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As obligate intracellular parasites, viruses have co-evolved with their respective hosts, and eukaryotic host organisms have developed diverse and sophisticated defenses to protect themselves against viral infections and their associated diseases. In turn, viruses have also developed a remarkable variety of strategies to avoid or subvert these host defenses. Antiviral immunity in higher animals is complex, and reflects a combination of innate and acquired (adaptive) immune response mechanisms, although there is considerable interplay between these two broad categories. Cytokines, dendritic cells, natural antibodies and certain T lymphocytes (γδ T cells) provide especially important bridging linkage between innate and adaptive immune responses. Antiviral immunity in animals is mediated by both cellular and humoral factors, and the nature of the immune response generated by different individuals infected with the same virus can be different, depending on the individual’s genetic constitution, environmental influences, and other factors that can determine the course and pathogenesis of the infection.

Innate immune defenses (also referred to as native or natural immunity) are constantly present to protect multicellular organisms against viral infections, and previous exposure to a particular virus is not required to activate these mechanisms. In contrast, adaptive immunity develops only after exposure to a virus, and is specific to that particular virus and, sometimes, its close relatives. Adaptive immunity involves cellular and antibody (humoral) effector mechanisms, mediated respectively by T and B lymphocytes. In further contrast to innate immunity, adaptive immune responses exhibit memory, such that the response may be quickly reactivated after re-exposure to the same virus. With many systemic viral infections, immunological memory after natural infection confers long-term, often life-long, protection against the associated disease.

The development of efficacious vaccines has substantially reduced the deleterious impact of viral diseases of humans and animals. The goal of vaccination is to stimulate the adaptive immune responses that protect animals after reinfection with specific viruses. An increasing variety of vaccine types are now commercially available for use in animals, especially companion and production animal species, including livestock, poultry and fish; these include inactivated (syn. “killed”), live-attenuated (syn. modified-live), and various types of recombinant and genetically engineered vaccines. Vaccines are used extensively in regulatory programs for the control of individual viral diseases of livestock, often in combination with specific management procedures. Other strategies for antiviral treatment and prophylaxis include drugs that interfere with viral infection and/or replication, as well as molecules that stimulate or mimic protective host responses.

**HOST IMMUNITY TO VIRAL INFECTIONS**

**Innate Immunity**

Innate immune defenses exhibit neither antigen specificity nor memory, but they provide a critical line of first defense against viral infections because they are constantly present and are operational immediately after viral infection. Innate immunity...
is often considered separate from acquired immune responses, but they are inextricably linked, and innate responses modulate subsequent acquired responses in many ways. Several distinct activities mediate innate immune defense, including: (1) epithelial barriers; (2) antimicrobial serum proteins such as complement; (3) natural antibodies produced by B1 lymphocytes; (4) the activities of phagocytic cells such as neutrophils, macrophages, and dendritic cells; (5) natural killer (NK) cells that can lyse virus-infected cells; (6) various cell types present at sites of virus invasion that possess receptors that generically recognize and quickly respond to invading viruses by transcriptional activation that results in production of a wide variety of protective molecules, the interferon (IFN) system being an especially critical and central component of antiviral resistance; (7) apoptosis, a process of programmed cell death that can eliminate virus-infected cells; (8) small RNA molecules that interfere with virus replication (RNAi).

Viruses that are transmitted horizontally between individuals must first breach the barriers at their portal of entry before they can cause infection in their respective hosts. For example, the epithelial lining of the skin and respiratory, gastrointestinal, and urogenital tracts provides a mechanical barrier against infection at these common sites of virus entry. Secretions and other activities at mucosal surfaces provide further non-specific protection against viral infection. For example, surfactant and the mucociliary apparatus confer non-specific antimicrobial protection to the respiratory tract. Similarly, antimicrobial protection in the gastrointestinal tract is mediated by, amongst others, the mucous barrier, regional pH extremes, sterilizing action of secretions (e.g., from the liver (bile) and pancreas), and specific antimicrobial peptides such as defensins that are present within the mucosa and its secretions.

A variety of plasma proteins exert antimicrobial activity, including the various complement proteins, C-reactive protein, mannose-binding protein, and broadly reactive natural antibodies. These proteins can exert either a direct antimicrobial effect, or they can promote the uptake of microorganisms into phagocytic cells by coating their surface to facilitate receptor binding (opsonization).

Phagocytic cells—macrophages and neutrophils—provide a critical antimicrobial function, as they are attracted to sites of inflammation, where they efficiently ingest and digest foreign materials, including microorganisms. These cells possess the intracellular machinery to destroy ingested microbes, particularly bacteria, through the actions of hydrolytic lysosomal enzymes as well as the production of activated oxygen and nitrogen metabolites within phagocytic vacuoles. Various soluble mediators can both attract these cells to sites of inflammation, and activate them to enhance their antimicrobial activity.

Dendritic cells are key players in both adaptive and innate immunity to viral infections (and will be discussed in detail in the section below on Adaptive Immunity). In addition to being highly efficient antigen-presenting cells, dendritic cells are an especially important source of type I IFN and various other cytokines that inhibit viral infection and replication. Interdigitating dendritic cells are abundant at portals of virus entry (such as the respiratory, urogenital, and gastrointestinal tracts, and skin), and they are endowed with pattern recognition receptors that allow them quickly to initiate protective innate immune responses to invading viruses.

Natural Killer Cells

Natural killer cells are specialized lymphocytes that are capable of rapid killing of virus-infected cells; thus they provide early and non-specific resistance against viral infections. Specifically, natural killer cells recognize host cells that express altered levels of major histocompatibility complex (MHC) class I molecules and/or heat shock (or similar) proteins. The function of natural killer cells is stringently regulated by the balance of activating and inhibitory signals expressed on the surface of target cells. Virus-infected cells typically express reduced levels of inhibitory class I MHC molecules and increased levels of ligands specific for activating receptors on natural killer cells. In summary, natural killer cells are not antigen specific; rather, their activation requires differential engagement of cell-surface receptors in combination with stimulation by proinflammatory cytokines (Figure 4.1).

Natural killer cells mediate death of virus-infected cells via apoptosis; this cytoidal activity is central to the control of viral infections, because it can eliminate infected cells before they release progeny virions. Natural killer cells also possess surface receptors for the Fc portion of immunoglobulin molecules, which allows them to bind and lyse antibody-coated target cells through the process of antibody-dependent cell-mediated cytoxicity. Lastly, natural killer cells synthesize and release a variety of cytokines, including type II IFN and several interleukins that stimulate their own proliferation and cytolytic activity.

Cellular Pattern Recognition Receptors

Cells at portals of virus entry possess surface receptors (pattern recognition receptors (PRRs)) that recognize specific pathogen-associated molecular patterns (PAMPs), which are macromolecules present in microbes but not on host cells. These pattern recognition receptors are expressed on and in a variety of different cells, including macrophages, dendritic cells, neutrophils, natural killer cells, endothelial cells, and mucosal epithelial cells. The binding of microbial macromolecules (PAMPs) to these receptors immediately triggers innate immