumor necrosis factor superfamily, this is a key molecule in the induction of cell death by apoptosis.

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CD181-CD185 CXCR1-CXCR5 chemokine receptors. CD184 is a coreceptor for feline immunodeficiency virus.

CD191-CD199 CCR1-CCR9 chemokine receptors.

CD206 The mannose-binding receptor found on mature macrophages and immature dendritic cells.

CD209 DC-SIGN found on a subset of dendritic cells. This molecule permits transient binding between T cells and dendritic cells. It is a C-type lectin whose ligand is ICAM-3 (CD50).

CD210 The IL-10 receptor α chain.

CD212 The IL-12 receptor β chain.

CD213 The IL-13 receptor α chain (and a member of the IL-4 receptor complex).

CD215 The IL-15 receptor α chain.

CD217 The IL-17 receptor.

CD218a and b The α and β chains of the IL-18 receptor.

CD230 The prion protein (PrP). A large membrane protein found on neurons. The abnormal form of this protein PrPsc is a transmissible agent causing spongiform encephalopathies.

CD233 An erythrocyte membrane protein that functions as an anion (chloride and bicarbonate) exchanger. Also called band 3 protein. It plays a major role in the removal of aged cells.

CD240 The Rhesus blood group molecules found in humans and some primates.

CD247 The T cell antigen receptor zeta (ζ) chain.

CD256 APRIL, a B cell stimulating cytokine from macrophages and dendritic cells.

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CD257 BAFF, a B cell stimulating cytokine expressed by B cells, macrophages, and dendritic cells. It is active in a cell-bound form or as a soluble fragment.

CD281-CD290 The CD designations of the toll-like receptors, TLR1 through TLR10.

CD295 The leptin receptor.

CD314 NKG2D, the NK cell receptor for the cellular stress proteins MIC-A and MIC-B.

CD327-CD329 Siglec-6, siglec-7, and siglec-9.

CD335 Also called NKp46. A member of the KIR family and an important molecule expressed on NK cells.

CD360 The IL-21 receptor.

CD369 Dectin-1. A C-type lectin important in antifungal immunity.

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APPENDIX 2

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Some Selected Cytokines

Adiponectin A glycoprotein secreted exclusively by adipocytes. It has antiinflammatory activities since it inhibits macrophage development and the production of TNF-α. Adiponectin regulates both glucose and lipid metabolism (Chapter 40).

APRIL (A proliferation-inducing ligand) A member of the TNF superfamily produced by monocytes, dendritic cells, T cells, and enterocytes. It stimulates the proliferation of B cells and inhibits B cell apoptosis (Chapter 15).

BAFF (B cell activating factor) A member of the TNF superfamily produced by T cells, macrophages, dendritic cells, and neutrophils. It is expressed on the surfaces of producing cells but can be cleaved off to act as a soluble cytokine. Like APRIL, it is an essential survival factor for B cells (Chapter 15).

Eotaxins A family of CC chemokines that selectively attract eosinophils and mobilize these cells from the bone marrow (Chapter 29).

Granulocyte colony-stimulating factor (G-CSF) A growth factor produced by macrophages, endothelial cells, and fibroblasts. It regulates the maturation of granulocyte progenitors into mature neutrophils. The term *colony-stimulating factor* refers to its ability to promote the growth of bone marrow stem cell "colonies" in tissue culture (Chapter 5).

Granulocyte-macrophage colony-stimulating factor (GM-CSF) Produced by T cells, macrophages, fibroblasts, and endothelial cells. It is the major regulator of granulocyte and macrophage stem cells. It induces M1 differentiation, phagocytosis, superoxide production, and ADCC by neutrophils (Chapter 18).

High mobility group box-1 (HMGB1) A chromatin-binding protein

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that is either actively secreted by inflammatory cells such as macrophages, or escapes from necrotic cells. HMGB-1 acts through TLRs to promote macrophage cytokine release, thus enhancing inflammation. It has bactericidal activity and is a potent inducer of fever (Chapter 7).

Interferon-α A protein family of at least 23 different isoforms. They are produced in large quantities by plasmacytic dendritic cells and in much smaller amounts by lymphocytes, monocytes, and macrophages. They activate NK cells and stimulate the differentiation of monocytes into dendritic cells, as well as the maturation and activity of dendritic cells. IFN-α also drives certain γ/δ T cell responses. They have, of course, potent antiviral activities (Chapter 27).

Interferon-β Produced by most nucleated cells and coded for by a single gene in most mammals. Produced in response to viral infections, it has similar properties to IFN-α (Chapter 27).

Interferon-γ The only type II interferon, a glycoprotein mainly produced by CD4+ Th1 cells, by some CD8+ T cells, and by NK cells. IFN-γ acts on B cells, T cells, NK cells, and macrophages and is the key mediator of cell-mediated immune responses (Chapter 14).

Interferon-λ A collective name for IL-28A, IL-28B, and IL-29. All are distantly related to IL-10. They use a distinct receptor system to trigger antiviral defenses (Chapter 27).

Interleukin-1 A family of proinflammatory cytokines produced by macrophages, dendritic cells, T cells, B cells, NK cells, vascular endothelium, fibroblasts, and keratinocytes. The two most important forms of IL-1 (α and β) act on Th2 cells, B cells, NK cells, neutrophils, eosinophils, dendritic cells, fibroblasts, endothelial cells, and hepatocytes (Chapter 3).

Interleukin-2 An immunoregulatory glycoprotein produced by Th1 and NK cells. It targets T cells, B cells, and NK cells. IL-2 activates helper and cytotoxic T cells and NK cells. IL-2 stimulates T cell

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proliferation and cytotoxicity (Chapter 14).

Interleukin-3 Produced by activated T cells, NK cells, eosinophils, and mast cells. It stimulates the growth and maturation of bone marrow stem cells for eosinophils, neutrophils, and monocytes (Chapter 29).

Interleukin-4 Produced by activated Th2 cells, mast cells, and activated basophils. It regulates the activities of B cells, T cells, macrophages, endothelial cells, fibroblasts, and mast cells. IL-4 stimulates the growth and differentiation of B cells and promotes type 2 immune responses (Chapter 14).

Interleukin-5 A growth factor produced by activated Th2 cells, mast cells, and eosinophils. In humans, it promotes eosinophil production (Chapter 29).

Interleukin-6 Produced by activated macrophages, T and B cells, mast cells, vascular endothelial cells, fibroblasts, keratinocytes, and mesangial cells. It acts on T cells, B cells, hepatocytes, and bone marrow stromal cells, as well as the brain, where it induces a fever (Chapter 7).

Interleukin-7 A growth factor produced by bone marrow and thymic stromal cells. It generates lymphoid stem cells. Its major role, however, is to control lymphocyte function by regulating *V(D)J* recombination in both B and T cells.

Interleukin-8 A proinflammatory chemokine (CXCL8). Like other chemokines, it is a relatively small (8.4 kDa) protein produced by macrophages and endothelial cells. IL-8 attracts and activates neutrophils (Chapter 3).

Interleukin-9 A growth factor produced by Th2 cells activated by IL-2. It promotes the growth of helper T cells and mast cells. It also potentiates the effects of IL-4 on IgE production.

Interleukin-10 An immunosuppressive and antiinflammatory cytokine. It suppresses T cell, NK cell, and macrophage activities. IL-10 is mainly produced by T cells, especially Treg cells but also

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by M2 macrophages, NK cells, and some dendritic cells. Its targets are Th1 cells, B cells, macrophages, NK cells, and mast cells (Chapter 20).

Interleukin-11 A growth factor produced by bone marrow stromal cells, epithelial cells, and fibroblasts. It stimulates B cell growth in association with IL-6.

Interleukin-12 An immunoregulatory cytokine consisting of two disulfide-linked subunits (p35 and p40). It is produced by monocytes and macrophages, dendritic cells, B cells, and keratinocytes. IL-12 acts on Th1 cells and NK cells to stimulate IFN-γ production as well as promoting cDC2 cell activities (Chapter 14).

Interleukin-13 An immunoregulatory cytokine produced by activated Th2 cells, cytotoxic T cells, NK cells, mast cells, and dendritic cells. It has biological activities similar to those of IL-4 because it acts through a receptor (CD213) that shares a common α chain with the IL-4R (Chapter 14).

Interleukin-14 Produced by T cells. It is a B cell growth factor that inhibits immunoglobulin secretion and selectively expands some B cell subpopulations.

Interleukin-15 Produced by activated macrophages, dendritic cells, endothelial cells, and fibroblasts. It shares many biological activities with IL-2. IL-15 acts as a T cell, B cell, and NK cell growth factor. IL-15 is essential for the prolonged survival of memory T cells (Chapter 14).

Interleukin-16 Produced by CD8+ T cells, eosinophils, dendritic cells, and mast cells. Its receptor is CD4 through which IL-16 regulates CD4+ T cell recruitment and activation.

Interleukin-17 A family of at least six proteins (IL17A-F) produced by Th17 helper cells and other innate immune cells. IL-17 stimulates macrophages and endothelial cells to secrete proinflammatory cytokines and chemokines leading to the

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recruitment and activation of neutrophils. Members of the IL-17 family play a key role in the development of acute inflammation, in autoimmune diseases, and in cancer (Chapter 20).

Interleukin-18 An IL-1 family member produced by macrophages and monocytes. IL-18 activates Th1 cells to promote the production of IFN-γ, TNF-α, IL-1, CD95L, and several chemokines. This can lead to positive feedback where the IL-18 and IFN-γ reinforce each other's activities (Chapter 3).

Interleukin-19 A member of the IL-10 family produced by B cells and activated monocytes. It is a proinflammatory cytokine that acts on monocytes to stimulate production of IL-1, IL-6, and TNF-α.

Interleukin-20 A regulatory cytokine and a member of the IL-10 family. It is produced by monocytes and keratinocytes and acts as a hematopoietic growth factor.

Interleukin-21 Produced by activated Th2 cells and is structurally related to IL-2 and IL-15. It promotes differentiation of B cells into plasma cells and memory B cells. It regulates NK, B, and T cell function (Chapter 15).

Interleukin-22 A member of the IL-10 family produced by activated Th17 cells, NK cells, and mast cells. It suppresses IL-4 production by Th2 cells and induces acute-phase protein production in the liver. It acts on B cells to promote lymphoid tissue development (Chapter 20).

Interleukin-23 IL-23 consists of a p40 subunit paired with an IL-12p19 subunit. It is produced by activated macrophages, dendritic cells, and γ/δ T cells. It stimulates Th17 cells to produce IL-17 and IL-22. These Th17 cells promote acute neutrophil-mediated inflammation (Chapter 20).

Interleukin-24 A member of the IL-10 family produced by activated monocytes and Th2 cells. It stimulates apoptosis in many tumor cell lines and stimulates acute-phase responses in hepatocytes.

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Interleukin-25 A member of the IL-17 family produced by Th2 cells and mast cells and is also called IL-17E. It plays an important role in intestinal immunity where it promotes Th2 and Tuft cell cytokine responses and resistance to helminths (Chapter 28).

Interleukin-26 A member of the IL-10 family produced by activated T cells (especially Th17 cells), memory cells, and NK cells. It induces the proliferation of keratinocytes and T cells. It has direct antimicrobial activity against many Gram-negative bacteria.

Interleukin-27 A heterodimer with one chain (p28) also called IL-30 and the other related to IL-12p40. IL-27 is expressed by activated monocytes and dendritic cells. IL-27 suppresses the activation of all three helper T cell subsets and prevents neutrophil activation. It induces T cell production of IL-10. It thus serves a major antiinflammatory role (Chapter 20).

Interleukin-28 Two antiviral proteins produced by virus-infected cells. They are also called IFN-λ2 (IL-28A) and IFN-λ3 (IL-28B). They share a common three-dimensional structure with IL-10 but have limited sequence similarity (Chapter 27).

Interleukin-29 (IFN-λ1) A member of the IL-10 family produced by virus-infected cells. It is closely related to IL-28A and IL-28B. It has antiviral activity.

Interleukin-30 Produced by antigen-presenting cells. It forms the p28 subunit of heterodimeric IL-27. IL-30 acts on naïve CD4 T cells and synergizes strongly with IL-12 to promote IFN-γ production by Th1 cells.

Interleukin-31 Produced by activated Th2 cells and is related to IL-6. Its receptor is expressed on keratinocytes and induced on monocytes by IFN-γ. It is involved in the pathogenesis of allergic skin diseases (Chapter 30). A monoclonal antibody against IL-31 prevents itch in dogs with atopic dermatitis.

Interleukin-32 Produced by activated human lymphocytes, NK cells, and endothelial cells. It induces monocytes to develop into

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macrophages and enhances macrophage production of the proinflammatory cytokines, TNF-α, IL-1β, IL-6, and IL-8. It has significant antiviral activity.

Interleukin-33 A proinflammatory member of the IL-1 family that is found within the cell nucleus. It is secreted by epithelial and endothelial cells as well as released by injured cells. (It thus functions as an alarmin.) Its targets include Th2 cells, ILC2 cells, basophils, and mast cells. It drives production of IL-4, IL-5, and IL-13 by Th2 cells, ILC2 cells, and mast cells. IL-33 plays a major role in antihelminth immunity (Chapter 28).

Interleukin-34 Expressed in many different tissues: spleen, liver, heart, brain, lung, etc. It is a growth factor that acts through the macrophage colony-stimulating factor receptor to promote monocyte maturation and macrophage colony formation in the bone marrow.

Interleukin-35 Produced by B cells. It is a heterodimer consisting of IL-12p35 and a peptide related to IL-12p40. It stimulates the growth of Treg cells while suppressing Th17 cells. It thus has antiinflammatory effects and regulates immune responses in infectious and autoimmune diseases.

Interleukin-36 A family of three IL-1–like cytokines (IL-36α, -36β, and -36γ) that signal through a common receptor. They are highly expressed in epithelial tissues and macrophages in response to bacteria or bacterial products. They act on keratinocytes and immune cells to trigger inflammation. It thus is implicated in skin and pulmonary diseases.

Interleukin-37 An antiinflammatory member of the IL-1 family produced by monocytes and dendritic cells. It acts by suppressing the production of proinflammatory cytokines such as IL-17 by inhibiting NF-κB and some protein kinases such as mTOR.

Interleukin-38 Also a member of the IL-1 family. It binds to the IL-36 receptor and acts as a receptor antagonist.

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Interleukin-39 A member of the IL-12 family. It is a heterodimeric protein that mediates inflammation in lupus-like mice.

Leptin A protein produced by adipocytes that suppresses appetite by signaling through a receptor in the hypothalamus. It exerts a strong proinflammatory effect and promotes dendritic cell activation and Th1 responses (Chapter 40).

Macrophage colony-stimulating factor (M-CSF) A growth factor produced by lymphocytes, macrophages, fibroblasts, epithelial cells, and endothelial cells. These act on monocyte stem cells to induce their proliferation and promote their differentiation into M2 cells.

Macrophage migration inhibitory factor (MIF) A protein produced by macrophages and T cells. It acts on macrophages to prevent their random migration, hence its name. It also activates lymphocytes. MIF promotes the production of the proinflammatory mediators TNF-α and IFN-γ.

Thymic stromal lymphopoietin (TSLP) A member of the IL-2 family that activates antigen-presenting cells, resulting in the release of monocyte chemoattractants. It is produced by fibroblasts, enterocytes, epithelial cells, mast cells, keratinocytes, and Th2 cells. It promotes mast cell production of Th2 cytokines. It is also a potent pruritogen causing severe itching (Chapter 30) and acts as a B cell growth factor (Chapter 21).

Transforming growth factor-β (TGF-β) A family of five signaling proteins. They are produced by platelets, activated macrophages, neutrophils, B cells, and T cells and act on most cell types, including T and B cells, dendritic cells, macrophages, neutrophils, and fibroblasts. The TGF-βs inhibit T and B cell proliferation and macrophage function, and are immunosuppressive (Chapter 20).

Tumor necrosis factor-α (TNF-α) A proinflammatory cytokine produced by macrophages, mast cells, T cells, endothelial cells, B cells, adipocytes, and fibroblasts. It is toxic to many tumor cells. TNF-α is the central inducer of inflammation (Chapter 3).

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Tumor necrosis factor-β (TNF-β) Produced by activated T and B cells and has similar properties to TNF-α. It is either secreted in a soluble form or forms a heterodimer with lymphotoxin-β in the T cell membrane. TNF-β kills tumor cells and activates neutrophils, macrophages, endothelial cells, and B cells (Chapter 18).

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APPENDIX 3

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Some Important Abbreviations

Immunologists and cell biologists are incorrigible users of acronyms. While convenient abbreviations, they can readily overwhelm students and newcomers to the subject. Here are some that are very widely employed.

ADCC antibody-dependent cell-mediated cytotoxicity

AhR aryl hydrocarbon receptor

AIDS acquired immune deficiency syndrome

AITP autoimmune thrombocytopenia

ANA antinuclear antibody

APC antigen-presenting cell

APRIL a proliferation-inducing ligand

BAFF B cell activating factor

BALT bronchus-associated lymphoid tissue

BCG bacillus Calmette-Guérin *(Mycobacterium bovis)*

BCR B cell (antigen) receptor

BLAD bovine leukocyte adherence deficiency

BLV bovine leukemia virus

BoLA bovine leukocyte antigen

C complement

CAM cell adhesion molecule

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CBH cutaneous basophil hypersensitivity

CD cluster of differentiation

cDC classical dendritic cell

CDw cluster of differentiation (provisional designation)

CDR complementarity determining region

CFT complement fixation test

CID combined immunodeficiency

CLL chronic lymphoid leukemia

cM centimorgans, a unit of genetic distance

Con A concanavalin A

CR complement receptor

CRP C-reactive protein

CSF colony-stimulating factor (or cerebrospinal fluid)

DAF decay accelerating factor

DAG diacylglycerol

DAMP damage-associated molecular pattern

DC dendritic cell

dsRNA double-stranded RNA

DTH delayed-type hypersensitivity

EAE experimental allergic encephalitis

EAN experimental allergic neuritis

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ELISA enzyme-linked immunosorbent assay

EPO eosinophil peroxidase

Fab antigen-binding fragment

Fc crystallizable fragment (of immunoglobulin)

FcR Fc receptor

FeLV feline leukemia virus

FoxP3 forkhead box P3

FPT failure of passive transfer

FITC fluorescein isothiocyanate

FIV feline immunodeficiency virus

GALT gut-associated lymphoid tissue

GM-CSF granulocyte-macrophage colony-stimulating factor

GPI glycosyl-phosphatidylinositol

GVH graft-versus-host (disease)

HAT hypoxanthine aminopterin thymidine (medium)

HDN hemolytic disease of the newborn

HEV high endothelial venule

HI hemagglutination inhibition

HIV human immunodeficiency virus

HLA human leukocyte antigen

HMGB1 high mobility group box protein-1

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HSP heat-shock protein

ICAM intercellular adhesion molecule

IDDM insulin-dependent diabetes mellitus

IDO indoleamine 2,3-dioxygenase

IEL intraepithelial lymphocytes

IFA indirect fluorescence assay

IFN interferon

Ig immunoglobulin

IK immunoconglutinin

IL interleukin

ILC innate lymphoid cell

IMHA immune-mediated hemolytic anemia

ISCOM immune-stimulating complex

ISG immune serum globulin

ITAM immunoreceptor tyrosine-based activation motif

IU international unit

IVIG intravenous (human) immunoglobulins

J joining

JAK janus tyrosine kinases

kb kilobases, a measure of gene size

kDa kilodalton

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KIR killer inhibitory receptor

LAD leukocyte adherence deficiency

LAK lymphokine-activated killer (cells)

LD_{50} lethal dose 50

LE lupus erythematosus

LFA leucocyte function-associated antigen

LGL large granular lymphocyte

lpr lymphoproliferation

LPS lipopolysaccharide

LT lymphotoxin (or leukotriene)

β_2M β_2-microglobulin

MAMP microbial-associated molecular pattern

MBL mannose-binding lectin

M-CSF macrophage colony-stimulating factor

MHC major histocompatibility complex

MIP macrophage inflammatory protein

MLD minimal lethal dose

MLR mixed lymphocyte reaction

MLV modified live virus

MPGN membranoproliferative glomerulonephritis

mTOR mechanistic target of rapamycin

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NF-κB nuclear factor-κB

NK natural killer (cell)

NKT natural killer T (cell)

NLR nucleotide-binding oligomerization domain, leucine-rich repeat (receptor)

NOS nitric oxide synthase

NOX NADPH oxidase

NS natural suppressor cell

PAF platelet-activating factor

PAMP pathogen-associated molecular pattern

PCA passive cutaneous anaphylaxis

pDC plasmacytoid dendritic cell

PF protective fraction

PFC plaque-forming cell

PG prostaglandin

PHA phytohemagglutinin

pIgR receptor for polymeric immunoglobulin

PKC protein kinase C

PPD purified protein derivative of tuberculin

PRR pattern-recognition receptor

PWM pokeweed mitogen

R receptor (e.g., IL-2R)

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RAST radioallergosorbent test

RF rheumatoid factor

RIA radioimmunoassay

RLR retinoic acid-inducible gene-like receptor

ROS reactive oxygen species

RNS reactive nitrogen species

S19 strain 19 *Brucella abortus* vaccine

SAA serum amyloid A (protein)

SAP serum amyloid P

SCID severe combined immunodeficiency

SID single intradermal test

SIRS systemic inflammatory response syndrome

SLA swine leukocyte antigen

SLE systemic lupus erythematosus

SMAC supramolecular activation cluster

ssRNA single-stranded RNA

STAT signal transducers and activators of transcription

TAP transporter for antigen processing

TCC terminal complement complex

$TCID_{50}$ tissue culture infective dose 50

TCR T cell (antigen) receptor

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TdT terminal deoxynucleotidyl transferase

TGF transforming growth factor

Th cell helper T cell

TIL tumor infiltrating lymphocytes

TLR toll-like receptor

TK thymidine kinase

TNF tumor necrosis factor

TSLP thymic stromal lymphopoietin

Treg regulatory T (cell)

WC workshop cluster

ZAP zeta-associated protein

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Glossary

A

Actinobacteria A major phylum of Gram-positive bacteria with a high G+C content. They include important pathogens such as the Mycobacteria, Corynebacteria, and Rhodococcus.

Activated macrophage A macrophage in a state of polarized metabolic and functional activity.

Active immunity Immunity produced as a result of administration of an antigen, thus triggering an immune response.

Acute inflammation Rapidly developing inflammation of recent onset. It is characterized by tissue infiltration by neutrophils.

Acute-phase proteins Proteins, synthesized by the liver and other tissues, whose level in serum rises rapidly in response to acute inflammation and tissue damage.

Adjuvant Any substance that, when given with an antigen, enhances the immune response to that antigen.

Adaptive immunity Immunity that recognizes foreign invaders and adapts to them. As a result it improves with experience.

Adoptive immunity Immunity that results from the transfer of cells from an immunized animal to an unimmunized recipient.

Affinity The strength of binding between two molecules such as an antigen and antibody. Usually expressed as an association constant (Ka).

Affinity maturation The progressive increase in antibody affinity

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for antigen that occurs during the course of an immune response as a result of somatic mutation in *V* genes.

Agammaglobulinemia The absence of γ-globulins in blood.

Agglutination The clumping of particulate antigens by antibodies.

Agnatha A class of jawless fish. It includes the cyclostomes, an order containing the hagfish and lamprey.

Alarmins Molecules released by dead or damaged tissues that trigger innate immune responses, especially inflammation. See also Damage-associated molecular patterns (DAMPs).

Albumin The blood protein largely responsible for maintaining plasma osmotic pressure.

Alleles Different forms of a gene that occupy the same polymorphic locus.

Allelic exclusion The expression of only one allelic protein by a cell from a heterozygous individual that has the genes to express both allelic proteins.

Allergens Antigens that provoke allergic reactions. Usually type I hypersensitivity.

Allergic contact dermatitis An inflammatory skin reaction mediated by Th1 cells responding to low-molecular-weight chemicals bound to skin cells.

Allergy A hypersensitivity reaction initiated by specific immunological mechanisms.

Allogeneic Genetically dissimilar animals of the same species.

Allograft An organ graft between two genetically dissimilar animals of the same species.

Allotype Phenotypic (antigenic and structural) differences between the proteins of different individuals of the same species as a result of transcription of different alleles.

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Alternative complement pathway The complement pathway triggered by the activation of C3 by the presence of an activating surface.

Amyloid An extracellular, amorphous, waxy protein deposited in the tissues of individuals with a chronic inflammation or a myeloma. It consists of misfolded insoluble fibrillar proteins.

Analog An organ or tissue that has the same function as another but is of different evolutionary origin.

Anamnestic response A secondary immune response.

Anaphylactoid response An inflammatory response that resembles anaphylaxis but is not mediated by IgE.

Anaphylatoxins Complement fragments that stimulate mast cell degranulation and smooth muscle contraction.

Anaphylaxis A severe, life-threatening generalized or systemic reaction due to an acute type I hypersensitivity response.

Anergy The failure of a sensitized animal to respond to an antigen —a form of immunological tolerance.

Antibiotic A chemical compound, usually obtained from microorganisms, that can prevent growth or kill bacteria. Do not confuse this with antibody.

Antibody An immunoglobulin molecule synthesized on exposure to antigen, which can combine specifically with that antigen.

Antibody-dependent cellular cytotoxicity (ADCC) The killing of antibody-coated target cells by cytotoxic cells with surface Fc receptors.

Antigen Any foreign substance that can bind to specific lymphocyte receptors and so induce an immune response.

Antigen-presenting cells Cells that can ingest, process, and present antigens to T cells. These cells express MHC class I or class II molecules on their surface. This forms a complex with antigenic

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peptides that can bind to TCRs.

Antigen processing The series of events that degrade protein antigens into short peptides that bind to MHC molecules so that they can be recognized by T cells with appropriate TCRs.

Antigen-sensitive cells Cells that can bind and respond to a specific antigen.

Antigenic determinant See Epitope.

Antigenic variation The progressive changes in surface antigens exhibited by viruses, parasites, and some bacteria in order to evade immune destruction.

Antigenicity The ability of a molecule to be recognized by an antibody or lymphocyte.

Antiglobulin Antibody made against an immunoglobulin, usually by injecting immunoglobulin into an animal of another species.

Antiglobulin test A technique for detecting the presence of nonagglutinating antibody on the surface of a particle.

Antiserum Serum that contains specific antibodies. Synonymous with immune globulin.

Antitoxin Antiserum directed against a toxin and used for passive immunization.

Anurans An order of advanced amphibians that includes the frogs and toads.

Apoptosis The controlled self-destruction of a cell; one form of programmed cell death. (*Apoptosis* is a Greek word describing the falling away of petals from flowers or leaves from trees.)

Arthus reaction Local inflammation due to a type III hypersensitivity reaction; it is induced by the injection of antigen into the skin of an immunized animal.

Asthma A hypersensitivity disease characterized by a reduction in

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airway diameter, leading to difficulty in breathing (dyspnea).

Atopy A genetic predisposition to become sensitized and produce IgE antibodies in response to allergens commonly occurring in the environment.

Attenuation The reduction of virulence of an infectious agent.

Autoantibodies Antibodies directed against epitopes on normal body tissues.

Autoantigen A normal body component that acts as an antigen.

Autograft A tissue or organ graft made between two sites within the same animal.

Autoimmune disease Disease caused by an immune attack against an individual's own tissues.

Autoimmunity An immune response against normal body components.

Autophagy A process of cellular self-digestion by which cells can ingest and destroy intracellular microbes or damaged organelles.

B

B lymphocytes (B cells) Lymphocytes that have undergone a period of processing in the bursa or its mammalian equivalent. They are responsible for antibody production.

Bacille Calmette-Guérin (BCG) vaccine An attenuated strain of *Mycobacterium bovis.* This may be used as a specific vaccine or as a nonspecific immune stimulator.

Bacterin A preparation of killed bacteria used for immunization.

Basophil A polymorphonuclear cell that contains granules with a high avidity for basic dyes such as hematoxylin. It participates in type I hypersensitivity reactions.

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BCG See Bacille Calmette-Guérin (BCG) vaccine.

Bence Jones protein Immunoglobulin light chains found in the urine of patients with myelomas. They precipitate out of solution when the urine is warmed and redissolve at higher temperatures.

Benign tumor A tumor that does not spread from its site of origin.

Blast cells Actively dividing cells with large amounts of cytoplasm.

Blastogenesis The stimulation of cell division.

Blocking antibody A noncytotoxic, non–complement-activating antibody that, by coating cells, can protect them against immune destruction.

Blood groups Antigens found on the surface of red blood cells. Their expression is inherited.

Bursectomy Surgical removal of the bursa of Fabricius.

C

C-terminus The end of a peptide chain with a free carboxyl (COO−) group.

C3 convertases Enzymes that can cleave native C3 into C3a and C3b fragments.

Capsid The protein coat around a virus.

Carcinoma A tumor originating from cells of epithelial origin.

Carrier An immunogenic macromolecule to which a hapten may be bound, making the hapten immunogenic.

Cascade reactions A linked series of enzyme reactions in which the products of one reaction catalyze a second reaction, and so forth.

Caspases Proteolytic enzymes that play key roles in inflammation and apoptosis.

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Cell-mediated cytotoxicity The killing of target cells induced by contact with cytotoxic T cells, NK cells, or macrophages.

Cell-mediated immunity A form of immune response mediated by T lymphocytes and macrophages; it can be conferred on an animal by adoptive transfer.

Cestodes Parasitic tapeworms.

Checkpoint therapy A cancer treatment that uses monoclonal antibodies to enhance T cell cytotoxicity and to destroy cancer cells.

Chemokine A family of proinflammatory and chemotactic cytokines with a characteristic sequence of four cysteine residues. They regulate the emigration of leukocytes from blood into tissues.

Chemotaxis The directed movement of cells under the influence of a chemical concentration gradient.

Chimera An animal that contains cells from two or more genetically different individuals.

Chondrichthyes The class that contains the cartilaginous fishes, including sharks, skates, and rays.

Chromosome translocation A form of mutation in which portions of two chromosomes switch positions.

Chronic inflammation Slowly developing or persistent inflammation characterized by tissue infiltration with macrophages and fibroblasts.

Class The five major forms of immunoglobulin molecules common to all members of a species (see Isotype). Each has its own set of heavy chain genes.

Class switch The change in immunoglobulin class that occurs during the course of an immune response as a result of heavy chain gene rearrangement.

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Classical complement pathway The complement pathway triggered by activation of C1 by antigen-antibody complexes.

Clonal deletion The elimination of self-reactive T cells in the thymus.

Clonal selection A key concept in immunology. The proliferation of specific lymphocyte clones in response to a specific epitope. The response is triggered through specific antigen-binding receptors.

Clone The progeny of a single cell.

Clonotype A clone of B cells with the ability to bind a single epitope.

Cluster of differentiation (CD) The set of monoclonal antibodies that recognize a single protein on a cell surface. A CD antigen is by extension, therefore, a defined protein on the surface of a cell.

Coelomocyte A phagocytic cell found in the coelomic cavity of invertebrates.

Collectins A family of carbohydrate-binding lectins that depend on calcium for their adhesion.

Colostrum The secretion that accumulates in the mammary gland in the last weeks of pregnancy. It is very rich in immunoglobulins.

Combined immunodeficiency A deficiency in both the T cell– and B cell–mediated components of the immune system.

Commensal Two species that live together and share resources.

Complement A group of serum and cell surface proteins activated by factors such as the combination of antigen and antibody and results in the generation of enzyme cascades that have a variety of biological consequences, including cell lysis and opsonization.

Complementarity determining region Those areas within the variable regions of antibodies and T cell antigen receptors that

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bind to antigens and determine the molecule's antigen-binding specificity. Synonymous with hypervariable region.

Concanavalin A (Con A) A lectin extracted from the jack bean that makes T cells divide.

Conglutinin A bovine mannose-binding protein that also combines with C3b.

Constant domains Structural domains with little sequence variability found in antibodies and TCRs.

Constant region The portion of immunoglobulin and TCR peptide chains that consists of a relatively constant sequence of amino acids.

Convertase A protease that acts on a protein to cause its activation.

Cortex The outer region of an organ such as the thymus or lymph node.

Corticosteroids Steroid hormones released from the adrenal cortex that have profound effects on the immune system. Some corticosteroids may be synthetic in origin.

Co-stimulators Molecules required to stimulate an antigen-sensitive cell simultaneously with antigen in order to initiate an effective immune response.

Cross-reaction The reaction of an antibody or an antigen receptor directed against one specific antigen, with a second antigen. This occurs because the two antigens possess an epitope in common.

Cutaneous basophil hypersensitivity A form of delayed hypersensitivity reaction in skin associated with an extensive basophil infiltration.

Cytokine storm The pathological effects induced by the massive activation of T cells and, as a result, the unregulated production of many different cytokines.

Cytokines Secreted proteins that mediate cellular interactions and

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regulate cell growth and secretion. As a result, they regulate many aspects of the immune system.

Cytolysis Destruction of cells by immune processes.

Cytotoxic lymphocyte A lymphocyte that can bind to target cells through its TCR and trigger its killing by apoptosis.

D

Damage-associated molecular patterns (DAMPs) Conserved molecular structures derived from damaged cells and tissues that trigger inflammation.

Defensins Antibacterial peptides that play a key role in innate immunity.

Delayed hypersensitivity A cell-mediated inflammatory reaction in the skin, so called because it takes 24 to 48 hours to reach maximum intensity.

Dendritic cells Specialized antigen-processing cells. They possess long cytoplasmic processes (dendrites), and their primary role is to function as antigen-trapping and antigen-presenting cells.

Desensitization The prevention of allergic reactions through the use of multiple injections of allergen.

Diapedesis The emigration of cells from intact blood vessels during inflammation.

Disseminated intravascular coagulation Activation of the clotting cascade within the circulation.

Disulfide bonds Bonds that form between two cysteine residues in a protein. They may be either interchain (between two peptide chains) or intrachain (joining two parts of one chain).

Domain Discrete structural units from which protein molecules are constructed. Their sizes, structures, and sequences are very diverse.

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Dysbiosis A disturbance or imbalance in the types and numbers of species in the normal microbiota.

Dysgammaglobulinemia The abnormal production of γ-globulins in blood.

E

Effector cell A cell that is able to "effect" an immune response. These cells include cytotoxic T cells and natural killer cells.

Eicosanoids A family of lipid-signaling molecules mainly synthesized from arachidonic acid. They include the prostaglandins and leukotrienes and are involved in inflammation, and allergic diseases.

Electrophoresis The separation of the proteins in a complex mixture by subjecting them to an electrical potential. They thus migrate in a substrate such as a gel or paper at a rate determined by their charge.

ELISA Enzyme-linked immunosorbent assay. A serological test that uses enzyme-linked antiglobulins and substrate bound to an inert surface.

Endocytosis The uptake of extracellular substances by cells.

Endogenous antigen Foreign antigen synthesized within body cells. Examples include newly formed virus proteins.

Endosomes Cytoplasmic vesicles formed by invagination of the outer cell membrane (endocytosis).

Endothelium The cells that line blood vessels and lymphatics.

Endotoxins Lipopolysaccharide components of Gram-negative bacterial cell walls.

Enhancement Improved survival of grafts or cancer cells mediated by some antibodies.

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Eosinophil A polymorphonuclear leukocyte containing characteristic granules that stain intensely with the dye eosin.

Eosinophilia Increased numbers of eosinophils in the blood.

Epithelioid cells Macrophages that accumulate around a tubercle and resemble epithelial cells in histological sections.

Epitope A site on the surface of an antigen that is recognized by an antigen receptor. As a result, immune responses are directed against specific epitopes. Synonymous with antigenic determinant.

Erythema Redness due to inflammation.

Eukaryotic organism An organism characterized by cells possessing a distinct nucleus and containing both DNA and RNA.

Eutherians The placental mammals; the order to which humans and domestic mammals belong.

Exocytosis The export of material from a cell by the fusion of cytoplasmic vesicles with the outer cell membrane.

Exogenous antigen A foreign antigen that originates at a source outside the body; for example, bacterial antigens.

Exon An expressed region within a gene.

Exotoxins Soluble protein toxins, usually produced by Gram-positive bacteria, that have a specific toxic effect.

F

Fab fragment The antigen-binding fragment of a partially digested antibody. It consists of a light chain and the N-terminal halves of a heavy chain.

Facultative intracellular organism An organism that can, if necessary, grow within cells.

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Fc receptor A cell surface receptor that specifically binds antibody molecules through their Fc region.

Fc region That part of an immunoglobulin molecule consisting of the C-terminal half of heavy chains. It is responsible for the biological activities of the molecule.

Firmicutes A bacterial phylum consisting mainly of Gram-positive bacteria characterized by having a low G+C ratio. They include important bacteria such as the Clostridia, Listeria, Erysipelothrix, and Bacillus

Fluorescent antibody An antibody chemically attached to a fluorescent dye.

Framework regions The parts of a variable region of immunoglobulins and TCRs that have a relatively constant amino acid sequence and so form a structure on which the hypervariable complementarity determining regions may be constructed.

G

G-proteins Guanosine triphosphate (GTP)–binding proteins that act as signal transducers for many cell surface receptors.

Gamma-globulins (γ–globulins) Serum proteins that migrate toward the cathode on electrophoresis. They contain most of the immunoglobulins.

Gammopathies Abnormal increases in γ-globulin levels.

Gel diffusion An immunoprecipitation technique that involves letting antigen and antibody meet and precipitate in a clear gel, such as agar.

Gene complex A cluster of related genes occupying a restricted area of a chromosome.

Gene conversion The exchange of blocks of DNA between

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different genes.

Gene segment Another term for exon. It tends to be used exclusively to denote the exons that code for immunoglobulin and TCR V, D, and J regions.

Genes Units of DNA that code for the amino acid sequence of a protein.

Germinal center A structure characteristic of many lymphoid organs, in which rapidly dividing B cells form a pale-staining spherical mass surrounded by a zone of dark-staining cells. This is the site where somatic mutation occurs and memory cells are generated.

Globulins Serum proteins precipitated by the presence of a half-saturated solution of ammonium sulfate.

Glomerulonephritis Pathological lesions in the glomeruli of the kidney.

Glycoform Differing molecular forms of a protein resulting from differences in glycosylation.

Glycoprotein A protein that contains carbohydrate.

Graft-versus-host disease Disease caused by an attack of transplanted lymphocytes (usually in the form of a bone marrow allograft) on the cells of a histoincompatible and immunodeficient recipient.

Granulocyte A myeloid cell containing prominent cytoplasmic granules. Granulocytes include neutrophils, eosinophils, and basophils.

Granuloma An inflammatory lesion characterized by chronic inflammation with mononuclear cell infiltration and extensive fibrosis.

Granzyme A family of proteases found in the granules of cytotoxic T cells.

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Growth factors Molecules that promote cell growth.

H

Haplotype The complete set of linked alleles within a gene complex. They are inherited as a group and determine a specific phenotype.

Hapten A small molecule that cannot initiate an immune response unless first bound to an immunogenic carrier molecule.

Heat-shock proteins Proteins synthesized by cells in response to many different physiological stresses. Their function is to act as chaperones and carry proteins within different subcompartments of a cell.

Helminths Worms, many of which are parasites and stimulate immune responses.

Helper T cells The subpopulation of T cells that promote immune responses by providing co-stimulation from cytokines and co-stimulatory receptors.

Hemagglutination The agglutination of red blood cells.

Hematopoietic organ An organ in which blood cells are produced.

Hemocytes Phagocytic cells found in invertebrate hemolymph.

Hemolymph The fluid that fills the body cavities of invertebrates. It has functions analogous to those of blood.

Hemolysin An antibody that can lyse red blood cells in the presence of complement.

Hemolytic disease Disease occurring as a result of the destruction of red blood cells by antibodies transferred to the young animal from its mother.

Herd immunity Immunity conferred on a population as a result of the presence of immune individuals within that population.

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Heterodimer A molecule consisting of two different subunits.

Heterophile antibodies Antibodies that react with epitopes found on a wide variety of unrelated molecules.

High endothelial venule A specialized blood vessel lined with high epithelium, found in the paracortex of lymph nodes and other lymphoid organs.

Hinge region The region between the first and second constant domains in some immunoglobulin molecules that permits them to bend freely.

Histiocytes Tissue macrophages.

Histocompatibility molecules Cell membrane proteins that are required to present antigen to antigen-sensitive cells.

Homodimer A molecule consisting of two identical subunits.

Homolog A part similar in structure, position, and origin to another organ.

Homology The degree of sequence similarity between two genes (nucleotide sequences) or two proteins (amino acid sequences).

Humoral immunity An immune response mediated by antibodies.

Hybridoma A cell line formed by the fusion of a myeloma cell with a normal antibody-producing cell.

Hypersensitivity Reproducible clinical signs initiated by exposure to an antigen at a dose tolerated by normal individuals.

Hypersensitivity pneumonitis Inflammation in the lung caused by a type III hypersensitivity reaction to inhaled antigen within the alveoli.

Hypervariable regions Areas within immunoglobulin or TCR variable regions where the greatest variations in amino acid sequence occur and which therefore bind antigens.

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Hypogammaglobulinemia Low levels of γ-globulins in blood.

I

Idiotype The collection of idiotopes on an immunoglobulin molecule.

Immediate hypersensitivity The hypersensitivity reaction mediated by IgE and mast cells, otherwise known as type I hypersensitivity.

Immune complex Another term for antigen-antibody complexes.

Immune elimination The removal of an antigen from the body by circulating antibodies and phagocytic cells.

Immune exclusion The prevention of binding of antigens to mucosal surfaces by immunoglobulin A.

Immune globulin An antibody preparation containing specific antibodies against a pathogen and used for passive immunization.

Immune paralysis Tolerance induced by very high doses of antigen.

Immune stimulants Compounds, commonly bacterial in origin, that stimulate the immune system by promoting cytokine release from macrophages.

Immune surveillance The concept that lymphocytes survey the body for cancerous or abnormal cells and then eliminate them.

Immune synapse An organized molecular structure that forms by receptors clustering in the area of contact between a T cell and an antigen-presenting cell or a target cell.

Immunity The state of resistance to an infection.

Immunization The administration of an antigen to an individual in order to confer immunity.

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Immunoconglutinins Autoantibodies directed against activated complement components.

Immunodeficiency Diseases in which immune function is partially or totally deficient.

Immunodiffusion Another name for the gel diffusion technique.

Immunodominant The epitope on a molecule that provokes the most intense immune response.

Immunoelectrophoresis A procedure involving electrophoresis in gel followed by immunoprecipitation; it is used to identify the proteins in a complex solution, such as serum.

Immunofluorescence Immunological tests that make use of antibodies conjugated to a fluorescent dye.

Immunogenetics That portion of immunology that deals with the direct effects of genes on the immune system.

Immunogenicity The ability of a molecule to elicit an immune response.

Immunoglobulin A glycoprotein with antibody activity.

Immunoglobulin superfamily A family of proteins that contain characteristic immunoglobulin domains.

Immunological paralysis A form of immunological tolerance in which an ongoing immune response is inhibited by the presence of large amounts of antigen.

Immunoperoxidase Immunological test that makes use of antibodies chemically conjugated to the enzyme peroxidase.

Immunosuppression Inhibition of the immune system by drugs or other processes.

Inactivated vaccine A vaccine containing an agent that has been treated in such a way that it can no longer replicate in the host.

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Incomplete antibody An antibody that can bind to a particulate antigen but cannot make it agglutinate.

Indurated Hardened.

Inflammasome A multiprotein complex that forms in response to the triggering of certain pattern-recognition receptors and initiates synthesis of inflammatory cytokines.

Inflammation The responses of tissues to injury. These responses enhance tissue defenses and initiate repair.

Innate immunity Immunity present in all animals that need not be induced by prior exposure to an infectious agent. It is mediated by proteins encoded in the germline.

Innate lymphoid cells (ILCs) A family of lymphocytes that do not express antigen-specific receptors. However, they produce multiple cytokines, and have helper or cytotoxic functions.

Inoculation The administration of a vaccine by injection or scratching.

Integral membrane protein Cell surface proteins that are integral components of the cell membrane as opposed to proteins that are passively adsorbed to cell surfaces.

Integrins A family of adhesion proteins found on cell membranes that bind either to ligands on the surface of other cells or to connective tissue proteins such as fibronectin or collagen.

Interchain bond A bond between two different peptide chains. Usually formed by a disulfide linkage between two cysteine residues.

Interferons Cytokines that can interfere with viral replication. Some interferons play an important role in the regulation of immunity.

Interleukins Cytokines that act as growth and differentiation factors for the cells of the immune system.

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Intrachain bond A bond between two cysteine residues on a single peptide chain. Because disulfide bonds are short, its effect is to produce a fold in the peptide chain.

Intraepithelial lymphocytes Lymphocytes, mainly T cells, located among the epithelial cells in the intestinal wall.

Intron A region inserted within a gene that separates exons and is not expressed.

Isoform Different molecular forms of a protein that are generated by differential processing of RNA transcripts of a single gene.

Isogeneic (syngeneic) Genetically identical.

Isograft A graft between two genetically identical animals.

Isotype switching The change in immunoglobulin class that occurs during the course of the immune response as a result of heavy chain gene switching.

Isotypes Closely related proteins that arise as a result of gene duplication. They are found in all animals of a species. Thus the subclasses of immunoglobulins are actually isotypes.

J

J chain A short peptide that joins units in the polymeric immunoglobulins IgM and IgA.

Joining (J) gene segment A short gene segment that is located 3′ to the *V* gene segments in immunoglobulin and TCR *V* genes and codes for part of the variable region.

K

K antigens Capsular antigens of Gram-negative bacteria.

Killer cell See Natural killer cells.

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Kinins Vasoactive peptides produced in injured or inflamed tissue.

Kupffer cells Macrophages lining the sinusoids of the liver.

L

Lag period The interval between administration of antigen and the first detection of antibody.

Langerhans cells A population of specialized dendritic cells found in the skin. They are effective antigen-presenting cells.

Lectin A protein that can bind specifically to a carbohydrate. Some lectins of plant origin can induce lymphocytes to divide.

Leukemia A cancer consisting of white cells that proliferate within the blood.

Leukocytes White blood cells. This general term covers all the nucleated cells of blood.

Leukopenia The absence of leukocytes.

Leukotoxin A toxin produced by bacteria that kills leukocytes.

Leukotrienes Lipid mediators derived from arachidonic acid and responsible for potent proinflammatory responses. Released by degranulating mast cells.

Ligand A generic term for the molecules that bind specifically to a receptor.

Linkage disequilibrium The nonrandom association of two genes found at different loci.

Linked recognition The necessity for lymphocytes to receive two or more simultaneous signals to be activated.

Locus The location of a gene in a chromosome.

Looping out A method of excising a segment of intervening DNA (intron) in order to join two gene segments (exons).

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Lymph The clear tissue fluid that flows through lymphatic vessels.

Lymphadenopathy Literally, "disease of lymph nodes." In practice, it is used to describe enlarged lymph nodes.

Lymphoblast A dividing lymphocyte.

Lymphocyte A small mononuclear cell with a round nucleus containing densely packed chromatin found in blood and lymphoid tissues. Most have only a thin rim of cytoplasm. They recognize foreign antigens through specialized receptors.

Lymphokine-activated killer (LAK) cells Lymphocytes activated by exposure to cytokines such as IL-2 in vitro.

Lymphokines Cytokines secreted by lymphocytes.

Lymphopenia Abnormally low numbers of lymphocytes in blood.

Lymphotoxins Cytotoxic cytokines secreted by lymphocytes.

Lysosomal enzymes The complex mixture of enzymes, many of which are proteases, found within lysosomes.

Lysosomes Cytoplasmic organelles found within phagocytic cells that contain a complex mixture of potent proteases.

Lysozyme An enzyme present in tears, saliva, and neutrophils. It attacks carbohydrates in the cell walls of Gram-positive bacteria.

M

Macrophages Large phagocytic cells containing a single rounded nucleus.

Major histocompatibility complex The gene region that contains the genes for the major histocompatibility molecules, as well as for some complement components and related proteins.

Malignant tumors Tumors whose cells have a tendency to invade normal tissues, break away, and spread by lymphatics or blood

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to distant tissue sites.

Marsupials The order containing the pouched mammals. These include not only the Australian forms such as kangaroos and koalas but also the opossums.

Maternal antibodies Antibodies that originate in the mother but enter the bloodstream of her offspring either by transport across the placenta, as in primates, or by adsorption of ingested colostrum, as in other mammals.

Medulla The region in the center of lymphoid organs such as the thymus or lymph nodes.

Memory cells A subpopulation of long-lived lymphocytes formed in response to antigen. On second response to that antigen, they mount a faster and stronger immune response.

Memory response The enhanced immune response that is triggered as a result of exposing a primed animal to antigen.

Mesangial cells Modified muscle cells found within a glomerulus.

MHC molecules Proteins coded for by genes located in the major histocompatibility complex.

MHC restriction The necessity for a T cell to recognize an antigen in association with an MHC molecule. It is required for helper and cytotoxic T cells to recognize antigen and for helper T cells to cooperate with B cells.

Microbiome The collective term for all the microbial genomes contained within the microbiota.

Microbiota The collective term for the microbial populations that colonize body surfaces.

Microglia Macrophages resident within the brain.

Mitogen Any substance that makes cells divide.

Mixed lymphocyte reaction Lymphocyte proliferation induced by

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contact with foreign lymphocytes in vitro.

Modified live virus A virus whose virulence has been reduced so that it can replicate in the host but cannot cause disease in normal animals.

Molecular mimicry The development by parasites or other infectious agents of molecules whose structure closely resembles molecules found in their host. In this way the invaders may be able to evade destruction by the immune system or perhaps trigger autoimmunity.

Monoclonal Originating from a single clone of cells.

Monoclonal antibody Antibody derived from a single clone of cells and hence chemically homogeneous.

Monoclonal gammopathy The appearance in serum of a high level of a monoclonal immunoglobulin. This is commonly, but not always, associated with the presence of a myeloma.

Monocytes Immature macrophages found in the blood.

Monomer The basic unit of a molecule that can be assembled using repeating subunits.

Mononuclear cells Those leukocytes with a single round nucleus; for example, lymphocytes and macrophages.

Mononuclear-phagocytic system The cells that belong to the macrophage family and their precursors.

Monotremes The order containing the least complex egg-laying mammals. These include the platypus and spiny anteaters (Echidna).

Myeloid system All the granulocytes and their precursors. These precursor cells are found in the bone marrow.

Myeloma A tumor of plasma cells.

Myeloma protein The immunoglobulin product secreted by a

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myeloma cell.

N

N-terminus The end of a peptide chain with a free amino (NH_2) group.

Natural antibodies Antibodies against foreign antigens found in serum in the absence of known antigenic stimulation from immunization or an infection. Most probably arise as a result of exposure to cross-reacting bacterial antigens.

Natural killer cells Innate lymphocytes that are found in normal, unsensitized individuals and that can recognize and kill abnormal cells such as tumor- and virus-infected cells.

Natural suppressor cells A population of innate cells found in unimmunized individuals that have the ability to suppress some immune responses.

Necrosis Cell death due to pathological causes.

Negative feedback A control mechanism whereby the products of a reaction act to suppress their own production.

Negative selection The killing of T cells that have the potential to react to self-antigens. A key mechanism in the prevention of autoimmunity.

Nematode A roundworm.

NETosis The exocytosis of a DNA net by a leukocyte in order to trap nearby microorganisms.

Neutralization Blockage of the activity of an organism or a toxin by antibody.

Neutropenia Low numbers of neutrophils in blood.

Neutrophilia High numbers of neutrophils in blood.

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Neutrophils Polymorphonuclear neutrophil granulocytes.

Noncovalent bonds Chemical bonds, such as hydrogen or hydrophobic bonds, that reversibly link peptide chains. They play a key role in the binding of antigen with antibodies or with T cell antigen receptors.

Nucleocapsid The key structural component of a virus consisting of the viral nucleic acid and its protective capsid coat.

Nude mice A mutant strain of mice that have no thymus and are hairless.

O

O antigens Somatic antigens of Gram-negative bacteria.

Obligate intracellular parasite An organism that is absolutely required to grow inside cells. Viruses are excellent examples.

Oncofetal antigens Antigens found on fetal and tumor cells.

Oncogene A gene whose protein product plays a key role in cell division. As a result, its uncontrolled production leads to excessive cell growth and tumor formation. Oncogenes may be found in normal cells, as well as in cancer-causing viruses.

Oncogenic virus A virus that causes cancer.

Ontogeny The embryonic development of an organ or animal.

Opportunistic pathogen An organism that, although unable to cause disease in a healthy individual, may invade and cause disease in an individual whose immunological defenses are impaired.

Opsonin A molecule that facilitates phagocytosis by coating foreign particles.

Optimal proportions When antigen and antibody combine, this is the ratio of reactants that generates the largest immune

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complexes.

Orthologous Genes that are clearly descended from a common ancestral gene.

Osteichthyes The class containing the bony fish. It includes several orders of fish, the most highly evolved of which are the teleosts. The teleosts include such typical fish as the goldfish, catfish, and trout.

P

Paracortex The region located between the cortex and medulla of lymph nodes in which T cells predominate.

Paralogous Two genes or gene clusters that, although likely descended from a single ancestor, are located on different chromosomes and have diverged significantly.

Parasite An organism that lives on or in a host animal and consumes resources of host origin.

Passive agglutination The agglutination of inert particles by antibody directed against antigen bound to their surface.

Passive immunization Protection of one individual conferred by administration of antibody produced in another individual.

Pathobiont A member of the normal microbiota that has the ability to become a pathogen.

Pathogen-associated molecular patterns (PAMPs) Conserved molecular structures widely distributed among pathogenic microbes that trigger inflammation.

Pathogenesis The mechanism of a disease.

Pathogenic organism (Pathogen) An organism that causes disease.

Pattern-recognition receptors (PRRs) Cellular receptors that can recognize pathogens or their soluble components. PRRs include

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toll-like receptors, among others.

Perforin A family of proteins made by T cells and NK cells (and the complement component C9) that, when polymerized, can insert themselves into target cell membranes and provoke cell lysis.

Phagocytes Cells whose prime function is to eat foreign particles, especially bacteria. They include macrophages and related cells, neutrophils, and eosinophils.

Phagocytosis The ability of some cells to ingest foreign particles. Literally, "eating by cells."

Phagolysosome A structure produced by the fusion of a phagosome and a lysosome following phagocytosis.

Phagosome The cytoplasmic vesicle that encloses an ingested organism.

Phenogroup A set of blood group alleles that are consistently inherited as a group.

Phylogeny The evolutionary history of a plant or animal species.

Phytohemagglutinin (PHA) A lectin derived from the red kidney bean. It acts as a T cell mitogen.

Pinocytosis The endocytosis of small fluid droplets—drinking by cells.

Plasma The clear fluid that forms the liquid phase of blood.

Plasma cell A fully differentiated B cell capable of synthesizing and secreting large amounts of antibody.

Point mutation A mutation resulting from an alteration in a single base in a gene.

Polyclonal gammopathies The appearance in serum of a high level of immunoglobulins of many different specificities originating from many different clones.

Polymorphism Inherited structural differences among proteins

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from allogeneic individuals as a result of multiple alternative alleles at a single locus.

Polymorphonuclear neutrophil granulocytes Blood leukocytes possessing neutrophilic cytoplasmic granules and an irregular lobed nucleus.

Positive selection The enhanced proliferation of cells within the thymus that can respond optimally to foreign antigen.

Precipitation The clumping of soluble antigen molecules by antibody to reproduce a visible precipitate.

Premunition A form of immunity seen in some parasitic conditions that depends on the continued presence of the parasite in the host.

Prevalence The number of cases of a disease.

Primary binding tests Serological assays that directly detect the binding of antigen and antibody.

Primary immune response The immune response resulting from an individual's first encounter with an antigen.

Primary immunodeficiencies Inherited immunodeficiency diseases.

Primary lymphoid organ An organ that serves as a source of lymphocytes or in which lymphocytes mature.

Primary pathogen An organism that can cause disease without first suppressing an individual's immune defenses.

Primary structure The amino acid sequence of a protein.

Privileged sites Locations within the body where foreign grafts are not rejected. A good example is the cornea of the eye.

Prokaryotic organism An organism composed of cells whose genetic material is free in the cytoplasm and as a result do not contain a recognizable nucleus.

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Prostanoids A class of lipid mediators derived from arachidonic acid produced by the actions of the enzyme cyclooxygenase. They include prostaglandins, thromboxanes, and prostacyclin.

Proteasome A large complex multienzyme structure found in the cytosol. It acts on ubiquinated cellular proteins to cleave them into small fragments.

Protein kinase An enzyme that phosphorylates proteins.

Prozone The inhibition of agglutination by the presence of high concentrations of antibody.

Pseudogenes DNA sequences that resemble functional genes but that cannot be transcribed.

Pyrogen A fever-causing substance.

R

Radioimmunoassay An immunological test that requires the use of an isotope-labeled reagent.

Reaginic antibody An antibody of the IgE class that mediates type I hypersensitivity.

Recombinant vaccine A vaccine that contains antigen prepared by recombinant DNA techniques.

Respiratory burst The rapid increase in metabolic activity that occurs in phagocytic cells while particles are being ingested. It generates potent oxidants that can kill invading microorganisms.

Retrovirus An RNA virus that employs the enzyme reverse transcriptase to convert its RNA into DNA.

Reverse transcriptase An enzyme that reversely transcribes RNA to DNA. It is found in retroviruses such as FIV.

Rheumatoid factor An autoantibody directed against epitopes on

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the immunoglobulin Fc region. Classically found in the blood of patients with rheumatoid arthritis.

S

Sarcoma A tumor arising from cells of mesodermal origin.

Secondary binding tests Serological tests that detect the consequences of antigen-antibody binding, such as agglutination and precipitation.

Secondary immune response An enhanced immune response that results from second or subsequent exposure to an antigen.

Secondary immunodeficiencies Immunodeficiency diseases resulting from a known, nongenetic cause.

Secondary infections Infections by organisms that can invade only a host whose defenses are first weakened or destroyed by other infectious agents or toxins.

Secondary lymphoid organ A lymphoid organ whose function is to trap and respond to foreign antigens.

Secondary structure The way in which a peptide chain is made up of structural components such as α-helices and β-pleated sheets.

Secretory component A protein produced by mucosal epithelial cells; it functions as an IgA receptor and, on binding to IgA, protects IgA against proteases in the intestine.

Selectin A family of cell surface adhesion proteins that bind cells to glycoproteins on vascular endothelium.

Self-cure The elimination of intestinal worms by a localized type I hypersensitivity reaction in the intestinal tract.

Sensitivity A term that describes the ability of a diagnostic test to correctly identify negative results.

Sensitization The triggering of an immune response by exposure to

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an antigen.

Sepsis The systemic inflammatory response to an infectious agent.

Septic shock A severe disease condition that results from the massive release of cytokines such as TNF as a result of infection.

Seroconversion The appearance of antibodies in blood, indicating the onset of an infection.

Serology The science of antibody detection.

Serum The clear, yellow fluid that is expressed when blood has clotted and the clot contracts.

Serum sickness A type III hypersensitivity response to the administration of foreign serum as a result of the development of immune complexes in the bloodstream.

Signal transduction The transmission of a signal through a receptor to a cell by means of a series of linked reactions.

Skin test A diagnostic procedure that induces a local inflammatory response following intradermal inoculation of an antigen or allergen.

Somatic antigens Antigens associated with bacterial bodies.

Somatic mutation Mutations that occur in somatic rather than germline cells. In immunology, this refers to the extensive mutations that occur in the *V* genes of B cells during the course of an immune response.

Specificity A term that describes the ability of a test to give true-positive reactions.

Splice The joining of two DNA or RNA segments (exons) together.

Stem cell A cell that can maintain itself and serve as a source of many differentiated cell lines.

Stimulation index A measure of the extent to which a cell population is stimulated to divide. It is the ratio of thymidine

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uptake in a stimulated cell population to the thymidine uptake in an unstimulated population.

Subclass Different immunoglobulin isotypes closely related within a specific class.

Subisotype See Subclass.

Substrate modulation A method of controlling enzyme activity seen in the complement system, by which a protein cannot be cleaved by a protease until it first binds to another protein.

Superantigen A molecule that, as a result of its ability to bind to certain TCR variable regions, can cause certain T cells to divide.

Superfamily A grouping of protein molecules that share common structures. For example, the members of the immunoglobulin superfamily all contain characteristic immunoglobulin domains.

Symbiosis The relation between species that live together

Synapse The area of contact between cells. Within the synapse, cell surface molecules are arranged in a well-defined pattern designed to optimize signaling between the cells.

Syndrome A group of symptoms and lesions that together are characteristic of a specific disease.

Syngeneic (isogeneic) Genetically identical.

T

T lymphocyte A lymphocyte that has undergone a period of processing in the thymus and is responsible for mediating cell-mediated immune responses. They are characterized by the presence of a TCR and its associated CD3 complex.

T cell receptor (TCR) The antigen-binding receptors of T lymphocytes. They are heterodimers of the antigen-binding subunits α and β or γ and δ. They associate with the CD3 complex.

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Terminal complement complex A multimolecular structure that formed by complement activation that generates pores in target cell membranes, leading to osmotic lysis and cell death.

Tertiary binding tests Serological tests that measure the protective ability of an antibody in living animals.

Thoracic duct The major lymphatic vessel that collects the lymph, draining the lower portion of the body.

Thymectomy Surgical removal of the thymus.

Thymocytes Developing lymphocytes in the thymus.

Thymus-dependent antigen An antigen that requires the assistance of helper T cells to provoke an immune response.

Thymus-independent antigen An antigen that can activate B cells and trigger an antibody response without help from T cells.

Titer The reciprocal of the highest dilution of a serum that gives a reaction in an immunological test.

Titration The measurement of the level of specific antibodies in a serum, achieved by testing increasing dilutions of the serum for antibody activity.

Tolerance A state of specific unresponsiveness to an antigen induced by prior exposure to that antigen.

Tolerogen A substance that induces tolerance.

Toxic shock A disease resulting from exposure to large amounts of staphylococcal superantigen.

Toxoid Nontoxic derivatives of toxins used as antigens.

Transcription The conversion of a DNA nucleotide sequence into an RNA nucleotide sequence by complementary base-pairing.

Transcription factors Specialized proteins that regulate gene activity by binding to the promoter region of genes. They thus

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turn gene transcription on or off.

Transduction The conversion of a signal from one form to another.

Translation The conversion of the RNA nucleotide sequence into an amino acid sequence in a ribosome.

Transporter protein Proteins that bind fragments of endogenous antigen and carry them to newly assembled MHC class I molecules in the endoplasmic reticulum.

Trematode A helminth known as a fluke. Trematodes are important human and animal parasites.

Tubercle A persistent inflammatory response to the presence of mycobacteria in the tissues.

Tuberculin An extract of tubercle bacilli used in a diagnostic skin test for tuberculosis.

Tumor necrosis factors Macrophage- and lymphocyte-derived cytokines that can exert a direct toxic effect on neoplastic cells.

Tunicates Complex marine invertebrates possessing characteristic outer cuticular coverings, whose embryonic stages possess features that resemble those found in some vertebrates.

Type 2 immune responses Immune responses mediated by Th2 cells, ILC2 cells, M2 macrophages, and type 2 cytokines. It involves antibody production and protects against helminths and toxins.

Tyrosine kinase An enzyme that phosphorylates tyrosine residues in proteins. It plays a key role in signal transduction.

U

Urodeles The most primitive order of amphibians; it includes the newts and salamanders.

Urticaria Inflammatory and edematous skin reactions due to

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allergic mechanisms and associated with intense itching.

V

Vaccination The administration of an antigen (vaccine) to stimulate a protective immune response against an infectious agent. The term is synonymous with immunization.

Vaccine A suspension of living or inactivated organisms used as an antigen to confer immunity.

Variable region That part of the immunoglobulin or TCR peptide chains in which the amino acid sequence shows significant variation among molecules.

Vasculitis Inflammation of blood vessel walls.

Vasoactive molecules Molecules that cause changes in local blood flow such as those observed during inflammation.

Virion A virus particle.

Virokine Proteins encoded by viruses that mimic cytokine activities.

Virulence The ability of an organism to cause disease.

X

Xenograft A graft between two animals of different species.

Xenohybridoma A hybridoma formed by fusing plasma cells and myeloma cells from two different species (for example, mouse and bovine).

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Index

Page numbers followed by "*f*" indicate figures, "*t*" indicate tables, and "*b*" indicate boxes.

A

Ablastins, 311

Acantholysis, 414–415

Accelerated rejection, 379–380

Acetylcholine, 219–220

  receptors, 419, 420*f*

Acidosis, 65–66, 224–225

Acquired immune system, Adaptive immune system

Acrodermatitis, 445–446

Actinobacillus pleuropneumoniae, 65, 291, 365

Actinobacillus suis, 17

Actinobacteria, 224

Activation

  eosinophils, 332

  macrophages, 52–54

    classical, 53*f*

    alternative, 53*f*, 54

  neutrophils, 42

Activation-induced cytidine deaminase, Cytidine deaminase

Activator protein-1 (AP-1), 79

VetBooks.ir

Active immunization, 263–265
Acute-phase proteins, 16–17, 62–65, 252, 418
Acute allograft rejection, 379–380
Adaptive immunity
  basic features, 4–7
  evasion of, 292–293
  in fish, 494–496
  mechanisms, 7
  neonatal, 258–259
  receptors in, 4
  to bacteria, 287–289
  to fungi, 295–296, 295*f*
  to helminths, 316–321
  to protozoa, 311–313
  to viruses, 301–302
Addressins, 242
Adenosine, 15–16, 57–58
Adenosine deaminase, 447
Adherence molecules, 136
Adiponectin, 29, 456
Adipose tissue, 456, 457*f*
Adjuvants, 270–273, 271*t*
  aluminum, 270–271
  combined, 273
  depot, 271*f*
  emulsions, 271–272
  immunostimulatory, 272–273

VetBooks.ir

nanoparticles, 272
oil-based, 273
particulate, 272
saponin-based, 271
Adrenaline, (epinephrine)
Adrenoceptors, 330–331, 331*t*, 407–408
Adrenocorticotrophic hormone, 220
Adverse events to vaccines, 280–283, 281*f*
Affinity, 156–157
Affinity maturation, 156–157
Aflatoxins, 455
African horse sickness, 265
African trypanosomes, 313
Agammaglobulinemia, 439
Age and immunity, 460–461, 460*f*
B cell responses, 461
innate immunity, 460–461
inflammaging, 460–461
lymphoid organs, 461
memory cells, 461
Tcell responses, 461
Agglutination tests, 264, 294–295, 417, 484–485
passive, 472, 483–484
Akabane virus, 250
Alarmins, 15, 329–330
Aleutian disease, 159, 304, 307, 307*f*
Alkaline phosphatase, 479–480

VetBooks.ir

Alkylating agents, 264–265, 465
Allergen-specific immunotherapy, 342–343, 346–347
Allergic contact dermatitis, 371–374, 372*f*, 373*t*
Allergic rhinitis, 340
Allergic triad, 340
Allergies
  anaphylaxis, 336–338, 337*t*
  diagnosis, 345–346
  dermatitis, 340–343
  environmental influences, 341
  genetics, 340–341
  loop, 326*f*
  to arthropods, 322, 344
  to drugs, 343–344
  to food, 338–339
  to milk, 338
  to parasites, 344
  to vaccines, 280, 343–344
  respiratory disease, 339–340
  treatment, 346–347
Allograft rejection, 378–379
  adaptive mechanisms, 380–381
  bone, 383
  bone marrow, 383–384
  cardiac, 382
  corneal, 382–383
  definition, 377

VetBooks.ir

destruction, 381
direct pathway, 380
ethical issues, 378
fish, 496
indirect pathway, 380
innate mechanisms, 380
invertebrates, 492
liver, 382
mechanisms, 380*f*
pathogenesis, 380–381
prevention, 382
renal, 378–382, 379*f*, 467
skin, 6, 7*f*, 374, 382, 452
tumors, 389–390
Allotypes, immunoglobulin, 168
Alopecia areata, 414
Alpacas, 256
Alpha-1 acid glycoprotein, 64–65
Alpha-antitrypsin, 65
Alpha-1 antichymotrypsin, 65
Alpha-2 macroglobulin, 65
Alternative macrophage activation, 196–197
Aluminium adjuvants, 268–271, 293
Amphibians
anurans, 497–498
B cells, 497
immunity, 496–498

VetBooks.ir

immunoglobulins, 497
MHC, 498
T cells, 498
thymus, 497
urodeles, 496–497
ventral cavity bodies, 497
Amyloidosis, 35–36, 69, 69*f*, 365, 438
transmissible, 70*b*
Anacultures, 293
Anamnestic (memory) response, 5–6
Anaphylatoxins, 32–33, 349
Anaphylaxis
basic features, 336–338
bovine, 336–338, 337*f*
cat, 338
dog, 338
horse, 336
pig, 338
sheep, 338
Anaplasma marginale, 292, 352
Anemia
of infection, 64, 356
foal immunodeficiency syndrome, 441–442
intravascular, 417, 417*f*
hemolytic anemia, 417–419, 425
Anergy, 210–211, 370
Ankylosing spondylitis, 405

VetBooks.ir

Anthrax vaccine, 265, 271
Antibodies
  antiviral, 301
  assays, 277, 472
  asymmetrical, 499
  blocking, 386–387
  class switch, 152, 154
  colostral, 253
  diversity, 178*b*
  genes, 174–175
  half-life, 259
  labels, 479–480
  maternal, 212, 212*f*, 258–260, 258*f*, 269, 276, 279
  monoclonal, 157
  opsonins, 43, 43*f*
  production sites, 120*f*
  receptors for, 43, 54–55, 73
  regulation of immune responses, 212
  structure, 167–168
  titration, 484, 484*f*
Antibody-dependent, cell-mediated cytotoxicity (ADCC), 193, 301
Antibody-mediated immune response, 5–6
Antigen-antibody complexes, Immune complexes
Antigen-presenting cells, 103, 210, 378
Antigen-processing cells, 96
  pathways, 96–99
Antigen receptors, 174–175, 183–185

VetBooks.ir

Antigenicity, 84–86
Antigens
autoantigens, 84
bacterial, 82–83
basic features, 5
binding site, MHC, 103*f*
blood group, 84, 87–88, 348–349
capsular (K), 82–83, 287
cell surface, 84
cryptic, 402–403
dietary, 214, 338–339
endogenous, 83, 187–188, 302
exogenous, 83
flagellar, 82–83, 294–295
foreign, 85–86
helminth, 321
hidden, 323, 406–407
histocompatibility, 84
lipids, 85, 206
microbial, 82–84
nonmicrobial, 84
nucleic acids, 85
polysaccharide, 85
processing, 89, 212
proteins, 85
receptors, 174
regulation by, 212

VetBooks.ir

somatic, 294–295
viral, 83
Antigenic determinants, Epitopes
Antigen specific immunotherapy, 346–347
Antigenic variation
bacterial, 292
helminths, 321
protozoa, 313–314
trypanosomes, 313–314, 314*f*
viral, 303
Antiglobulin tests, 352, 356, 418, 484, 485*f*
Antiglobulins, 472
Antihistamine drugs, 346
Antilymphocyte serum, 469–470
Antimicrobial peptides, 18, 24–25
Antinuclear antibodies, 424–427, 427*b*, 428*f*
Antiserum, Immune globulins
Antitoxins, 83
Apicomplexans, 311–312
Apoptosis, 15*b*, 188–189, 189*f*–190*f*
death receptor pathway, 188
defects in, 425
extrinsic pathway, 188
inhibition of, 305
intrinsic pathway, 188
mitochondrial pathway, 188–189
neutrophil, 47–48

VetBooks.ir

regulation of, 218
T cell, 215–216
Virus infected cells, 299
Apoptosome, 15, 189, 192
APRIL, 154*b*, 240, 244, 334
Arachidonic acid, 23–24, 328
Arcanobacteria, 224, 418
Arginase, 196, 218, 393
Artemin, 344
Artemis mutation, 443, 443*f*
Arthritis
autoimmune, 429–433
erosive, 429–431
immune-complex, 366
nonerosive, 432–433
rheumatoid, 429–431
Arthropods
allergies to, 322, 344
immunity to, 322–323
saliva, 322
vaccines, 323
Arthus reaction, 358, 358*f*–359*f*
Aryl hydrocarbon receptor (Ahr), 199–200, 344*b*
Asialo-GM_1, 204–205
Aspergillus flavus, 291–292
Asymmetric cell division, 190, 197
Assays in living systems, 486–487

VetBooks.ir

Asthma, 338, 340*f*, 360–361
Ataxia telangiectasia, 447–448
Atopic dermatitis, 325, 340–343, 340*t*, 341*f*
  aryl hydrocarbon receptor, 344*b*
  barrier dysfunction, 343
  genetic background, 340–341
  infections, 343
  smoking, 344
Atopy, 324–325
Atrophic lymphocytic pancreatitis, 411
Attenuation, 265, 267
Aureolysin, 290
Autogenous vaccines, 293–294
Autografts, 377
Autoimmune
  adrenalitis, 411
  blood diseases, 417–419
  dermatitis, 414–417
  diabetes mellitus, 410–411
  endocrine disease, 409–411
  eye disease, 412–414
  hemolytic anemia, immune-mediated hemolytic anemia
  hepatitis, 421–422
  masticatory myositis, 420–421
  muscle disease, 419–421
  nephritis, 417
  neurologic disease, 411–412

VetBooks.ir

orchitis, 414
polyendocrine disease, 403–404
polyglandular syndrome, 409, 411
reproductive diseases, 414
skin diseases, 414–417
thrombocytopenia, 283, 419
Autoimmune regulator (AIRE), 209
Autoimmunity
breed predispositions, 406–407
genetic predisposition, 406
induction of, 402
infection-induced, 403–405
mechanisms, 407–408
predisposing factors, 406–407
spaying and neutering, 406*b*
vaccine associated, 283
Autoinflammatory disease, 423
Autonomic nervous system, 220
Autophagy, 45*b*, 47*f*, 96, 111, 240–241, 288, 293
Autophagosome, 47*f*, 293
Avidin-biotin, 479
Azathioprine, 307, 382, 466
Azotemia, 364–365
Azurocidin, 51–52

B

Babesiosis, 311, 313–315, 352

VetBooks.ir

Bacille Calmette-Guérin (BCG), 130, 265, 320, 391–392, 394, 468–469, *Mycobacterium bovis*

Bacillus anthracis, 25, 63, 287

Bacillus subtilis, 227

Bacteria

- acid-fast, 10*f*
- adverse consequences of immunity, 294
- antigens, 82–83
- as immunostimulants, 468–469
- capsules, 287
- evasion of immunity by, 289–293, 291*f*
- exotoxins, 83
- fimbriae, 83
- flagella, 83
- Gram-negative, 10
- Gram-positive, 10
- immunity to, 285, 287–289, 287*f*
- modification by immunity, 288–289
- nucleic acids, 85
- pili, 83
- segmented filamentous, 199–200, 224, 230, 231*f*, 239, 245
- septic shock, 65–67
- serology, 294–295
- vaccines, 293–294

Bactericidal permeability-increasing protein, 25

Bacterins, 264–265, 293–294

Bacteroides fragilis, 227–228, 391

VetBooks.ir

Bacteroides thetaiotamicron, 228
Bacteroidetes, 224
BAFF, 154*b*, 240, 244
Band 3, 402–403, 417
Bartonella vinsonii, 427
Basal transcription complex, 80*f*, 81
Base deletion, 177
Base insertion, 177
Basement membrane diseases, 415–417
Basophils, 318, 331–332, 331*f*, 368
B cells, 111
  activating factor (BAFF), 154*b*, 240, 244
  in the aged, 461
  viruses and, 303–304
  anergy, 211
  antigen processing, 96, 150, 150*f*
  antigen receptors, 148–150, 148*f*, 150*f*
    antigen-binding component, 148–149, 149*f*
    features, 125–127
    signal transduction, 75, 78, 79*f*
  apoptosis, 154–155
  co-stimulation, 150–151
  class switch, 179*f*
  corticosteroids, 465
  costimulation, 150–151
  deficiencies, 441–442, 444–445, 447–448
  fish, 495–496

VetBooks.ir

germinal centers, 156–157
identifying features, 124*t*
intestinal, 242
location, 147–148
lymph nodes, 113
mammary gland, 236
memory, 154–156
microbiota and, 229
mitogens, 130
responses to antigens, 151–152, 154, 156*f*
regulatory, 218
signal transduction, 149–150
spleen, 119
subpopulations, 157
surface receptors, 126*f*
tolerance, 210–211
tumors, 399–400
B1 cells, 157
B2 cells, 157
bcl-2, 192, 218
Behavioral fever, 504–505
Beige mice, 436
Bence-Jones proteins, 158
Beta-galactosidase, 479–480
Beta$_2$-microglobulin, 101–103
Beta-propiolactone, 264–265
Betamethasone, 465–466

VetBooks.ir

Bidirectional flow ELISA, 478–479
Bifidobacterium, 255, 349
Biofilms, 290–291
Biotin, 479
Birbeck granules, 92
Birds
  allografts, 502
  antibody diversity, 502
  bursa of Fabricius, 111–112
  immunoglobulin classes, 501–502
  immunoglobulin A, 501–502
  immunoglobulin M, 501
  immunoglobulin Y, 491–492, 501*f*
  immunity in, 501
  lymphoid organs, 500, 500*f*
  maternal immunity, 502
  MHC molecules, 500–501, 500*f*
  NK cells, 501
  T cells, 502
Black hair follicular dysplasia, 427
Blistering skin diseases, 414–415
Blockade, 55–56
Blocking antibodies, 386–387, 393
Blood coagulation, 24
Blood groups, 87–88, 348–349, 350*t*
  cats, 354–355
  cattle, 351–353

VetBooks.ir

dogs, 354
graft rejection, 378
horses, 350–351
pigs, 353–354
sheep, 353
Blood transfusions, 349–355
Blue-eye, 305, 306*f*, 360
Bluetongue, 250, 276
Body strike, 323
BoLA system, 101
Bo-lysin, 192
Bone marrow, 113
allografts, 377, 444
antibody production, 120*f*
primary lymphoid organ, 109
stem cells, 109
structure, 109
Boophilus microplus, 107, 323
Bordetella bronchiseptica, 285, 382
Borna disease, 305
Borrelia burgdorferi, 44, 266, 287, 323, 404, 412–413
Bovine
anaphylaxis, 336–338, 337*f*
blood groups, 351–352
dendritic cells, 95
diabetes mellitus, 411
genome, 24*b*

VetBooks.ir

hemolytic disease, 352
herpesvirus-1, 299, 300*f*, 303, 450
immunodeficiencies, 442
immunodeficiency virus, 455
immunoglobulins, 170–171, 339
immunoglobulin genes, 181
leukemia virus, 399
leukocyte adhesion deficiency, 437–438
leukocyte antigens (BoLA), 101
leukosis, 107
lymphocytes, 129–130
mastitis, 236
MHC, 105*f*, 107
neonatal pancytopenia, 352–353, 352*f*
NK cells, 204–205
NKT cells, 206
persistent lymphocytosis, 399
respiratory syncytial virus, 305
spongiform encephalopathy, 70–71
Treg cells, 213
tuberculin tests, 369–370, 369*t*
viral diarrhea, 250, 250*f*, 352–353, 449–450
WC1, 125, 129
Bradykinin, 23
Breed predispositions
atopy, 340–341
autoimmunity, 406–407, 417

VetBooks.ir

diabetes mellitus, 410–411
enteropathies, 232
hemolytic disease, 353–354
myasthenia gravis, 419
thrombocytopenia, 419
vaccine responses, 280–281
Brittany Spaniels, 35–36
Bronchoalveolar cells, 237–238, 238*t*
Bronchus-associated lymphoid tissue, 237*f*, 241
Brucella abortus, 46, 87–88, 194, 288, 291–293
Brucella buffered antigen tests, 295
Brucella canis, 414
Brucella skin tests, 370
Brucella vaccines, 264
Brugia pahangi, 320
Bruton-type agammaglobulinemia, 447–448
Bullous pemphigoid, 416
Bursa of Fabricius, 111–112
function, 111–112
hormones., 111–112
structure, 111
Bursectomy, 110*t*, 111, 123–124
Bursin, 111–112
Bystander activation, 405, 405*f*

C

C-reactive protein, 16–17, 31, 62–63, 418

VetBooks.ir

Cadherin, E, 99
Calcineurin, 79, 137, 150
  inhibitors, 79, 466–467, 467*f*
Calcitonin gene-related peptide, 23
Calcium cyanamide, 372
Calcium ions, 75
Calf
  immune system development, 248, 248*f*
  vaccination, 260*f*
Calprotectin, 25, 230, 235
Camel, immunoglobulins, 172*b*
Campylobacter fetus, 238, 292
Campylobacter jejuni, 288–290
Canarypox vectored vaccines, 260, 268
Cancer/testis antigens, 389
Candida albicans, 17, 43–44, 296
Canditatus salvagella, 230
Canine
  atopic dermatitis, 325, 340–344, 340*t*, 341*f*, 344*b*
  cardiomyopathy, 421
  C3 deficiency, 35–36
  cyclical neutropenia, 438
  distemper, 429
    encephalitis, 305
    immunosuppression, 450, 450*f*
  glomerulopathy, 366
  graft-versus-host disease, 384, 384*f*

VetBooks.ir

hepatitis, 403
immunoglobulins, 172
inflammatory bowel disease, 232–233
juvenile polyarteritis, 433
leukocyte adhesion deficiency, 436–437
lupus, 412, 426–427
necrotizing meningoencephalitis, 412
neutrophil dysfunction, 436–438
polyarthritis, 432
polyneuritis, 411
Caninized monoclonal antibodies, 160–161, 470
Caprine arthritis-encephalitis (CAE), 107, 304
Capsids, 84*f*
Carbohydrates, immunostimulating, 469
Carboxymycobactin, 64
Carcinoembryonic antigen, 389
Card agglutination tests, 264
Cardiomyopathy, canine, 421
Carrier molecules, 86–87
Caseous lymphadenitis, 107
Caspases, 12, 19–20, 78, 192
effector, 15, 188*f*, 189
inflammatory, 188*f*
initiator, 188, 188*f*
Cathelicidins, 24–25, 46, 51–52, 222–223, 239, 286, 290, 331
Cathepsins, 40–41, 328
Cats, feline

VetBooks.ir

acute-phase proteins, 65
allograft rejection, 382
anaphylaxis, 338
blood groups, 84, 354–355
dendritic cells, 96, 96*f*
food allergies, 339
graft-versus-host disease, 384
hemolytic disease, 355
histiocytosis, 99
immune system development, 249–250
immunodeficiencies, 446, 452–454
immunoglobulins, 162–163, 172
immunoglobulin genes, 182, 182*t*
lymphocytes, 130
NK cells, 206
retrovirus infections, 451–455
transfusion reactions, 354–355
Cattle, Bovine
CD system, 14*b*, 84, 125
CD1, 14, 85, 95–96, 103, 145, 206
CD2, 129–130, 192
CD3, 127
complex, 134
CD4, 127, 130, 134, 213
CD4/8 ratio, 453–454, 461
CD5, 157
CD8, 127, 134, 173

VetBooks.ir

CD11a/CD18 (LFA-1), 32, 128–129, 136
CD11b/CD18 (CR3), 54–55
CD11c/CD18 (CR4), 32
CD14, 14, 63, 236
CD16 (FcγRIII), 54–55, 128, 204
CD18, 436–438
CD19, 32–34, 153*f*
CD20, 395
CD21 (CR2), 32, 153*f*, 252
CD23, (FcεRII), 128
CD25 (IL-2R), 65, 213, 288–289
CD28, 135–136, 138, 178–179, 192–193, 213
CD29, 128–129
CD31, 57–58, 58*f*
CD32 (FcγRII), 43, 54–55, 128, 212, 213*f*
CD35 (CR1), 32, 43, 54–56, 128
CD36, 14
CD40, 55, 94–96, 135, 151, 152*f*, 154, 178–179
CD43, 136–137
CD44, 129, 197
CD45, 127, 129, 136–137
CD48, 192
CD49, 128–129
CD50 (ICAM-3), 93
CD54 (ICAM-1), 41, 129
CD55, 32, 320
CD56, 204

VetBooks.ir

CD58, 129, 192
CD62E (E-selectin), 41
CD62L (L-selectin), 125
CD64 (FcγRI), 54–56, 128
CD72, 157
CD79, 149, 150*f*
CD80, 94, 135, 178–179
CD86, 93–94, 135, 140, 192
CD89 (FcαR), 125, 128
CD95, 188, 192–193, 193*f*, 197, 203, 218, 403
CD95L (fas-ligand), 73, 192–193, 218, 373, 382–383, 393
CD106, (VCAM_1), 129
CD120 (TNF-αR), 128
CD121 (IL-1R), 20
CD126 (IL-6R), 125
CD134, 453–454
CD152, CTLA-4
CD154, 55, 73, 135, 151, 178–179
CD158, *See* KIR
CD163, 218
CD206, 17, 54
CD209 (DC-SIGN), 93
CD210 (IL-10R), 128
Cecum, 225
Cell-mediated cytotoxicity, 193–194, 193*t*, 200–206
Cell-mediated immunity
  antibacterial, 288

VetBooks.ir

antifungal, 296
antiviral, 302
colostrum, 257–258
features, 6–7
measurement, 374–376
in vitro, 374
in vivo, 374–376
to helminths, 319–320
to protozoa, 312
Cell membranes, exchange, 154*b*
Centroblasts, 113
Ceramide, 75–76, 192, 343
Cerebellar degeneration, 412
Ceruloplasmin, 65
Checkpoint inhibitors, 391
Chédiak-Higashi Syndrome, 307, 436, 436*f*
Cheetahs, 70*b*, 107
Chemoattractants, 42
Chemokines
classification, 20, 21*f*
functions, 19–22, 73, 93, 115–116
nomenclature, 21*t*
receptors, 43, 92
Chemotaxis, 42
Chicken lymphoid tumors, 400
Chimera, 208, 208*f*, 268, 383
Chimpanzees, 172

VetBooks.ir

Chitin, 322, 325, 344
Chitinases, 316, 329
Chlamydophilia, psittaci, 291–292
Chondroitin sulfate, 327
Chromium release assays, 374, 375*f*
Chronic active hepatitis, 421–422
Chronic airway obstructive disease, 360*f*
Chronic graft rejection, 379–380
Chronic granulomatous disease, 447
Chronic superficial keratitis, 429
Chytridiomycosis, 497*b*
Circadian rhythms, 54*b*, 220
Circovirus infections, 455
Citrullinated antigens, 431
Citrulline, 431
Class II-associated Ii peptide (CLIP), 97
Class switch, 248
Classically activated macrophages, M1 macrophages
Clonal anergy, 210–211
Clonotype, 154
Clostridial clusters, 228, 228*f*
Clostridium difficile, 230–231
Clostridium perfringens, 255*f*, 261–263, 285
Clostridium tetani, 83, 261–263, 285
Clusterin, 32
Coccidioidin, 370
Coccidiosis, 315

VetBooks.ir

Codfish, 496*b*
Coggins' test, 482
Coiling phagocytosis, 44
Cold agglutinins, 418
Collagens, antibodies to, 416–417, 430–431
Collagenase, 40–41, 52
Collectins, 9, 17
Colony-stimulating factors, 73, 391–392
Colostrometer, 256
Colostrum
  absorption, 254–255
  antibodies in, 276
  cell-mediated immunity and, 257–258
  composition, 253, 254*f*, 254*t*
  hemolytic disease, 349
  micro RNAs, 255
Commensal bacteria, 2
Common variable immunodeficiency, 441
Comparative cervical test, 370
Competitive immunoassays, 472–473, 473*f*
Complementarity determining region (CDR), 134, 148–149, 174, 178, 179*f*, 248
Complement system
  activation, 27–32, 349
  allotypes, 430
  bacterial killing, 286
  basic features, 26

VetBooks.ir

B cells, 151
blood coagulation, 33–34
cell removal, 33
chemotaxis, 34
classical pathway, 63, 384
components, 28*t*
consequences of activation, 33–35
convertases, 28
C5a, 23, 42, 51–52, 65, 359
deficiencies, 35–37, 365–366, 424–425
fish, 494
fixation test, 264, 485, 486*f*
functions, 27*f*
genes, 35
immune regulation, 34–35
inactivators, 32
inflammation, 25, 33
inhibitors, 31–32, 290, 322–323
invertebrates, 492
opsonization, 33, 44
pathways, 27*f*
  alternative, 27–29, 28*f*, 296, 322–323
  amplification, 31–32
  classical, 30–31, 287
  lectin, 29–30
proteins, 27
receptors (CR), 32–33, 43, 54–55, 128, 326, 343

VetBooks.ir

regulation, 32–33, 384
viruses, 301
Complotype, 35–36
Conduits reticular, 113–115
Concanavalin A, 130, 257–258, 374
Conglutinin, 9, 63, 299
Constant domains
immunoglobulin, 149
MHC, 101–102
TCR, 132–134
Contact allergens, 373*b*
Contagious ecthyma, 265
Convertase, C3, 28, 30*f*
Coonhound paralysis, 411
Copper deficiency, 456–457
Core antigens, 294
Core vaccines, Essential vaccines
Cornea, allografts, 382–383
Coronavirus, 258
Cortex
bursa, 111
lymph node, 113, 117–118
thymus, 109–110
Corticosteroids, 56, 78, 99, 304, 339, 346, 431, 464–465, 464*b*, 464*f*
Corticotrophin-releasing factor, 219
Corynebacterium diphtheriae, 25
Corynebacterium pseudotuberculosis, 288, 291, 366

VetBooks.ir

Costimulation
of B cells, 150–151
of T cells, 134–136, 191
Coughing, 2–3
Coxiella burnetii, 287–288
CpG nucleotides, 14–15, 53–54, 211, 268–269, 272–273
CR, Complement receptors
CR1, 32
CRIg, 32
CR2 (CD21), 32–33, 326
CR3, 33
CR4, 33
C-reactive protein, 62–63, 62*f*
Cross-matching, 354–355
Cross-priming, 99
Cross-reactions, 88*t*
Cruciate ligament rupture, 433
Cryptdins, 25
Cryptococcus neoformans, 17, 58–59, 296
Cryptopatches, 226, 243
Cryptosporidium parvum, 25, 311–312, 314, 439
CTLA-4, (Cytotoxic T lymphocyte antigen-4), 135–136, 193, 213, 229–230, 391, 395
Culicoides midges, 107, 344
Cutaneous basophil hypersensitivity, 322, 369, 369*f*
Cutaneous histiocytosis, 99
CXCL8, interleukin-8

VetBooks.ir

CXCR2 defects, 43
Cyclin B1, 391
Cyclooxygenase, 19., 23–24, 61
Cyclophilin, 466–467
Cyclophosphamide, 307, 383, 465
Cyclosporine, 79, 99, 346, 382–383, 418, 466–467, 467*f*
Cyclostomes, immunity in, 493–494
Cysteinyl leukotrienes, 24
Cysticercus bovis, 316
Cytidine deaminase, 176, 176*f*, 179–180, 243–244
Cytochrome C, 189, 192
Cytokines
  assays, 374–375
  costimulatory, 136
  classification, 74*t*
  effects on macrophages, 197*t*
  functions, 73–74
  group I, 74
  group II, 74
  group III, 74
  group IV, 74
  nomenclature, 72–73
  properties, 73*b*
  receptors, 74–76, 74*f*, 128
  regulation, 76–77, 302–303
  signal transduction, 77–80
  sources

VetBooks.ir

dendritic cells, 93
eosinophils, 334*t*
macrophages, 51
mast cells, 329, 334*t*
neutrophils, 46–47
sentinel cells, 19–22
T cells, 151
storm, 65, 68–69
structure, 74
therapy, 469–470
Cytomegalovirus, 204
Cytotoxic drugs, 465–466
Cytotoxic T cells, Tcells, cytotoxic
Cytotoxicity tests, 485

D

Dal blood group, 354
Damage-associated molecular patterns (DAMPs), 9–10, 15–16, 18, 51, 380
Dapsone, 416
DC-SIGN (CD209), 9, 92–93, 296, 468
Death-inducing signaling complex, 192–193
Death receptor, 192–193
Decay accelerating factor, 32, 320
Dectins, 9, 296
Defective neutrophil functions, 435–438
Defense collagens, 29*b*
Defensins, 22, 24–25, 46, 51–52, 220, 222–223, 239–240, 290, 299

Deficiencies, immunodeficiencies
canine C3, 35–36
leukocyte adhesion, 436–438
mannose binding lectin, 37
porcine factor H, 36–37
Degenerative myelopathy, 412
Delayed hypersensitivity reactions, 197, 367–368
Delayed immunoglobulin synthesis, 441, 445
Demodectic mange, 322, 344
Demyelinating leukoencephalomyelitis, 450
Dendrites, 90
Dendritic cells
basic features, 7, 17, 90–95
bovine, 95
canine, 95–96
classical, 89
cDC1 cells, 94
cDC2 cells, 94, 140, 329
equine, 95
feline, 96
follicular, 92, 117, 138, 212, 244
immature, 92–93
intestinal, 241*f*, 246
lymph nodes, 113–116
mature, 61, 92–93, 93*f*
old age, 460–461
origin, 90

VetBooks.ir

pigs, 95
plasmacytoid, 92, 299
skin, 92
structure, 90
subpopulations, 90–92, 91*f*
classical, 90–91
cDC1 cells, 94–95, 288
cDC2 cells, 94–95
follicular, 90–91
Langerhans cells, 90–92
plasmacytoid, 90–91
tolerance induction, 93–94, 218, 385
Dense deposit disease, 36
Deoxyadenosine, 292
Deoxyguanosine, 14
Deoxynivanol, 455
Dermatitis
allergic contact, 371–374, 372*f*, 373*t*
atopic, 340–343
herpetiformis, 416
lupus, 427
Dermatomyositis, 405–406, 421, 421*f*
Dermatophagoides mites, 341
Desmocollin, 415
Desmoglein, 414–415
Devil facial tumor disease, 398
Dexamethasone, 465

VetBooks.ir

Diabetes mellitus, autoimmune, 410–411
Diacylglycerol, 75, 76*f*, 79, 328
Diarrhea, 2–3, 339
Dicer, 301
Dichelobacter nodosus, 294
Dichlorvos, 372, 372*f*
Dictyocaulus viviparous, 276, 321
DiGeorge anomaly, 447
Direct fluorescent antibody tests, 473, 473*f*
Dirofilariasis, 344, 365
Discoid lupus erythematosus, 428
Disposable immunoassay devices, 478–479
Disseminated intravascular coagulation (DIC), 66–68, 67*f*, 306, 349
DIVA (Differentiate between immunized and vaccinated animals) vaccines, 267
DLA complex, 101
DNA
  bacterial, 14
  ligase, 176
  nets, 292
  synthesis inhibitors, 466
  vaccines, polynucleotide vaccines
DNA-dependent protein kinase, 440, 440*f*, 443
Dogs, Canine
  acute-phase proteins, 65
  allergies, 336–345
  anaphylaxis, 338

VetBooks.ir

blood groups, 354
complement deficiency, 35–36
cutaneous histiocytosis, 99
dendritic cells, 95–96
enteropathies, 232
food allergies, 339
hemolytic disease, 354
histiocytoma, 99
immune system development, 249
immunodeficiencies, 443–446
immunoglobulin genes, 182*t*
leukocyte antigens (DLA), 101
lymphocytes, 130
lymphoid tumors, 400
microbiota, 223*f*, 225*f*
NK cells, 205–206
Peyer's patches, 112
retroviruses, 455
severe combined immunodeficiency, 443–444
systemic histiocytosis, 99
T cell deficiency, 445–446
vaccination, 280–281
Dolphins, lymph nodes, 118
Dopamine, 329, 336–338
Dot blot, 477–478
Drugs, hypersensitivities to, 343–344, 356, 366, 373
Dscam, 492

VetBooks.ir

Duration of immunity, 276–277, 277*t*
Dysbiosis, 230–232, 407

VetBooks.ir

E

Early secretory antigenic target (ESAT), 367, 374–375
East Coast Fever, 312
Echinococcus granulosus, 316, 321
Edrophonium chloride, 419–420
Effector sites, intestinal, 242–243
Efferocytosis, 57–58
Egg yolk, antibodies, 472
Ehrlichia canis, 159, 427
Eicosanoids, 23–24
Eimeria, 315
Eimeria bovis, 43–44, 44*f*
ELA system, 101
Elastase, 40–41, 52
Electrophoresis, serum, 163, 163*f*
Elimination diets, 339
ELISA assays
  antibody sandwich, 475–476, 476*f*
  antigen capture, 452
  bidirectional flow, 478–479, 479*f*
  competitive, 476, 477*f*
  dipstick, 257
  membrane filter test, 257, 309, 452
  microwell, 474–476, 475*f*
  indirect, 474–475
  interferon release assay, 374–375

VetBooks.ir

labeled antigen, 476, 476*f*
rapid, 277
sandwich, 374–375
tests, 345–346, 454
ELISpot assay, 375–376, 376*f*, 476
Ellipsoids, 118–119
Endogenous antigens, 90
processing, 188*f*
Endometrial cups, 386
Endorphins, 220
Endosomes, 52
Endotoxins, Lipopolysaccharides
Endurance races, 459
Enhancement, tumor, 393
viral disease, 305–306
Enhancer elements, 80*f*, 81
Enkephalins, 220
Enterochelin, 64, 287
Enterocytes, 228–229, 239–240
Enteropathy, 232–233
Enterotoxins, 68
Enterococcus faecalis, 32
Environmental allergens, 341
Environmental toxins, 455
Enzyme-linked immunosorbent assay, ELISA
Eosinophilic asthma, 339–340
Eosinophilic enteritis, 345

VetBooks.ir

Eosinophilic granuloma complex, 344–345, 345*f*
Eosinophilic plaques, 342
Eosinophilic ulcers, 345
Eosinophils
  activation, 332
  cationic protein, 316–317, 333–334, 344
  cytokine release, 334*t*
  degranulation, 332–334
  extracellular NETs, 317, 332–333
  functions, 317, 317*f*, 332–334
  granules, 317, 332–333
  major basic protein, 316–317, 333–334
  mediators, 332–334, 333*f*
  mobilization, 333*f*
  neurotoxin, 316–317, 333–334, 344
  peroxidase, 333–334, 345
  receptors, 316
  resistance to helminths, 316–317
  structure, 332*f*–333*f*
Eotaxins, 316, 332
Epidermal barrier dysfunction, 343
Epidermolysis bullosa acquisita, 416–417
Epigenetic regulation, 185–186
Epigonal organ, 495
Epinephrine, 220, 330–331, 336–338, 346
Epistatic effects, 353
Epithelioid cells, 58–59, 371

VetBooks.ir

Epitopes, 86–87
Epitope spreading, 405
Equibactin, 292
Equine, horse
Equine infectious anemia, 305, 308, 482
Equine recurrent uveitis, 412–413
Equivalence zone, 481–482
Erysipelothrix rhusiopathiae, 294
Erythema multiforme, 373
Erythrophagocytosis, 417
ESAT-6, 367, 374–375
Escherichia coli, 17, 31, 46, 56*f*, 63, 291–293
Ethyleneimine, 264–265
Evasion of the immune response by
  bacteria, 289–293
  helminths, 320
  protozoa, 313–314
  viruses, 302–305
Exchange transfusion, 351
Exercise and immunity, 459–460, 459*f*
Exocytosis, 332–333
Exfoliative lupus dermatitis, 427
Exogenous antigens, 90
  processing, 96–99
Exosomes, 51–52, 92, 255, 381
Exotoxins, 83
Experimental allergic encephalomyelitis, 273, 411

VetBooks.ir

Extracellular invaders, 4–5
Extracellular traps
  eosinophils, 332–333
  macrophages, 52
  neutrophils, 43–44, 360–361
Exsheathing antigens, 317
Extrinsic atopic dermatitis, 340–341

F

Fab exchange, 167–168
Fab region, 148–149, 148*f*, 167
Fab'2 fragment, 263
Factor H, 290–291
Failure of passive transfer, 442
  diagnosis of, 256–257
  management of, 257
  tests for, 256–257
F antigens, 83
Farmer's lung, 360
Fas-ligand, *See* CDL
Fasciola hepatica, 320–321
Fatty acids
  immunosuppressive, 465*b*
  omega-3, 346
  omega-5, 346
Fc receptors
  basic features, 128, 128*t*, 149

VetBooks.ir

B cells, 128
basophils, 332
dendritic cells, 92, 128
eosinophils, 316
FcαRI, 128, 244–245
FcεRI, 128, 316, 325–326, 326*f*, 332
FcεRII (CD23), 128, 325–326
FcγR1, 128, 128*t*
FcγRII (CD32), 128, 128*t*, 151, 258
FcγRIII, 128, 128*t*, 203, 212
FcγRIV, 128, 128*t*
Fcγ2R, 54–55, 128
FcRn, 103, 236, 238, 253–254, 255*f*
macrophages, 54–55
mast cells, 325–326, 327*f*
neutrophils, 43
NK cells, 203
parasites, 320
viral entry, 305–306
Fc region, 148, 148*f*, 167, 263
Feline, cats
coronavirus, 306
Feline immunodeficiency virus, 452–454
diagnosis, 454
immunity, 454
immunosuppression, 453–454
pathogenesis, 453, 453*f*

VetBooks.ir

secondary infections, 453
vaccine, 454
Feline infectious peritonitis (FIP), 65, 87–88, 107, 306–307, 306*f*
Feline leukemia
anemia, 418
arthritis, 433
basic features, 451–452
diagnosis, 452, 478–479
immunity, 452
immunosuppression, 452
B cell defects, 452
T cell defects, 452
lupus, 427
panleukopenia, 449–450
pathogenesis, 451–452
polyarthritis, 433
rabies vaccine, 396–397
sarcoma, 396–398, 396*f*, 451
syncytia-forming virus, 433
tumors, 452
vaccines, 265–266, 396–397, 452
Fell pony immunodeficiency syndrome, 441–442
Ferritin, 63
Ferroportin, 63–64, 64*f*
Fever, 61, 62*f*, 108, 503–505, 504*f*
Fibrinogen, 65
Fibrinopeptide B, 42

VetBooks.ir

Fibroblast-like growth factor, 196–197
Fibroblasts, 58, 96
Fibronectin, 43, 55–56, 196–197
Fibroreticular cells, 113–115
Ficolins, 9, 29
Filaggrin, 343
Fimbriae, 83
Finnish-Landrace glomerulopathy, 365–366
Firmicutes, 224
Fish
  adaptive immunity, 494–496
  autoimmunity, 495*b*
  cell-mediated immunity, 496
  complement, 494
  cytokines, 496
  immunoglobulins, 495–496
  innate immunity, 3–4, 494
  lymphoid organs, 492
  MHC molecules, 496
Flagellin, 85, 272–273
Flea bite dermatitis, 322, 344, 369
Flocculation, 481
Flow cytometer, 125, 480–481, 480*f*–481*f*
Fluorescein isothiocyanate (FITC), 473–474
Fluorescent antibody tests, Immunofluorescence
Foal, horse
  failure of passive transfer, 257

hemolytic disease in, 350
immune system development, 248
immunodeficiency syndrome, 441–442
FOCMA antigen, 390
Folic acid antagonists, 465–466
Food
allergy to, 214, 338–339
immunity to, 246
responsive enteropathy, 233
Foot-and-mouth disease, 107
vaccines, 265, 271
Foreign material, fate, 55–57
Formylated methionine, 42
Formyl peptides, 15
Fowlpox, 268
Foxp3, 79, 227, 229, 393, 454
Fractalkine, 22
Framework regions, 148–149
Freund's complete adjuvant, 273, 404–405, 414
Fucose-mannose ligand, 315
Fumonisins, 455
Fungi
immunity to, 295–296, 295*f*
PAMPs, 296
Fusobacterium necrophorum, 291

G

VetBooks.ir

Galectins, 17, 213, 386
Gamma globulins, 163
Gammopathy
  monoclonal, 157, 159, 307
  polyclonal, 159, 425–426
Ganciclovir, 394–395
Gastrointestinal tract, immunity in, 239–241
Gelatinase, 40–41
Gel diffusion, Immunodiffusion
Gender differences, 279–280
Genes
  cloning, 265–267
  complement, 104
  conversion, 102, 104, 179–180
  gun, 269
  immunoglobulins, 175
  MHC molecules, 102
  rearrangement, 176–177
  recombination, 175, 183
  TCR molecules, 183
  transcription, 81
Genetic predisposition
  atopy, 340–341
  autoimmunity, 406–407, 417
  vaccine responses, 280–281
Genitourinary system, microbiota, 224
German Shepherd dogs

VetBooks.ir

diarrhea, 13*b*
pyoderma, 444–445
vaccination, 280
Germ-free animals, 226
German shepherd pyoderma, 446
Germinal centers, 92, 113, 115*f*, 156–157, 157*f*, 177–179, 211, 381
Ghrelin, 227–228
Giant cells, 50–51, 58–59, 371
Giardia sp., 314–315
gld mutation, 193, 403, 406
Glial fibrillary acidic protein, 412
Glomerulonephritis, 362–363
clinical features, 363–366
membranoproliferative, 305, 362–363
Glomerulopathy
Canine, 366
Finnish-Landrace, 364*f*, 365–366
Immunoglobulin A, 365
swine, 36, 365
Glucans, 469
Glucocorticoids, Corticosteroids
Glutamic acid decarboxylase, 410
Glutathione, 46
Glutathione peroxidase, 320
Glutathione-S-transferase, 317, 320–322
Gluten-sensitive enteropathy, 233
Glycocalyx, 240, 321

VetBooks.ir

Glycolipids, 354
Glycopeptidolipids, 51
Glycophorins, 417
Glycosyltransferases, 353
Gold salts, 431
G-proteins, 75, 76*f*
Graft rejection, 6
Graft vascular disease, 382
Graft-versus-host disease, 69, 248, 383–384, 383*f*–384*f*
Granulation tissue, 58–59
Granulocytes, classification, 39
Granulocyte colony-stimulating factor, 40, 438
Granulocyte-macrophage colony-stimulating factor (GM-CSF), 57, 95–96, 143, 145, 193–194, 386
Granulomas, 120*f*, 144–145, 289, 316, 319–320, 371
  pathogenesis, 58–59, 58*f*
  type I, 59
  type II, 59
Granulomatous meningoencephalitis, 412
Granulysin, 192, 197, 203, 205, 288, 373
Granzymes, 192, 288
Gray collie syndrome, 438
Group 1 species, 112–113
Group 2 species, 112–113
Growth factors, 73
Growth hormone deficiency, 445
GTP-binding proteins, G-proteins

VetBooks.ir

Guanosine triphosphate, 291
Gulf coast itch, 344
Gullain-Barré syndrome, 283, 411
Gut-associated lymphoid tissue (GALT), 227, 241

H

H antigens, 83
Haemonchus contortus, 316, 318, 320–321
Haemophilus, influenza, 292–293
Haemophilus parasuis, 17
Hair follicle diseases, 414
Haplotypes, 101, 410–411
Haptens, 82., 86–87, 86*f*
Haptoglobin, 63–65
Hassall's corpuscles, 109–111
HAT medium, 160
Hay fever, 338
Hay sickness, 360
Heart, allografts, 382
Heartworm infection, 344, 365
Heat shock proteins, 15–16, 53–54, 83, 92, 287–288, 291, 367, 404–405
Heaves, 360
Heavy chain disease, 157
Heavy chains, immunoglobulin, Immunoglobulin heavy chains
Helicobacter, 224
Helminths
  adaptive immunity, 316–320

VetBooks.ir

antigens, 321
allergies to, 245
defense molecules, 321
eosinophils and, 316–317
cell-mediated immunity, 319–320
intestinal immunity to, 245–246
evasion of immunity, 320
immunity, 315–322
innate immunity, 318*b*
microbiota and, 315–322
mast cells and, 245–246, 327
vaccination, 321–322
variation, 319
Helper T cells, *See* T helper cells
Hemagglutination inhibition, 309
Hematopoiesis, immune suppression of, 419
Hematoxylin bodies, 425
Hemoglobin A, 316
Hemoglobinuria, 349, 417–418
Hemolymph nodes, 118
Hemolysis, 417, 417*f*
Hemolytic anemia, 417–419, 425
Hemolytic disease of the newborn, 349, 350*f*
cats, 355
cattle, 315, 352
dogs, 354–355
horses, 350–351

VetBooks.ir

diagnosis, 351
management, 351
humans, 355
pigs, 353–354
Hemopexin, 64
Hemophagocytic syndrome, 355
Heparan sulfate, 93
Heparin, 327, 329, 336–338
Hepatocytes, 62
Hepcidin, 63–64, 64*f*
Herd immunity, 275
Hereditary parakeratosis, 442
Herpesviruses, 305
Heterozygote advantage, 106
Hibernation, 505
High endothelial venules, 113, 115*f*, 117, 128–129
High mobility group box protein-1 (HMGB-1), 15, 16*f*, 51, 61–62, 65, 93, 189, 380, 431
Histamine, 23, 41, 319, 327, 329, 331–332, 336, 342
Histocompatibility antigens, MHC antigens
Histiocytes, 50
Histiocytic ulcerative colitis, 233
Histiocytoma, 99
Histiocytosis, 99
Histones, 185
Histoplasmin, 370
HMGB1, High mobility group box protein-1

Hog cholera vaccination, 353–354
Horseradish peroxidase, 478–480
Horses
  acute-phase proteins, 65
  allergies, 339
  anaphylaxis, 336
  asthma, 360–361
  blood groups, 350
  common variable immunodeficiency, 441
  dendritic cells, 94
  EqWC1, 95, 129
  foal immunodeficiency syndrome, 441–442
  hemolytic disease
    diagnosis, 351
    management, 351
  immune-mediated hemolytic anemia, 418–419
  immune system development, 248
  immunodeficiencies, 440*f*
  immunoglobulin deficiencies, 441
  immunoglobulin genes, 174
  immunoglobulins, 170
  keratitis, 429
  leukocyte antigens (ELA), 101
  lymphocytes, 129
  lymphosarcomas, 400
  MHC, 102*f*, 105*f*, 107, 107*b*
  NK cells, 204

VetBooks.ir

polyneuritis, 411
regulatory T cells, 215
sarcoids, 398, 468–469
severe combined immunodeficiency, 439–442
tuberculin tests, 370
uveitis, 412–413
Hot spots, 341
House dust mites, 340, 341*f*, 342
Humans
hemolytic disease, 355
leukocyte antigens (HLA), 101
immunodeficiencies, 447–448
Humoral immune response, *See* antibody mediated immunity
H-Y antigens, 378
Hybridomas, 159–161, 161*f*
Hydrogen bonds, 174
Hydrogen peroxide, 42, 45–46
5-hydroxytryptamine, Serotonin
Hydatid disease, 344
Hygiene hypothesis, 251, 335–336, 336*f*
Hyperacute graft rejection, 379–381
Hypereosinophilic syndrome, 345
Hypergammaglobulinemia, 308
Hypersensitivity pneumonitis, 360–361
Hyperthyroidism, 410
Hypersensitivity, type I, (allergies)
allergic disease, 336–345

VetBooks.ir

in autoimmunity, 407
breed predisposition, 324–325, 340–341
cytokines in, 334*t*
diagnosis, 345–346
induction, 324–325
milk allergy, 407
to arthropods, 322, 344
to bacteria, 294
to drugs, 343–344
to environmental allergens, 341
to fleas, 322, 344
to helminths, 318, 344
to protozoa, 314
to vaccines, 281–282, 343–344
to viruses, 305
to yeasts, 343
treatment, 342*b*, 346–347
Hypersensitivity type II
in autoimmunity, 407–408
to bacteria, 294
to drugs, 356
in infectious diseases, 356
polyarthritis, 366
to protozoa, 314
to red cells, 348
to viruses, 305
Hypersensitivity, type III

VetBooks.ir

in autoimmunity, 408
classification, 357–358
dietary, 366
generalized reactions, 361–363
infectious diseases, 364*t*
local reactions, 358–361
pneumonitis, 360–361
to bacteria, 294
to drugs, 366
to protozoa, 314
to vaccines, 282
to viruses, 305
vasculitis, 433–434
Hypersensitivity, type IV
delayed reactions, 367
in autoimmunity, 408
pathological consequences, 371–374
to bacteria, 294
to fungi, 296
to protozoa, 315
to vaccines, 282
Hypervariable regions
BCRs, 148–149
genes, 178
TCRs, 134
variable lymphocyte receptors, 493
Hyperviscosity syndrome, 158

VetBooks.ir

Hypoallergenic diets, 339
Hypochlorite, 45, 359
Hypobiosis, 315–316
Hypoderma infestation, 323
Hypogammaglobulinemia, 441
Hypothalamic-pituitary axis, 61–62, 220
Hypothalamus, 61
Hypothyroidism, 410
Hypotrichosis, 446, 446*f*

I

ICAM-1, 136–137, 192
ICAM-3, 93
Idiopathic hypereosinophilic syndrome, 345
Idiotypes, 168
IKK complex, 78, 464
Imidazoquinolines, 272–273
Imidiazoleacetic acid, 332
Immune checkpoint therapy, 193, 395, 395*f*
Immune-complexes, 92, 317, 357, 359–360, 362–363, 362*f*, 364*f*, 425, 454, 482
Immune-complex lesions, 306, 314, 358, 360, 364–366, 408, 429, 459
Immune elimination, 56, 237*f*, 245–246
Immune exclusion, 237*f*, 243–245
Immune globulins, 261–263
Immune-mediated hemolytic anemia, 283, 407–408, 417–419
  classification, 417–418, 418*t*
  diagnosis, 418

VetBooks.ir

Immune-mediated keratoconjunctivitis, 413–414
Immune-mediated vasculitis, 282, 305, 412, 433–434
Immune paralysis, 210
Immune reconstitution inflammatory syndrome, 67*b*
Immune responses
  adaptive, 4–7
  adverse consequences, 294
  innate, 3–4
  regulation, 211
  time course, 5, 6*f*
  type 1, 288–289
  type 2, 288–289, 314, 317, 332, 336
Immune-stimulating complexes (ISCOMS), 271, 281
Immune surveillance, 389
Immune system
  development, 248–251
  stimulation, 468–470
  suppression, 463
Immunization, vaccination
  active, 261, 263–265
  passive, 261–263
Immunochromatography, 479, 480*f*
Immunoconglutinins, 403
Immunodeficiencies
  B cells, 441–442, 444–445, 447–448
  cats, 446
  cattle, 437–438, 442

VetBooks.ir

combined, 439–444, 447
dogs, 436, 438, 443–446
horses, 439–442
humans, 447–448
immunoglobulin, 441–442, 444–445, 447–448
mice, 446–447
pigs, 442–443
primary, 435
secondary, 449
T cells, 445–447
variable, 441
virus-induced, 449
Immunodiffusion tests, 482, 483*f*
Immunoelectrophoresis, 160*f*, 483–484, 483*f*–484*f*
Immunoenhancing drugs, 479
Immunoenzyme assays, 474–478
Immunofluorescence assays, 427, 452, 453*f*, 473–474
Immunoglobulins, 162–163, 170–172
allotypes, 168, 168*f*, 170
camels, 172*b*
cats, 162–163, 172
cattle, 170–171, 171*f*–172*f*.
class switch, 169
classes, 162–167, 170*t*
colostral, 276
constant regions, 149
deficiencies, 441–442, 444–445, 445*b*

VetBooks.ir

diversity, 175, 180, 182*t*
dogs, 172
domains, 45, 148, 169–170
fish, 495–496
framework regions, 148–149
genes, 172
heavy chains, 148., 168–170, 180
  clans, 503
  genes, 168–170, 171*f*, 172, 175, 175*t*, 176*f*, 177
hinge region, 149, 167
horses, 170
idiotypes, 168
intravenous therapy, 411, 468, 468*f*
kappa chains, 148, 175
lambda chains, 148, 175
levels, 164*t*
light chains, 148, 163–164
  genes, 176–177
milk, 170–171
pigs, 172
primates, 172
rabbits, 168
receptors, 165
rodents, 172
sheep, 171
structure, 167–168
subclasses, 168

VetBooks.ir

variable regions, 148–149, 169
variants, 168
Immunoglobulin superfamily, 132, 133*f*
Immunoglobulin A
basic features, 165, 245*f*, 246
birds, 501–502
cervicovaginal mucus, 238
class switch, 243–245
colostrum, 253
costimulation, 243–245
deficiencies, 325, 444–445, 445*b*, 447–448
eggs, 502
intestinal, 229, 243–245, 318
levels, 243*f*, 243*t*, 249*b*
nephropathy, 365
proteases, 292–293
receptors, 128, 244–245
regulation, 244*f*
secretory, 237, 244
structure, 165*f*, 244
wolves, 445*b*
Immunoglobulin D
amphibians, 497
birds, 501
fish, 496
on B cells, 152
microbiota, 167*b*

VetBooks.ir

monotremes, 503
reptiles, 499
structure, 165–167, 166*f*
Immunoglobulin E, 325–326
allergies, 342–343
atopic dermatitis, 342–343
colostrum, 276
costimulation, 325
half-life, 165, 325
intestinal, 245–246, 245*f*
against parasites, 318
production, 325
receptors, 325–326
structure, 165, 165*f*
vaccines, 281–282
Immunoglobulin G
agglutination, 484*t*
basic features, 163–164
colostrum, 253, 256
deficiency, 441–442
half-life, 254–255
hemolytic anemia, 417
intestinal, 246
maternal, 253
receptors, 54–55, 103, 128, 128*t*, 151, 203, 212, 236, 238, 253–254, 255*f*, 258
structure, 164*f*, 167*f*

VetBooks.ir

Immunoglobulin M
agglutination, 484*t*
B cells, 152
birds, 501
complement activation by, 287
deficiency, 441, 444
foals, 439–440
hemolytic anemia, 417
intestinal, 245
opsonization, 287
structure, 164*f*
Immunoglobulin Y
birds, 472, 491–492, 501*f*
reptiles, 499
Immunohistochemistry, 478
Immunologic memory, 5, 7, 204, 461
Immunological "big-bang", 493–494, 494*f*
Immunological synapses, 136–137, 190
Immunological tests
diagnostic applications, 487–489
sensitivity, 488–489
specificity, 488–489
Immunoproliferative enteropathy, 233
Immunoreceptor tyrosine-based activation motifs (ITAMs), 79, 137, 150
Immunosenescence, 460–461, 460*f*
Immunostimulants, 468–470

VetBooks.ir

Immunosuppression
age related, 460
drugs, 464–466
myeloma, 158
nonspecific, 463–466
parasite-induced, 313, 321
pregnancy, 387, 470
radiation induced, 463–464
selective, 466–468
stress induced, 459, 461–462
ticks, 323
toxin-induced, 455
tumor mediated, 392–393, 392*f*, 462*f*
vaccine-induced, 282–283, 308
virus-induced, 449–451
Immunotherapy
allergies, 346
tumors, 393–396
active, 394
antibody-mediated, 395
approaches, 394*b*
checkpoint therapy, 395
cytokine therapy approaches, 394
passive, 394–395
Incompatible transfusions, 349
Incomplete antibodies, 484
Indoleamine 2,3-dioxygenase (IDO), 93–94, 136, 218, 288, 334, 382–

VetBooks.ir

383, 389
Indirect fluorescent antibody tests, 473, 474*f*
Infectious bursal disease, 111, 449
Infectious bovine rhinotracheitis, 250
Inflammasome, 12*b*, 13*f*, 46, 240

VetBooks.ir

Inflammation
acute, 18
adipose tissue, 456
chronic, 57–59
complement, 25
eosinophils, 332–334
fish, 494
immune-complexes, 358–361
macrophages, 17
mast cells, 17
mediators, 22–24
neutrophils, 38
resolution, 58*f*
signs, 19, 22, 22*f*
suppression, 463
systemic effects, 60
T cell induced, 367
Tumors, 390
Inflammatory airway disease, 361
Inflammatory bowel diseases, 232–233
Inflammatory myopathy, 420–421
Influenza
antigenic drift, 304
antigenic shift, 304
avian, 303
equine, 303
hemagglutinins, 303

VetBooks.ir

neuraminidases, 303
strains, 304*t*
virus, 303
Injection-site-associated sarcomas, (Sarcomas)
Innate Immunity
aged animals, 460–461
antimicrobial molecules, 3–4
basic features, 3–4, 9
complement system, 9
evasion, 289–292, 320
fish, 494
inherited defects, 435–438
in invertebrates, 491–492
macrophages, 49–51
mediators, 18
natural killer cells, 198
neutrophils, 38
on body surfaces, 234–241, 235*f*
recognition of invaders, 9
sentinel cells, 17
subsystems, 1, 3*f*
systemic responses, 60
to bacteria, 286–287
to fungi, 296
to helminths, 199, 315–316
to protozoa, 311
to viruses, 263–265

VetBooks.ir

Innate lymphoid cells, 127, 198, 199*f*
cytotoxic, 200–206, Natural Killer cells
group 1, 199, 199*f*
group 2, 199, 200*f*, 317–318, 339–340
group 3, 199–200, 200*f*, 229
helper, 199–200
lymph nodes, 115–116
Inosine monophosphate dehydrogenase inhibitors, 468
Inositol trisphosphate, 75, 79, 328
Insects
hypersensitivity to, 344
stings, 344
Insulin-dependent diabetes mellitus, 407
Integrins, 41–42, 51–52, 55, 117, 128–129, 136, 437
Intercellular adhesion molecule-1(ICAM-1), 129
Interferon-alpha, 12, 25, 73, 286, 299, 301, 470
Interferon-beta, 12, 73, 299
Interferon-delta, 299, 387
Interferon-epsilon, 299
Interferon-gamma, 73, 140, 141*f*, 154, 199, 251, 299, 370, 374–375, 376*f*, 387, 398, 467
macrophage activation, 53, 193–194, 196*f*, 288, 293, 302
NK cells, 53–54, 197, 203, 203*f*
Interferon-epsilon, 238
Interferon-kappa, 299
Interferon-lambda, 73, 92, 250–251, 299
Interferon-omega, 299, 387, 470

VetBooks.ir

Interferon-tau, 299, 387
Interferon-X, 24
Interferon-zeta, 299
Interferons
  antiviral activities, 299–301, 301*f*
  basic features, 73, 299
  measurement, 300*b*
  pregnancy, 385–387
  receptors, 300*f*
  therapy, 470
  type I, 12–13, 19, 61, 73, 92, 339
  type II, interferon-gamma
  type III, interferon-lambda
Interleukins, 72–73
Interleukin-1, 12, 19–20, 20*f*, 51, 60, 62, 93
  family, 19–20, 74
  receptor antagonist (IL-1RA), 18, 196
  receptors, 18, 92
Interleukin-2, 74, 140, 142*f*, 470
  receptor, 74*f*, 197, 443
Interleukin-3, 332
Interleukin-4, 140–143, 142*f*–143*f*, 151, 154, 289, 325
Interleukin-5, 140, 142*f*, 151, 199, 332, 333*f*
Interleukin-6, 12, 20, 21*f*, 60, 62, 390
  receptor, 20, 125, 151
Interleukin-7, 145–146
Interleukin-8 (CXCL8), 21–22, 51, 73, 324, 361, 431

VetBooks.ir

Interleukin-9, 140, 142*f*

Interleukin-10, 48, 93–94, 143–145, 196, 204, 213–215, 214*f*, 218, 293, 308, 370

family, 74

Interleukin-12, 53–54, 94–95, 136, 189–190

family, 74, 94–95, 140, 142–143

Interleukin 13, 24, 140–142, 142*f*, 151, 199, 318, 394–395

Interleukin-15, 145–146, 197

Interleukin-16, 368

Interleukin-17, 25, 40, 48, 136*f*, 143–144, 143*b*, 199–200, 216–218, 217*f*, 286, 371–372, 412, 424, 431

family, 74, 216, 361

Interleukin-18, 25, 51, 62

Interleukin-21, 143, 151

Interleukin-22, 25, 143–144, 199–200, 230, 235, 386

Interleukin-23, 38, 41, 48, 94–95, 136, 143–144, 199, 216*b*, 286

Interleukin-25, 317–318, 320*f*, 329

Interleukin-26, 81*b*

Interleukin-27, 94–95

Interleukin-31, 160–161, 342

Interleukin-33, 15, 140, 199, 317–319, 329, 330*f*, 338–340, 344, 371–372, 431

Interleukin-35, 94–95, 143–144

Interleukin-36, 20

Interleukin-37, 20

Interleukin-39, 94–95

International biological standard, 263

Intestinal dendritic cells, 229

VetBooks.ir

Intestinal epithelial cells, enterocytes
Intestinal inflammatory disease, 232–233
Intestinal permeability
Intestine, protective mechanisms, 243–246
Intracellular adhesion molecule-1 (ICAM-1), 51–52, 128–129
Intracellular bacteria, 288, 291–292, 292*t*
Intracellular invaders, 4–5
Intradermal skin tests, 56, 343*f*, 345, 374
Intraepithelial lymphocytes, 226, 242–243, 242*f*
Intranasal vaccines, 275
Intrapalpebral test, 370
Intrauterine infections, 250–251
Intravenous immunoglobulin therapy (IVIG), 411, 468, 468*f*
Intravenous johnin test, 370
Intrinsic atopic dermatitis, 340–341
Invariant (Ii) chain, 97, 103
Invertebrates
  adaptive immunity, 492
  antimicrobial peptides, 492
  graft rejection, 492
  immunity, 491–492
  innate immunity, 491–492
  phagocytosis, 491–492
  physical barriers, 491
  prophenoloxidase system, 491
  RNA interference, 492
IRAK, 78

VetBooks.ir

IRF3, 12
Iron binding molecules, 63–64
Iron deficiency, 456–457
ISCOMs, 271
ISG pathway, 300
Isoantibodies, 349
Isografts, 377
Isoproterenol, 330–331, 336–338
Ixodes ricinus, 323
Ixodes scapularis, 322–323

J

JAK-STAT pathway, 80, 140, 195–196, 300, 342, 406
  inhibition, 80*b*, 293, 299
J blood group antigen, 352
Janus kinases (JAK), 80
Jaundiced foal agglutination test, 351
J chain, 164–165
J genes, 74, 175*f*
J5 mutant vaccine, 236, 294
Johne's disease, 288–289, 289*f*–290*f*, 293–294
Johnin reactions, 370
Junctional diversity, 176–178
Juvenile polyarthritis syndrome, 432

K

K antigens, 82–83, 293

VetBooks.ir

Kai blood group, 354
Kallikreins, 23
Kaposi's sarcoma, 389
Kappa light chains, 66–67
Keratinocytes
  antibacterial peptides from, 235
  as antigen-presenting cells, 96, 235
  pattern-recognition receptors, 235
Keratoconjunctivitis sicca, 428–429
Ketoconazole, 382
Kidney, fish, 495
Kidney grafts, 379–382
Killer cell immunoglobulin-like receptors (KIR), 201–203, 205
Kindlin, 436–437
Kininogens, 18
Kinins, 18
Klebsiella pneumoniae, 31, 289–290, 405
Kupffer cells, 50, 55, 417–418

L

Lactobacilli, 255
Lactoferrin, 25, 40–41, 46, 57, 63, 236
Lactoperoxidase, 236
Lamb, immune system development, 248–249, 461
Lambda light chains, 65–66
Lamellipodia, 42
Lamin B, 436

VetBooks.ir

Laminitis, 68–69, 230–231
Langerhans cells, 9, 56, 92, 99, 212, 220, 235, 322, 341, 371–372, 382
Langerin, 92
Latency, 305
Late-phase reaction, 329, 331
Lateral flow chromatography tests, 309, 479
Latex agglutination test, 257
Lectin, mannose-binding, 9, 17, 29, 37, 43, 55–56, 63, 297
Lectin pathway, 29–30
Lectins, 16
  C-type, 16–18, 92, 201–203, 236–237, 299
  P-type, 16, 62–63
  S-type, 16
Leflunomide, 99, 382, 431, 468
Legionella pneumophila, 44
Leishmaniasis, 313, 427
Leishmania amazonensis, 43–44, 310–311, 315
Lentivirus, 308, 453*f*
Leptin, 62, 231, 456
Leptospira interrogans, 144–145, 405, 412–413
Leptospirosis, 295
Lethal dose 50, 486, 486*f*
Lethal hit, 192
Leukemia, 394
  acute lymphoid, 400
  chronic lymphoid, 400
  chronic myeloid, 400

VetBooks.ir

Leukocytes
Adherence deficiency, 33, 436–438
Basic features, 9
classification, 39
differentiation, 40*f*
origins, 39*f*
swarming, 42
Leukocyte function-associated antigen-1 (LFA-1), 41, 128–129, 136–137, 192, 201, 305, 378, 404
Leukocyte immunoglobulin-like receptors (LILR), 202–203, 205
Leukocytoclastic vasculitis, 433–434
Leukotoxins, 42, 291, 293
Leukotrienes, 23–24, 42, 57, 65–66, 328, 332, 336–338
LFA-1, *See* Leukocyte function associated antigen-1
Leydig organ, 495
Light chains, immunoglobulin, *under* Immunoglobulins
Light chain disease, 157–158
Limitin, 299
Linear IgA dermatosis, 416
Lipocalin 2, 64, 230
Lipopeptides, 206
Lipopolysaccharides, 10, 14, 53–54, 82–83, 130
Lipopolysaccharide-binding protein, 14, 63, 236
Lipoproteins, 206
Liposomes, 271, 272*f*
Lipotechoic acids, 10, 14, 82–83
Lipoxins, 57

VetBooks.ir

Lipoxygenase, 23–24
Listeria monocytogenes, 17, 46, 192, 194–196, 288, 291–292
Listeriolysin, 291–292
Liver, 55, 382
Liver fluke, 59
Lokivetmab, 342
Looping out deletion, 169
lpr (lymphoproliferative) mutation, 193, 403, 406
Lung, microbiota, 224
Lungworm vaccines, 321
Lupus band, 425, 425*f*
Lupus erythematosus (LE) cells, 412, 425, 425*f*, 427
Lupus polyarthritis, 432
Ly49, 201, 202*f*, 203–204
Lyme disease, 364–365
Lymph, 113
Lymphadenopathy, 453
Lymph nodes
  B cells, 113
  cortex, 113, 117–118
  dendritic cells, 113, 116*f*
  function, 115–117
  medulla, 113, 117–118
  paracortex, 113, 117–118
  species differences, 117–118
  structure, 113–115
Lymphoblasts, 138*f*

VetBooks.ir

Lymphocytes, 108
adherence molecules, 128–129
antibody receptors, 128
antigen receptors, 125–127
blood, 124*t*
B cells, 147
circulation, 117
colostral, 257–258
complement receptors, 128
cytokine receptors, 128
cytotoxic, 187
fish, 492
identifying features, 124*t*
immunoglobulin receptors, 128*t*
innate, 198
intraepithelial, 242–243, 242*f*
mitogens, 122
mucosal, 242–243
NK cells, 200
phenotypes, 129
populations, 123–124
receptors, 126*f*–127*f*
regulation, 127–128
sources, 109
species differences, 129–130
stem cells, 439*f*
structure, 122–123

VetBooks.ir

surface molecules, 125–129
T cells, 132
Lymphocytic pancreatitis, 411
Lymphocytic parathyroiditis, 410
Lymphocytic-plasmacytic enteritis, 232
Lymphocytic thyroiditis, 410
Lymphoglandular complexes, 56
Lymphoid, organs, 108
primary, 109–113
secondary, 113–121
tertiary, 121
Lymphoid leukosis, 109
Lymphoid tissue inducer cells, 200
Lymphoid tumors, 399–400, 403
Lymphokine-activated killer cells, 203, 391, 391*f*
Lymphomas
B cell, 389, 396
sheep, 400
T cell, 395
vaccines, 396
Lymphopenia, 454, 454*f*
Lymphosarcomas
bovine, 399–400
equine, 400
Lymphotactin (XCL1), 22, 145, 369
Lysosomal trafficking regulator, 436
Lysosomes, 46, 96, 99

VetBooks.ir

Lysozyme, 18, 40–41, 236, 299

LYST gene, 436

M

M cells, 229, 237, 241, 241*f*

M1 macrophages, 52–53, 194, 195*f*, 218, 273, 288, 293, 312, 371, 456

M2 macrophages, 52–53, 57, 57*f*, 194–197, 196*f*, 215, 218, 316, 386, 391–392

Mac-1, 436–437, 438*f*

Macroglobulinemia, 158

Macrophage inflammatory protein-1, 145

Macrophages

- activation, 215–216, 288, 300, 302, 312
- adipose tissue, 456
- aged, 460
- alternative activation, 196–197
- alveolar, 50, 56–57, 237–238
- antigen-processing, 96
- antitumor, 391–392
- colony-stimulating factor (M-CSF), 53
- classical activation, 53–54, 193–197
- cytokines from, 52*f*
- cytotoxic, 193–194
- extracellular traps (METs), 52
- feline infectious peritonitis, 306
- functions, 17, 51–55
- inflammatory, 51
- life history, 50–51

VetBooks.ir

lungs, 238
lymph nodes, 113
mannose receptor, 9, 218
newborns, 252
origins, 50–51
phagocytosis, 51–52
polarization, 52–53
products, 52
properties, 49–51
pulmonary intravascular, 50, 55, 55*f*, 238
receptors, 54–55
regulatory, 53, 218
rheumatoid arthritis, 431
sentinel cells, 51
structure, 50
MAdCAM-1, 116, 242
Maedi-visna, 304
Magnesium deficiency, 456–457
Major acute-phase protein, 65
Major histocompatibility complex (MHC), 90, 100–107, 107*b*
allograft rejection, 378–379
antibodies to, 386
antigen binding sites, 102, 103*f*, 104
autoimmunity, 406–407
body odors, 107
cattle, 105*f*
cats, 105*f*

VetBooks.ir

cell expression, 103
class Ia molecules, 90, 101–103
allograft rejection, 378
antibodies to, 352
antigen-processing pathway, 97–99
CD-binding, 189
genes, 102
graft versus host disease, 383
missing, 200
molecules, 378
nonpolymorphic molecules, 385–386
polymorphism, 102, 106
structure, 101–102
Class II molecules, 90, 103–104, 111
allograft rejection, 378, 381
antigen-processing pathway, 96–97
B cells, 150
genes, 104
graft versus host disease, 384
polymorphism, 104
structure, 103
Class III molecules, 104
region, 35
disease associations, 104–107
dogs, 105*f*
domestic animals, 104
gene loci, 102

VetBooks.ir

graft rejection, 103
haplotypes, 101, 410–411
horses, 102*f*, 105*f*
nonpolymorphic MHC Class 1 molecules, 102–103
odors, 107
pigs, 105*f*
regions, 100–101
sperm counts, 107*b*
MHC class II compartment (MIIC), 89
Malassezia pachydermatitis, 341, 343
Malignant catarrhal fever, 399
Mallein, 370
Malnutrition and immunity, 455–459
Mammary gland, immunity in, 236
Mange, 322
Mannheimia hemolytica, 25, 52, 219, 264–265, 291–293, 305, 449–451
Mannose-binding lectin, 9, 17, 29, 37, 43, 55–56, 63, 297
Mannose-binding receptor (CD206), 54, 92
Mannosylated lipoarabinomannan, 293
MAP kinase (MAPK), 79–80, 137, 289–290
Macaques, 450–451
Marek's disease vaccine, 264, 395–396
Marek's tumor-specific antigen, 390
Maresin, 57
Marginal zone, spleen, 119
Marker vaccines, 267
Marsupials

VetBooks.ir

immune system, 502–503
immunoglobulins, 503
Mast cells
connective tissue, 327
degranulation, 328, 328*f*–329*f*
infections, 331
intestinal, 245
life history, 327–328
location, 327
mediators, 329, 330*f*
mucosal, 327
receptors, 325–326
regulation, 330–331
response to antigen, 328–331
response to parasites, 318, 319*f*
sentinel cells, 17, 326–327
signal transduction, 328*f*
structure, 327, 327*f*
types, 327*t*
Mastitis, vaccines, 236
Maternal antibodies, 212, 212*f*, 258–260, 258*f*, 269, 276, 279, 454
Matrix gel agglutination, 484
Medulla
bursa, 111
lymph node, 113
thymus, 111
Megaesophagus, 419–421

VetBooks.ir

Melanin, 491
Melanocytes, 413, 413*f*
Melanoma
  swine, 398
  treatment, 394, 469
  vaccine, 396
Melanomacrophage centers, 495
Melphalan, 158–159
Membrane attack complex, 31, 192
Membranoproliferative glomerulonephritis, 362–363, 425
Memory cells
  aged, 461
  B cells, 5
  NK cells, 204
  T cells, 7
Mesangial cells, 362–363
Metalloproteases, 431
Methotrexate, 383–384, 431, 465–466
Methylprednisolone, 465
MIC-A, 103, 144, 203, 380
MIC-B, 103, 144, 203
Mice
  beige, 436
  immunodeficiencies, 446–447
  Moth-eaten mice, 447
  nude, 389, 446–447
  SCID mice, 447

VetBooks.ir

X-linked immunodeficiency, 447
Microbiome, 222
Microbiota, 182
allergic disease, 335–336
autoimmunity, 407
B cell repertoire, 182
blood groups, 349*b*
cancer surveillance, 391*b*
dysbiosis, 230–232
functions, 225–228
gastrointestinal tract, 224–225, 249
graft-versus-host disease, 384
IgA and, 245
IgD and, 167*b*
IgE and, 336
immune responses to, 228–230
immune system development, 226–227
importance, 222*f*
inflammatory bowel diseases, 232–233
large intestine, 225
locatiom, 222–225
newborns, 251
nutritional role, 225
odors, 232
nutritional role, 225
rabbits, 182
regulation of B cells by, 229

VetBooks.ir

regulation of T cells by, 229–230
respiratory tract, 223–224
rheumatoid arthritis, 429
rumen, 224–225
signals to the body, 227–228
skin, 222–223, 343
T cell functions, 229–230
Microchimerism, 405–406
Microglia, 50
Microparticles, 272
MicroRNAs, 185, 255
Microvesicles, 255
Milk
allergy to, 407
antimicrobial molecules in, 236
cells in, 236, 258
immunoglobulins, 170–171, 236, 255
ring test, 295, 295*f*
whey, 263
Mink
Aleutian disease, 159, 304, 307, 307*f*
antisperm antbodies, 409–411
Missing-self strategy, 200
Mitochondria, 15
Mitogens, 130, 130*b*
Modified live vaccines, 264, 277
Molecular mimicry, 404–405

VetBooks.ir

Monoclonal antibodies
antitumor, 395
atopic dermatitis, 342*b*
checkpoint inhibitors, 395
gammopathy, 157, 159, 307
passive immunization with, 263
production and use, 161*f*
serologic tests using, 374–375, 472
treatment with, 469–470
Monocyte chemoattractant protein-1, 51–52
Monocytes, 50, 90–92
Mononuclear phagocytic system, 50, 50*f*, 91–92
Monotremes
immunity in, 502–503
immunoglobulins, 503
immunoglobulin O, 503
lymphoid organs, 502–503
Moraxella bovis, 291
mTOR pathway, 218
Mucocutaneous lupus, 427
Mucosa-associated lymphoid tissue, 241–243
Mucosal disease, 250–251, 251*f*
Mucus layer, 237–239, 240*f*
Multiantigen print immunoassay, 478
Multibacillary disease, 288–289
Multiple myelomas, Myelomas
Multiple organ dysfunction syndrome, 68

VetBooks.ir

Muramyl dipeptide, 13, 273, 315, 468–469
Mutation, MHC, 102
Mx guanosine triphosphatase, 300
Mx proteins, 300
Myasthenia gravis, 403, 419–420, 420*f*
Mycobacterium avium, 111, 286–287, 370
Mycobacterium avium paratuberculosis, 25, 293, 370
Mycobacterium bovis, 289, 369
Mycobacterium phlei, 370
Mycobacterium tuberculosis, 44, 53–54, 58–59, 63, 192, 194, 288–292, 369
Mycophenolate mofetil, 383, 418, 468
Mycoplasma haemofelis, 418
Mycoplasma hyopneumoniae, 405
Mycoplasma mycoides, 291, 405
Mycotoxins, 455
MyD88, 12, 78, 312, 407, 467–468
Myelin, 411
Myeloid-derived suppressor cells, 386, 393
Myeloid stem cells, 40, 50–51
Myeloma, 70, 157–159, 158*f*, 212, 483
Myeloperoxidase, 40–41, 45
Myositis, 420–421

N

NADPH oxidase (NOX), 45
Nanonets, 240
Nanoparticles, 272, 272*f*

VetBooks.ir

Natural antibodies, 85, 349
Natural killer (NK) cells
activation, 53–54, 61, 92, 202*f*
allograft rejection, 380–381
antibacterial, 286, 288
antiprotozoal, 312
antitumor, 390–391
antiviral, 301
basic features, 200
contact dermatitis, 372
corticosteroids, 465
deficiency, 355
development, 124
effector mechanisms, 192, 200
evasion of, 303
functions, 103, 140, 203–206, 204*f*, 249
graft rejection, 380
location, 200
memory, 204
morphology, 200
origins, 200
receptors, 201–203, 202*f*
regulation, 300
species differences, 200, 204, 205*f*
structure, 201*f*
subsets, 204
target cell recognition, 200–201

VetBooks.ir

trophoblast, 385–386
tumor destruction by, 390–391
uterine, 386
Natural killer T (NKT) cells, 206
Natural suppressor (NS) cells, 218
Natural Treg cells, 213
N'Dama cattle, 311
Necroptosis, 15, 189
Necrotizing leukoencephalitis, 412
Necrotizing meningoencephalitis, 412
Negative acute-phase proteins, 65
Negative selection, 111, 208–210, 209*f*
Neisseria gonorrhoeae, 292–293
Neonatal immunoglobulin receptor, FcRn
Neonatal isoerythrolysis, Hemolytic disease of the newborn
Neonatal pancytopenia, 283, 352–353
Neonatal purpura, 354
Neonatal thrombocytopenia, 351, 354
Neospora caninum, 204–205
Nephrotic syndrome, 364–365
NETosis, 43–44, 424–425
Network transmission, 72
Neural regulation of immunity, 219–220
Neuritis of the cauda equina, 411
Neurokinin, 19, 220
Neuropeptides, 220
Neutering effects, 406*b*

VetBooks.ir

Neutralization, tests, 486
Neutropenia, immune-mediated, 419
Neutrophilia, 39–40
Neutrophils, 39–41
  activation, 42
  adhesion, 43
  apoptosis, 58*f*
  Arthus reaction, 358
  changes in, 41
  chemotaxis, 42–43
  corticosteroids and, 464–465
  cytokines from, 46–47
  defective function, 438
  destruction, 45–47
  emigration, 41–42
  enzymes, 46
  extracellular traps (NETs), 43–44, 44*f*, 292
  fate, 47–48
  granules, 14, 40–41
  ingestion, 44
  opsonization, 43
  origins, 39–40
  phagocytosis by, 42–47
  receptors, 47, 47*f*
  respiratory burst, 45–46
  structure, 40–41
  swarming, 42

VetBooks.ir

in synovial fluid, 431
Newborn animals
immunity in, 251–253
adaptive immunity, 251–252
innate immunity, 252–253
vaccination, 259–260
Newcastle disease vaccines, 268
New Zealand black mice, 403–404, 407, 424
NF-κB, 12, 76, 78, 78*f*, 137, 210, 216, 219, 289–290, 312, 336, 346, 464
NF-AT pathway, 79–80, 137, 216
Nitric oxide, 23, 52
Nitric oxide synthase, 19, 52–54, 65–66, 195–196, 288
Nitrogen dioxide, 53
NKG2D receptors, 201, 202*f*, 203, 286
NK-lysin, 204–205
NK2 cells, 204
NOD-like receptors, 13–14, 299
Noncovalent bonds, 174*f*
Nonobese diabetic (NOD) mice, 407
Nonsteroidal anti-inflammatory drugs, 431
Norepinephrine, 220, 330–331
Noroviruses, 232
Nuclear factor-kappa B (NF-κB), 150
Nucleic acids, viral, 15
Nucleotide oligomerization domain-like receptors, Nod-like receptors

O

VetBooks.ir

O antigens, 82–83
Obesity, 230–231, 456
Obstructive pulmonary disease, 361
Oclacitinib, 80, 342, 346
Occludin, 343
Ocular squamous cell carcinoma, 398, 468–469
Odors, 232
2′5′-oligoadenylate synthetase, 300, 300*f*
Onchocerca cervicalis, 412–413
Oncofetal antigens, 389
Onychodystrophy, 427
Ophthalmic test, 370
Opportunistic pathogens, 2
Opsonins, 43, 287
Opsonization, 43
Optimal proportions, 481–482
Oral tolerance, 214, 338–339
Organ grafts, 377–378
Ornithine, 196
Osteodystrophy, vaccine-induced, 283
Osteopontin, 196–197
Overdominant selection, 106
Oxalobacteriaceae, 223

P

Pancytopenia, bovine neonatal, 352–353
Paneth cells, 25, 239–240, 240*f*, 318

VetBooks.ir

Pannus, 429
Papillomas, 398
Paracortex, 113
Parainfluenza-3, 450
Parasites, Protozoa, Helminths and Arthropods
  allergies to, 344
  evasion of the immune response by, 313–314, 320
Parentage testing, 355
Particle concentration fluorescence assays, 474, 474*f*
Passive cutaneous anaphylaxis, 345, 345*f*
Passive agglutination, 484–485
Passive immunization, 261–263, 262*f*
Passive transfer of maternal immunity, 253–255
  absorption failure, 256
  diagnosis of, 256–257, 257*t*
  ingestion failure, 248–250
  management, 257
  production failure, 256
Pasteurella multocida, 63
Patch testing, 373
Pathobionts, 285
Pathogen-associated molecular patterns (PAMPS), 9–15, 11*t*, 18, 26, 203
Pathogens, 2
Pattern-recognition receptors, 10, 11*f*, 13*t*, 16–17, 51, 62–63, 73, 92, 299, 317–318
Paucibacillary disease, 288–289
Pelger-Huët anomaly, 436

VetBooks.ir

Pemphigus
erythematosus, 415
foliaceus, 415, 415*f*
panepidemal pustular, 415
paraneoplastic, 415
vegetans, 415
vulgaris, 414–415, 415*f*–416*f*
Penicillin
allergy, 343–344, 356
as hapten, 87
Pentraxins, 16–17, 31, 62–63
Peptidoglycans, 10, 10*f*, 13–14, 82–83
Peptidoglycan recognition proteins, 14
Perforin pathway, 191–192, 191*f*
Perforins, 191–192, 192*f*, 203
Periarteriolar lymphoid sheath, 118–119
Periodic ophthalmia, 412–413
Periodontal disease, 239*b*
Periparturient immunosuppression, 387, 470
Peroxiredoxin, 320
Peroxynitrite, 52
Persistent lymphocytosis, 399
Peyer's patches
development, 248
function, 113, 199, 229, 241
ileal, 112, 181, 227, 241
jejunal, 112, 227, 241

VetBooks.ir

responses, 339
structure, 112–113, 112*f*, 226
Phagocytosis
adherence, 43
basic features, 42–47, 43*f*
chemotaxis, 42–43
coiling, 44
complement-mediated, 33
destruction, 45–47
inhibition, 290
invertebrates, 491
macrophages, 51–52
neutrophils, 42–47
opsonization, 43
Type 1, 43–44
Type 2, 33, 44
Phagolysosomes, 46
Phagosomes, 44, 93, 96, 291
Phenogroups, 348–349, 351–352
Phenotypes, lymphocyte, 126*b*
Phenylephrine, 330–331
Phorbol myristate acetate, 258
Phosphatidylinositol, 75, 150
Phosphatidylserine, 47–48
Phosphocholine, 62–63
Phosphoglucomutase, 339
Phospholipase A, 328

VetBooks.ir

Phospholipase C, 75, 79
Phosphorylation of proteins, 75
Phylogeny, mammalian, 503, 504*f*
Physical barriers to invasion, 2–3
Phytohemagglutinin, 130, 374
Pigs
  acute-phase proteins, 65
  anaphylaxis, 338
  blood groups, 353–354, 353*b*
  combined immunodeficiency, 442–443
  dendritic cells, 95
  Factor H deficiency, 36–37
  gamma/delta T cells, 130
  germ-free, 182
  glomerulopathy, 365
  graft donors, 384
  hemolytic disease, 353–354
  immune system development in, 249
  immunoglobulins, 170
  immunoglobulin genes, 181
  leukocyte antigens (SLA), 101
  lymph nodes, 117–118, 117*f*
  lymphocytes, 130
  melanomas, 398
  MHC, 105*f*, 107
  NK cells, 205
  Peyer's patches, 112

VetBooks.ir

regulatory T cells, 215
T cell circulation, 118*f*
thrombocytopenia, 354
WC1, 130
xenografts, 384
Pilus antigens, 83
Pinocytosis, 93
Pituitary pars intermedia dysfunction, 460–461
Placenta
immunosuppression, 385*f*, 386–387
microbiota, 251*b*
structure, 253, 253*f*
Plasma, spray-dried, 263
Plasma cells
functions, 154, 211
intestinal, 154
memory, 156, 276
spleen, 119–120
structure, 155*f*
Plasma transfusions, 257
Plasmacytomas, Myelomas. p
Plasmid, 269
Plasmin, 24, 67
Plasminogen activator, 52, 195–196
Platelet-activating factor, 24, 41, 320, 332
Platelet-derived growth factor, 196–197
Pleiotropy, 73–74

VetBooks.ir

Pneumocystis, 296, 439, 445, 450
Pneumonitis, hypersensitivity, 360–361
Poison ivy, 87
Pokeweed mitogen, 130
Polyandroalbumin, 414
Polyarteritis, 433, 433*f*
Polyarthritis
  autoimmune, 429–433
  canine, 432
  chronic progressive, 433
  classification, 432*t*
  equine, 432
  erosive, 429–431
  feline, 433
  idiopathic, 432–433
  immune mediated, 366, 411–412
  lupus, 426, 432
  nonerosive, 432–433
  types, 432
  vaccine induced, 283
  with Polymyositis, 432
Polyclonal gammopathy, 159, 425–426
Polymerase chain reaction, 309, 437–438, 442, 452, 487
Polymeric immunoglobulin receptor (PIgR), 165, 165*f*, 237, 244, 244*f*
Polymorphism, MHC, 102, 104, 107
Polymorphonuclear neutrophil granulocyte, neutrophil
Polymyositis, 420–421

VetBooks.ir

Polynucleotide vaccines, 260, 268–270, 269*f*
Porcine circovirus, 96
Porcine enzootic pneumonia, 405
Porcine reproductive and respiratory syndrome (PRRS), 95, 308, 450
Porins, 83
Positive selection, 111, 208–209, 209*f*
Postdistemper demyelinating leukoencephalopathy, 411
Posttraumatic immune deficiency, 460
Postvaccinal distemper virus encephalitis, 282
Postweaning multisystemic wasting syndrome, 455
Precipitation tests, 481–484
Precocious strains, 315
Prednisolone, 346, 382, 418, 465–466
Pregnancy, immunosuppression, 281, 385–387
Premunition, 312–313
Preventable fraction, 278
Primary agammaglobulinemia, 441
Primary binding tests, 472–479
Primary granules, 40–41, 333–334
Primary follicles, 119–120
Primary immune response, 5, 165, 165*f*
Primary immunodeficiencies, 435
Primary lymphoid organs, 109–113
Primary pathogens, 2
Primates
  lentiviruses in, 450–451
  retrovirus infections, 450–451

VetBooks.ir

Prime-boost strategies, 269–270
Probiotics, 232*b*
Programmed cell death-1 (PD-1), 193, 393, 395
Profilin, 312
Proliferation assays, 374, 375*f*
Pronephros, 495
Properdin, 28–29, 33
Prophenoloxidase system, 491, 491*f*
Propionibacterium acnes, 272–273, 469
Propranolol, 330–331
Propylthiouracil, 428
Prostacyclins, 24
Prostaglandins, 23*f*, 24, 61, 65–66, 195–196, 328
Prostate specific antigen, 389
Proteasomes, 98–99
Protectin, 32, 57
Protection tests, 486–487
Protein A, 290
Protein kinase R, 300
Protein-losing enteropathy, 233
Protein misfolding diseases, 69–71
Proteinuria, 255
Proteobacteria, 223–224
Protozoa, immunity to, 311–315
Proximal signaling complex, 79–80
Prozone, 484
Pruriceptors, 342

VetBooks.ir

Pruritus, 322, 330, 340, 342, 342*b*, 342*f*, 344
Pseudogenes, 102, 170, 179–180
Pseudomonas aeruginosa, 32, 289–293
Pseudopelade, 414
Pseudorabies, 95, 219
Pulmonary intravascular macrophages, 55, 55*f*, 252
Puppy, canine and dog
  immune system development, 249
  vaccination, 259
Purified protein derivative (PPD), 141*f*, 367
Purpura hemorrhagica, 294, 366
Pus, 42
Pyroptosis, 15

Q

Quantitative precipitation curve, 481*f*
Quil A, 271, 352–353

VetBooks.ir

R

Rabbit, 172
Rabies, 297–298, 302
Rabies diagnosis, 473
Rabies vaccines, 265, 268, 280, 396–397, 411
Radial immunodiffusion, 482–483, 483*f*
Radiation, 463–464
Radioallergosorbent test (RAST), 345–346
Radioimmunoassays, 472–473
RAG genes, 210
RANTES (CCL5), 21–22
Rapamycin, 467–468
RA-shared epitope, 430
Reactive amyloidosis, 69–70
Reactive oxygen species (ROS), 46, 48, 51, 68, 287
Reactive nitrogen species (RNS), 52
Real-time PCR, 487
Receiver operating characteristic curve, 488–489, 488*f*
Receptors
  acetylcholine, 419, 420*f*
  assembly, 180
  B cells, antigen, 174
  complement, 32–33, 43, 54–55, 128, 326, 343
  cytokine, 74–76, 74*f*, 128
  editing, 177–178, 210, 403
  Fc, Fc receptors

VetBooks.ir

macrophages, 54–55
mannose, 54, 92
neutrophils, 47, 47*f*
T cells, antigen, 125–127, 132–134, 133*f*
toll-like, 10–13
Recombinant vaccines, 265–268
Recombinase activating gene (RAG), 389, 493–494
Recombination
class switch, 169, 248
TCR, 183–184, 184*f*
Recurrent airway obstruction, 360–361
Red blood cells
blood groups, 87–88, 348–349, 350*t*
hemolysis, 417, 417*f*
Refractometry, 257
RegIII, 229, 239
Regakine-1, 22
Regulation of immune responses
antibody-mediated, 212
antigen-mediated, 212
antigen processing and, 212
B cells, 218
dendritic cells, 218
failure of, 403
innate immunity by, 216–218
macrophages, 218
natural suppressor cells by, 218

VetBooks.ir

neural, 219–220
T cells by, 213–216
Regulatory cells, 213–216, under T cells, B cells and macrophages
Relapsing Polychondritis, 417
Renal allografts, 378–382, 379*f*, 467
Reoviruses, 339, 403–404
Reproductive disease, autoimmune, 414
Reptiles
allograft rejection, 499
immunity, 498–499
immunoglobulins, 499
lymphoid organs, 499
Residual virulence, 264, 280
Resistin, 318, 456
Resolvin, 57
Respiratory burst, 45–46, 68, 93, 460
Respiratory tract
allergic disease, 339–340
immunity in, 236–238
immunoglobulins in, 237
inhaled particles, 56–57, 237*f*
lymphoid tissue in, 236, 237*f*
microbiota, 223–224
Retained placenta, 386
Reticular dysgenesis, 447
Retinoic acid, 93–94, 213, 229–230, 457, 469
Retinoic acid-inducible gene receptors, RIG-like receptors

VetBooks.ir

Retroviruses, 298, 299*f*, 403–404, 426, 450–451, 455
Revaccination, 276–277
Reverse transcriptase, 298
Reverse vaccinology, 270
Rhesus antigen, 355
Rheumatic fever, 404
Rheumatoid arthritis, 429–431
  diagnosis, 431, 431*b*
  pathogenesis, 429–431, 430*f*
  susceptibility, 430
  treatment, 431
Rheumatoid factors, 403, 425, 430, 431*b*
Rhodococcus equi, 25, 206, 252*b*, 286, 288, 366, 432, 439
Rhus radicans, 372
Rinderpest
  eradication, 268
  vaccination, 265, 268
RIG-like receptors, 13, 299
RNA, double stranded, 272–273, 298
RNA, single stranded, 272–273, 298
RNA-induced silencing complex (RISC), 301
RNA interference, 301, 492, 492*f*
RNA polymerase, 81
Rocket electrophoresis, 483–484
Rodent ulcers, 345
ROC curves, 65
ROR-γ, 80, 131, 199

VetBooks.ir

RTX proteins, 291
Rumen, 224–225

S

S100 proteins, 15–16, 24–25
Saccharopolyspora rectivirgula, 360
Salbutamol, 330–331
Saliva
  arthropod, 322, 322*f*
  IgA, 239
Salmonella enterica Dublin, 294
  Pullorum, 295
  Typhimurium, 286, 290–291, 294
Salmonella enterica, 17, 288
Saponins, 271, 315
Saposins, 192
Sarcoids, 398
Sarcomas
  canine transmissible, 397
  injection site, 396
  prevalence, 396–397
  vaccine associated, 396
Scavenger receptor proteins
Schirmer tear test, 428
Scleroderma, 405–406
Scrapie, 70–71, 107, 264
Secondary binding tests, 472, 481–485

VetBooks.ir

Secondary granules, 40–41
Secondary immune response, 5
Secondary lymphoid organs, 113–121
Secretors, 349, 352
Secretory component, 253–254
Secretory IgA, IgA, secretory
Segmented filamentous bacteria (SFB), 199–200, 224, 230, 231*f*, 239, 245
Selectins, 9, 51–52, 117, 129
  E-, 41
  L-, 41, 219, 235–236
  P-, 41
Selenium deficiency, 456–457
Self-cure, 245–246
Seminal plasma, 238, 385
Sentinel cells, 17–22
Septic shock, 19, 65–68
Serine protease inhibitor (SLP1), 57
Serologic tests, reagents, 472, 472*t*, 484*t*
Serology, blood groups, 351–355
Serotonin, 23, 328–329, 336, 456
Serpins, 320, 323
Serprocidins, 25
Serum, 5, 472
Serum amyloid A, 65, 69–70, 230, 252
Serum amyloid P, 16–17, 62–63
Serum sickness, 263, 361, 362*f*

VetBooks.ir

Severe combined immunodeficiency
calves, 442
dogs, 443–444
foals, 439–441
pigs, 442–443
mice, 447
Sheep
anaphylaxis, 338
blood groups, 353
gamma/delta T cells, 185
immunoglobulins, 171
immunoglobulin genes, 181–182
lymphocytes, 130
lymphomas, 400
MHC, 107
NK cells, 205
Peyer's patches, 112, 181–182
WC1 (T19), 130
Shell coat, 503
Shipping fever, 219
Short chain fatty acids, 222, 224, 227, 227*f*, 336
Short thermal test, 370
Sialic acid, 28
Sickness behavior, 60–62, 220
sex differences, 61*b*
Sickness metabolic changes, 62
Siderocalin, 63–64

VetBooks.ir

Siderophores, 64
Signal transduction, 74
  pathways, 75*f*, 77*f*
  T cell, 79*f*, 137
  TLRs, 78*f*
Signaling. differential, 152
Simian immunodeficiency virus, 450–451
Simian retrovirus, 451
Single intradermal test, 369
Single radial immunodiffusion, 257
Sjögren's syndrome, 423–427
Skin
  atopic disease, 340–343
  autoimmune diseases, 412–414
  barrier protein defects, 343
  blistering diseases, 414–415
  defences, 234–236
  grafts, 382
  hair follicle diseases, 414
  microbiota, 222–223, 223*f*–224*f*
  tests, 367–371
  T cells in, 223, 235
SLA, 101
Small interfering RNAs, 301
Sodium myoinositol co-transporter, 442
Somatic mutation, 156–157, 178–179, 181, 185, 461
Spätzle, 492

VetBooks.ir

Spaying, effects of, 406*b*
Sperm
antibodies to, 385, 414
MHC and, 107*b*
rejection of, 385
Sphingomyelin, 75–76, 192
Sphingosine phosphate, 343
Spiral arteries, 386
Spleen, 118–120
function, 119–120
macrophages, 55–56
red pulp, 118
structure, 118–119
white pulp, 108
Splenomegaly, 352, 418
Splenectomy, 315, 418–419
Spring rise, 315–316
Squalene, 271–272
Squamous cell eye carcinoma, 107, 398
Src kinases, 150
Staphyloccal hypersensitivity, 361
Staphylococcus aureus, 25, 63, 68, 236, 251–252, 290–291
Staphylococcus epidermidis, 235, 289–290
Staphylococcus pseudintermedius, 343
Staphyloferrin, 64
Staphylokinase, 290
Starvation and immunity, 456

VetBooks.ir

STAT proteins, 80, 389
Stem cells, 62, 383
Stevens-Johnson syndrome, 373
Steroid-responsive meningitis-arteritis, 411–412
Stimulation index, 374
Stormont test, 370
Strain 19, 264–265, 294
Strain 45/20, 264
Strain J5, 236, 294
Strain RB-51, 264, 294
Streptococcus equi, 25, 86, 366
Streptococcus fecalis, 418
Streptococcus pneumoniae, 44, 62–63, 290–293
Streptococcus pyogenes, 32
Streptolysin, 291
Stress and immunity, 219–220, 279, 304
Stress proteins, 200–201
Sublingual immunotherapy, 347
Substance P, 220
Substrate modulation, 28, 33*f*
Superantigens, 138–139, 139*f*, 182, 302, 308, 405
Superoxide anion, 52
Superoxide dismutase, 45, 320
Suppressive active peptide, 460
Supramolecular activation clusters, 136–137, 137*f*, 190–191, 191*f*, 203
Surfactant proteins, 9, 29, 236–238, 251–252, 286–287, 299
Sweet itch, 344

VetBooks.ir

Swine, pigs
Synapses, immunological, 136–137, 137*f*, 190–191, 191*f*, 203
Synergy, 73–74
Systemic inflammatory response syndrome, 15, 65–69
Systemic innate responses, 62–65
Systemic lupus erythematosus, 423–427
  B cell abnormalities, 424–425
  canine, 412, 426–427
  diagnosis, 427, 427*b*, 428*f*
  equine, 426, 426*f*
  feline, 427
  genetic predisposition, 424, 426
  glomerulonephritis, 426
  hemophagocytic syndrome, 355
  inheritance, 426*f*
  pathogenesis, 32, 424–427, 424*f*
  T cell abnormalities, 424
  treatment, 427

T

T2- toxin, 455
T19, 130
Tacrolimus, 79, 346, 467
Taenia ovis vaccine, 321
Taenia solium, 321
Taeniastatin, 321
Target of rapamycin, 467–468

VetBooks.ir

inhibitors, 467–468, 467*f*
Tasmanian devil, 398
Taurine deficiency, 458–459
T cells, 110–111
aged, 461
allografts, 380–381
antigen receptors, 183–185
apoptosis, 215–216
blood, 123, 124*t*
corticosteroids, 465
deficiencies, 445–446
dysfunction, 393
effector, 197
exhaustion, 193, 314, 322, 393, 395
follicular helper, 243–244
helper, 189–190
identifying features, 124*t*
intestinal, 242–243
intraepithelial, 242–243
lymph nodes, 113
memory, 117, 145–146, 197, 386*b*
microbiota and, 229–230
mitogens, 130
origins, 123
pigs, 145
proliferation assays, 374, 375*f*
receptors, 127*f*

VetBooks.ir

regulatory, 213–216
skin, 7
subpopulations, 139–144, 193
therapy, 394–395
to tumors, 391
virus-infected, 304
T cell antigen receptors (TCR), 132–134, 133*f*
alpha/beta, 125–127, 132
basic features and functions, 125–127, 132–134
diversity generation, 183–185, 184*t*, 185*b*, 248
gamma/delta, 125–127, 130, 132, 185, 215, 235, 243, 368
-high species, 185
-low species, 185
genes coding for, 183
gene rearrangement, 183–184, 184*f*
hypervariable regions, 183
signal transduction, 75, 78, 79*f*, 137
T cell subpopulations
cytotoxic, 6, 190–193, 191*f*, 288, 302, 312, 375*f*, 378, 408
gamma/delta, 96, 144–145, 144*f*, 185, 289, 311, 318
helper
subpopulations, 139–144, 139*f*
helper-1T cells, (Th1 cells), 91, 97, 140, 197, 296, 346–347
helper 2 T cells, (Th2 cells), 91, 140–143, 150–151, 151*t*, 152*f*, 318, 332, 346–347
helper 17 T cells (Th17 cells), 143, 216–218, 216*f*–217*f*, 220, 229–230, 236, 286, 288, 296, 318, 331, 339–340, 456
helper 22 T cells (Th22 cells), 235

regulatory T cells, (Treg cells), 93–94, 143–144, 213–216, 213*f*–214*f*, 227, 228*f*, 229–230, 246, 346–347, 386, 393, 454, 467–468
Telodorsagia circumcincta, 316–318
Temperature-sensitive mutants, 308–309
Terminal complement complex, 31–32, 32*f*, 286
Testes, antigens, 403
Testosterone, 107*b*, 111
Tetanus
immune globulin, 263
toxin, 5, 486
toxoid, 152, 293
vaccination, 6*f*
Tetherin, 300
Theileria parva, 312–313, 315
Thermophilic actinomycetes, 360
Thoracic duct, 117
Thrombin, 24
Thrombocytopenia
autoimmune, 419, 425
drug-induced, 419
immune complex, 366
neonatal, 351
Thromboxanes, 24
Thymectomy, 110, 110*t*, 123, 419
Thymic hormones, 111
Thymic stromal lymphopoietin, 111, 136, 199, 240, 317–318, 320*f*, 329, 338, 341–342, 344
Thymidine kinase, 267

VetBooks.ir

Thymidine, tritiated, 130*b*, 374
Thymocytes, 109–110, 209–210
Thymoids, 493
Thymulin, 111
Thymus, 109–111
  atrophy, 461
  central tolerance in, 208–210
  function, 110–111
  gene rearrangement in, 185
  hormones, 111
  independent antigens, 152, 153*f*
  location, 109
  structure, 109–110, 494
Thyroid
  antibodies to, 409
  autoimmune diseases, 283, 410
  stimulating hormone, 407–408, 410
Ticks
  immunity to, 322–323
  immunosuppression, 322*f*
Tissue factor, 65–67
Titration, antibody, 484, 484*f*
T lymphocyte, Tcell
Tolerance, 208
  B cell, 210–211, 211*f*
  central, 208–210
  dendritic cells, 90, 93–94

VetBooks.ir

duration, 208*f*, 211
oral, 214, 338–339
immunity to, 246
peripheral, 210, 210*f*
T cell, 208–210, 209*f*
Tolerosomes, 246
Toll-like receptors (TLR), 10–13, 11*b*, 11*f*, 11*t*, 270, 286, 289–290, 406, 424–425
B cells, 151
cell surface, 11*t*
dendritic cells, 92–93
intestinal epithelial cells, 228
intracellular, 11, 11*f*, 11*t*
invertebrates, 492
ligands, 272
macrophages, 54
mast cells, 328
ruminal, 225
signal transduction, 12*f*, 78*f*, 79–80, 291*f*
stem cells, 13
viruses, 299
Tonsils, 239, 239*f*, 241
Toxic epidermal necrolysis, 373
Toxic shock syndrome, 68
Toxin-induced immunosuppression, 455
Toxins, neutralization, 287
Toxocara canis, 141*f*

VetBooks.ir

Toxoids, 83, 261–265, 293

Toxoplasma gondii, 194, 218, 311–313, 312*f*, 315, 370

Trace elements, 456–457

TRAIL, 213, 393

TRAF6, 78

Transcription factors

AP-1, 79, 79*f*

Foxp3, 79–80, 143–144, 213

GATA3, 80, 139*f*, 199

NF-AT, 79–80, 150

NF-κB, 78, 150

RORγT, 80, 131, 199

T-bet, 80, 140, 199

Transferrin, 63–64, 195–196

Transforming growth factor-beta, 46–47, 57–58, 93–94, 136–137, 143, 154, 196–197, 213, 215–216, 215*f*

Transfusion reactions, 349

Transglutaminase, 196–197

Transient hypogammaglobulinemia, 441

Translocon pattern, 495

Transmissible amyloidosis, 70*b*

Transmissible gastroenteritis (TGE), 87–88, 95, 258, 268

Transmissible venereal sarcoma, 397

Transportation stress, 459–460

Transporter proteins (TAP), 98, 98*f*, 104

Trapped neutrophil syndrome, 438

Treg cells, regulatory T cells

VetBooks.ir

Trehalose dimycolate, 468–469
Trichinella spiralis, 316–317
Trichohyalin, 414
Trichomonas vaginalis, 311
Trichostrongylus colubriformis, 107
Trichothecenes, 455
Trichuris muris, 319
Trickle infections, 315, 319, 321–322
TRIF, 15, 219
Tritiated thymidine, 374
Trophoblast, 385–387
Trypanosoma brucei, 310–311, 313–314
Trypanosoma congolense, 313–314
Trypanosoma cruzi, 312, 403–404
Trypanosoma lewisi, 313
Trypanosoma theileri, 313
Trypanosomiasis, 107, 311, 314
  antigenic variation, 313–314, 314*f*
Trypanotolerance, 311
Tryptophan, 218, 292
Tubercles, 371, 371*f*
Tuberculosis, 197, under Mycobacterium
  bovine, 289
  serology, 371
Tuberculin, purified protein derivative, 367
Tuberculin reaction, 197, 367–371, 368*f*
Tuberculin skin tests, 273, 369, 369*t*

VetBooks.ir

Tumor infiltrating lymphocytes, 394
Tuft cells, 318*b*, 320*f*
Tumor necrosis factors, 73
  superfamily, 73–74
Tumor necrosis factor-alpha, 12, 51, 53–54, 60, 62, 93, 140, 188, 199, 203, 302
  convertase, 19
  family, 19
  functions, 19, 42, 57, 117
  genes, 104
  monoclonal antibodies, 431
  neutrophil activation, 41
  receptors, 19, 92
Tumor necrosis factor-beta (TNF-β), 192
Tumor suppressor genes, 397
Tumors
  as allografts, 389–390
  antigens, 389–390
  cell selection, 392
  failure of immunity to, 392–393
  immunity, 390–392
  immunosuppression by, 392–393, 461–462, 462*f*
  immunotherapy, 393–396
  inflammation and, 390
  injection site-associated, 396–397
  lymphoid, 393–396
  neoantigens, 389–390

VetBooks.ir

vaccines against, 395–396
Type I hypersensitivity
allergic disease, 336–345
in autoimmunity, 407
breed predisposition, 324–325, 340–341
cytokines in, 334*t*
diagnosis, 345–346
induction, 324–325
milk allergy, 407
to arthropods, 322, 344
to bacteria, 294
to drugs, 343–344
to environmental allergens, 341
to fleas, 322, 344
to helminths, 318, 344
to protozoa, 314
to vaccines, 281–282, 343–344
to viruses, 305
to yeasts, 343
treatment, 342*b*, 346–347
Type II hypersensitivity
in autoimmunity, 407–408
to bacteria, 294
to drugs, 356
in infectious diseases, 356
polyarthritis, 366
to protozoa, 314

VetBooks.ir

to red cells, 348
to viruses, 305
Type III hypersensitivity
arthritis, 432
autoimmunity, 408
classification, 357–358
dietary, 366
generalized reactions, 361–363
infectious diseases, 364*t*
local reactions, 358–361
pneumonitis, 360–361
to bacteria, 294
to drugs, 366
to protozoa, 314
to vaccines, 263, 282
to viruses, 305
vasculitis, 433–434
Type IV hypersensitivity
delayed reactions, 367
in autoimmunity, 408
pathological consequences, 371–374
to bacteria, 288, 294
to fungi, 296
to protozoa, 315
to vaccines, 282
Type III secretion systems, 291
Type 1 immune responses, 94, 95*f*, 141*f*

VetBooks.ir

Type 2 immune responses, 94, 95*f*, 140, 141*f*
Type 1 membranoproliferative glomerulonephritis, 362–363
Type 2 membranoproliferative glomerulonephritis, 363
Type 3 membranoproliferative glomerulonephritis, 363
Type D simian retroviruses, 451
Tyrosinase, 396
Tyrosine kinases, 75–76, 78–79, 137

U

Ubiquitin, 98
Uric acid, 93
Uridine triphosphate, 57–58
Urogenital tract, immunity in, 238
Urticaria, 338, 338*f*, 340
Urushiol, 87, 372
Uveodermatological Syndrome, 413, 413*f*

V

Vaccination
  adverse consequences, 280–283
  against bacteria, 293–294
  against caries, 239
  against helminths, 321–322
  against protozoa, 315
  against viruses, 308–309
  antibody assays, 277
  assessment, 278

VetBooks.ir

failures, 278–280
neonatal, 258–259
on body surfaces, 275
schedules, 276–277
strategies, 277–278
young animals, 259–260
Vaccines
administration, 275–277
adverse effects, 280–283
aerosolization, 275
allergic reactions to, 343–344
antibacterial, 294
antiviral, 308–309
assessment, 278
attenuation, 265
autoimmunity induced by, 283
body size, 281*f*
category I, 265–267
category II, 267
category III, 267–268
category IV, 268–270
classification, 266*t*
contraceptive, 414
cost benefits, 268*b*
DIVA vaccines, 267
Dose, 275
efficacy, 275, 284

VetBooks.ir

essential (core), 275
failure, 278–280, 278*f*
genetically engineered, 265–267
helminth, 321–322
immunosuppressive, 282–283, 308
inactivated, 264–265
intranasal, 246
killed, 264, 264*t*
live, 264, 267–268
modified live, 246
multiple antigens, 275–276
optional (non-core) vaccines, 275
oral, 246
polynucleotide, 268–270
polyvalent, 293–294
predictive, 277–278
production, 283–284
production enhancing, 414
prophylactic, 277
protozoa, 315
reactive, 277
recombinant, 276–277
ring, 277–278
schedules, 259, 276–277
strategies, 277–278
surfaces, 246
yeast-based, 268

VetBooks.ir

Vaccinia, 267*f*, 268
Vagus nerve, 60, 219–220
Van der Waals force, 174
Variable regions
  genes, 169
  immunoglobulins, 148–149
  lymphocyte receptors, 491–492
  TCR, 138–139
Variant surface glycoproteins, 314
Vascular endothelial cells, 22, 51–52, 96
Vascular endothelial growth factor (VEGF), 57–58, 196–197, 393
Vasculitis, immune mediated, 282, 305, 412, 433–434
Vasoactive molecules, 22*t*, 23*f*
  amines, 23
  lipids, 23–24
  peptides, 23, 220, 244
Venoms, 12
Verrucobacteria, 225
Vesicular systemic lupus, 426–427
VH gene clans, 503
Vi antigens, 295
Vincristine, 419
Viperin, 300, 302–303
Virions, 83
Virokines, 303
Virome, 232
Virulence, 2

VetBooks.ir

Virus-like particles, 261, 309
Viruses, *See also* specific diseases
adaptive immunity, 301–302
adverse consequences of immunity, 305–306
antibody-mediated immunity, 301
antigens, 298
cell-mediated immunity, 302
endogenous, 97, 298
enhancement, 305–306
evasion of immunity, 302–305
hemagglutination, 485
immunosuppression by, 304, 449–451
infections, pathogenesis, 298–299
innate immunity, 299–301
latency, 305
neutralization, 301, 309
nucleic acids, 15
serology, 309
structure, 298
vaccination, 308–309
Vitamins, 457–459, 469
Vitamin A, 241–243, 457*f*, 458, 469
Vitamin D, 241–242, 286*b*, 286*f*, 458, 458*f*, 469
Vitamin E, 458, 469
Vitronectin, 32
Vogt-Koyanagi-Harada syndrome, 413
Volume transmission, 72

VetBooks.ir

Vomeronasal organ, 107
Vomiting, 2–3

W

Waldeyer's ring, 241
Water-in-oil emulsions, 271–272
WC antigens, 95, 125, 129, 144–145, 368
Weep and sweep, 319
West Nile virus, 268
Western blotting, 309, 345–346, 476–478, 477*f*
Wheal-and-flare response, 343*f*, 345
White blood cells, Leukocytes
White-nose syndrome, 67*b*, 505
Wiscott-Aldrich syndrome, 447–448
Worms, Helminths

X

Xenografts, 319, 378, 378*f*
Xerostomia, 428
X-Linked Immunodeficiency
  dogs, 443
  mice, 447

Y

Yeasts, 10
Yersinia enterocolitica, 87–88
Yersinia pestis, 289–290

VetBooks.ir

Z

Zeta-associated protein-70 (ZAP-70), 79, 79*f*

Zeta potential, 43

Zinc deficiency, 442, 445–446, 456–457

Zinc sulfate turbidity test, 256–257

Zinc treatment, 442

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Greek Letters

Lowercase Greek letters are widely used in immunology to denote peptide chains or other molecules. Below is a list of Greek letters with examples of their use.

Greek Letter		Immunology Examples
α	alpha	α heavy chains (IgA)
β	beta	β_2 microglobulin
γ	gamma	γ globulins, interferon-γ
δ	delta	δ heavy chains (IgD)
ε	epsilon	ε heavy chains (IgE)
ζ	zeta	ζ chain of CD3
η	eta	η chain of CD3
θ	theta	θ defensin
κ	kappa	κ light chains
λ	lambda	λ light chains
μ	mu	μ heavy chains (IgM)
υ	upsilon	υ heavy chain (IgY)
φ	phi	φX174, a bacteriophage
ψ	psi	A notation for a pseudogene
τ	tau	interferon-τ
ω	omega	interferon-ω

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Table of Contents

Table of Contents 3
Copyright 17
Dedication 19
Preface 20
Acknowledgments 23
1 Surviving in a Microbial World 24
The Microbial World 27
The Defenders 30
Where to Go for Additional Information 45
2 Innate Immunity 46
How Invaders Are Recognized 50
Pathogen-Associated Molecular Patterns 61
Damage-Associated Molecular Patterns 64
Soluble Pattern-Recognition Receptors 69
Sentinel Cells 72
3 Humoral Innate Immunity 74
Products of Sentinel Cells 76
Inflammatory Mediators 84
The Coagulation System 90
Antimicrobial Molecules 91
4 Humoral Innate Immunity 96
Complement Proteins 100
Activation Pathways 101
Regulation of Complement Activation 115
Other Consequences of Complement Activation 118
Complement Genes 121
Complement Deficiencies 122
5 Cellular Innate Immunity 127
Leukocyte Classification 131
Neutrophils 133

VetBooks.ir

Emigration From the Bloodstream 136
Phagocytosis 140
Surface Receptors 153
Fate 154
6 Cellular Innate Immunity 155
Macrophages 158
Functions 163
Fate of Foreign Material 173
Resolution of Inflammation 179
7 Sickness 185
Sickness Behavior 188
Systemic Innate Responses 192
Systemic Inflammatory Response Syndrome 201
Protein Misfolding Diseases 211
8 How Immune Cells Communicate 216
Cytokine Nomenclature 219
Cytokine Functions 221
Cytokine Structure 223
Cytokine Receptors 225
Cytokine Regulation 234
Signal Transduction 236
Gene Transcription 245
9 Antigens 247
Microbial Antigens 250
Nonmicrobial Antigens 255
What Makes a Good Antigen? 257
Epitopes 262
Cross-Reactions 267
10 Dendritic Cells and Antigen Processing 270
Dendritic Cells 275
Dendritic Cells in Domestic Animals 288
Other Antigen-Processing Cells 291
Antigen Processing 293

VetBooks.ir

Histiocytosis and Histiocytomas 299
11 The Major Histocompatibility Complex 300
Major Histocompatibility Complex 304
MHC Class Ia Molecules 306
MHC Class II Molecules 311
MHC Class III Molecules 314
MHC of Domestic Animals 315
MHC Molecules and Disease 317
MHC and Body Odors 322
12 Organs of the Immune System 324
Sources of Lymphocytes 329
Primary Lymphoid Organs 330
Secondary Lymphoid Organs 341
Tertiary Lymphoid Organs 360
13 Lymphocytes 361
Lymphocyte Structure 365
Lymphocyte Populations 368
Lymphocyte Surface Molecules 373
Species Differences 384
Lymphocyte Mitogens 386
14 Helper T Cells and Their Response to Antigens 388
Immunoglobulin Superfamily 393
T Cell Antigen Receptors 395
Co-Stimulators 400
Immunological Synapse Formation 404
Signal Transduction 406
Overall Considerations 407
Superantigens 410
Helper T Cell Subpopulations 412
Species Differences 422
Memory T Cells 425
15 B Cells and Their Response to Antigens 428
B Cell Antigen Receptors 432

VetBooks.ir

Antigen Presentation by B Cells 439
Co-stimulation of B Cells 441
B Cell Response 448
Cellular Responses 451
Plasma Cells 454
Memory B Cells 457
Germinal Centers 460
Myelomas 463
Hybridomas 469
16 Antibodies 473
Immunoglobulins 477
Immunoglobulin Classes 480
Three-Dimensional Structure of Immunoglobulins 489
Immunoglobulin Variants 491
Production of Immunoglobulin Heavy Chains 493
Immunoglobulins of Domestic Mammals 497
17 How Antigen-Binding Receptors Are Made 504
Receptor-Antigen Binding 509
Antigen Receptor Genes 512
Immunoglobulin Diversity 514
Gene Recombination 515
Generation of Junctional Diversity 517
Somatic Mutation 524
Gene Conversion 528
Species Differences 531
T Cell Receptor Diversity 536
γ/δ T Cell Diversity 542
Epigenetic Regulation 543
18 T Cells and the Destruction of Cell-Associated Invaders 545
Endogenous Antigens 549
Apoptosis 550
Cell Cooperation 554
Cytotoxic T Cell Responses 556

VetBooks.ir

Cytotoxic T Cell Subsets 563
Other Mechanisms of Cellular Cytotoxicity 564
Macrophage Activation 567
Effector T Cell Memory 574
19 Innate Lymphoid Cells 576
Innate Helper Cells 581
Innate Cytotoxic Cells 586
NKT Cells 601
20 Regulation of Adaptive Immunity 604
Tolerance 608
T Cell Tolerance 611
B Cell Tolerance 617
Duration of Tolerance 619
Control of Immune Responses 620
Antigen Regulation of Immune Responses 621
Antibody Regulation of Immune Responses 622
Inhibitory Receptors 624
Regulatory Cells 626
Regulation by the Innate Immune System 632
Regulation of Apoptosis 639
Neural Regulation of Immunity 640
21 The Microbiota and the Immune System 646
The Locations of the Microbiota 652
The Functions of the Microbiota 660
Immune Responses to the Microbiota 667
Dysbiosis 676
Odors 678
The Virome 679
Inflammatory Bowel Diseases 680
22 Immunity at Body Surfaces 683
Immunity on Body Surfaces 687
Mucosal Lymphoid Tissues 704
Adaptive Protective Mechanisms 711

VetBooks.ir

Immunity to Food 719
Vaccination on Body Surfaces 721
23 Immunity in the Fetus and Newborn 723
Development of the Immune System 727
Immune Response of Newborn Mammals 737
Transfer of Immunity From Mother to Offspring 742
Failure of Passive Transfer 752
Cell-Mediated Immunity and Colostrum 758
Development of Adaptive Immunity in Neonatal Mammals 760
Vaccination of Young Animals 763
24 Vaccines and Their Production 767
Types of Immunization Procedures 771
Passive Immunization 773
Active Immunization 776
Modern Vaccine Technology 782
Adjuvants 797
25 The Use of Vaccines 805
Administration of Vaccines 810
Vaccination Strategies 817
Vaccine Assessment 818
Failures in Vaccination 819
Adverse Consequences of Vaccination 824
Adverse Effect Principles 833
Production, Presentation, and Control of Vaccines 834
26 Immunity to Bacteria and Fungi 836
Innate Immunity 840
Adaptive Immunity 843
Evasion of the Immune Response 852
Some Antibacterial Vaccines 862
Adverse Consequences of the Immune Responses 865
Serology of Bacterial Infections 866
Immunity to Fungal Infections 869
27 Immunity to Viruses 871

VetBooks.ir

Virus Structure and Antigens 876
Pathogenesis of Virus Infections 877
Innate Immunity 880
Adaptive Immunity 888
Evasion of the Immune Response by Viruses 891
Adverse Consequences of Immunity to Viruses 898
Some Selected Virus Diseases 901
Some Antiviral Vaccines 908
Serology of Viral Diseases 910
28 Immunity to Parasites 911
Immunity to Protozoa 916
Immunity to Helminths 930
Immunity to Arthropods 948
29 Mast Cell and Eosinophil-Mediated Hypersensitivity 954
Induction of Type I Hypersensitivity 958
Immunoglobulin E 959
Mast Cells 964
Response of Mast Cells to Antigen 968
Basophils 976
Eosinophils 978
30 Allergic Diseases 984
The Hygiene Hypothesis 988
Specific Allergic Diseases 991
Diagnosis of Type I Hypersensitivity 1015
Treatment of Type I Hypersensitivity 1018
31 Red Cell Antigens and Antibody-Mediated Hypersensitivity 1022
Blood Groups 1026
Blood Transfusion and Incompatible Transfusions 1027
Hemolytic Disease of the Newborn 1029
Blood Groups, Blood Transfusion, and Hemolytic Disease in Domestic Animals 1030
Parentage Testing 1045
Hemophagocytic Syndrome 1046

VetBooks.ir

Type II Hypersensitivity Reactions to Drugs 1047
Type II Hypersensitivity in Infectious Diseases 1048
32 Immune Complexes and Neutrophil-Mediated Hypersensitivity 1049
Classification of Type III Hypersensitivity Reactions 1053
Local Type III Hypersensitivity Reactions 1054
Generalized Type III Hypersensitivity Reactions 1064
Clinical Features of Glomerulonephritis 1073
Other Immune Complex–Mediated Lesions 1079
33 T Cell–Mediated Hypersensitivity 1081
The Tuberculin Reaction 1084
Pathological Consequences of Type IV Hypersensitivity 1093
Measurement of Cell-Mediated Immunity 1102
34 Organ Graft Rejection and Pregnancy 1109
Grafting of Organs 1112
Allograft Rejection 1114
Renal Allografts 1118
Skin Allografts 1124
Liver Allografts 1125
Cardiac Allografts 1126
Corneal Allografts 1127
Bone Allografts 1128
Bone Marrow Allografts 1129
Xenografts 1133
Allografts and the Reproductive System 1135
35 Cancer Immunology and Immunotherapy 1143
Tumors as Allografts? 1148
Cellular Defenses 1154
Failure of Immunity to Cancer Cells 1158
Tumor Immunotherapy 1163
Some Selected Tumors 1171
Lymphoid Tumors 1178
36 Autoimmunity 1183

VetBooks.ir

Induction of Autoimmunity 1188
Normal Immune Responses 1189
Abnormal Immune Responses 1192
Predisposing Factors 1199
Mechanisms of Tissue Damage in Autoimmunity 1204
37 Organ-Specific Autoimmune Diseases 1206
Autoimmune Endocrine Disease 1210
Autoimmune Neurological Disease 1215
Autoimmune Eye Disease 1220
Autoimmune Reproductive Diseases 1224
Autoimmune Skin Diseases 1226
Autoimmune Nephritis 1234
Autoimmune Blood Diseases 1235
Autoimmune Muscle Diseases 1242
Dermatomyositis 1247
Chronic Active Hepatitis 1250
38 Immune-Mediated Inflammatory Diseases 1251
Systemic Lupus Erythematosus 1255
Discoid Lupus Erythematosus 1268
Sjögren's Syndrome 1269
Autoimmune Polyarthritis 1271
Immune Vasculitis 1283
39 Primary Immunodeficiencies 1286
Inherited Defects in Innate Immunity 1290
Inherited Defects in the Adaptive Immune System 1300
Immunodeficiencies of Horses 1301
Immunodeficiencies of Cattle 1308
Immunodeficiencies of Pigs 1310
Immunodeficiencies of Dogs 1312
Immunodeficiencies of Cats 1320
Immunodeficiencies of Mice 1321
Immunodeficiencies of Humans 1324
40 Secondary Immunological Defects 1326

VetBooks.ir

Virus-Induced Immunosuppression 1330
Retrovirus Infections in Cats 1335
Other Causes of Secondary Immunodeficiency 1347
Other Secondary Immunodeficiencies 1363
41 Drugs and Other Agents That Affect the Immune System 1365
Suppression of the Immune System 1368
Nonspecific Immunosuppression 1369
Selective Immunosuppression 1378
Stimulation of the Immune System 1385
42 Immunodiagnostic Techniques 1391
Reagents Used in Serological Tests 1396
Primary Binding Tests 1398
Antibody Labels 1417
The Flow Cytometer 1418
Secondary Binding Tests 1422
Assays in Living Systems 1436
Molecular Methods 1439
Diagnostic Applications of Immunological Tests 1442
43 Evolution of the Immune System 1446
Immunity in Invertebrates 1450
Immunity in Vertebrates 1456
Immunity in Cyclostomes 1457
Immunity in Jawed Fish 1460
Immunity in Amphibians 1468
Immunity in Reptiles 1474
Immunity in Birds 1477
Generation of Antibody Diversity 1484
Maternal Immunity 1486
Immunity in Monotremes and Marsupials 1488
Mammalian Phylogeny 1491
Fever 1494
Appendix 1 Annotated List of Selected CD Molecules 1497

VetBooks.ir

Appendix 2 Some Selected Cytokines 1509
Appendix 3 Some Important Abbreviations 1519
Glossary 1528
Index 1564
Greek Letters 1694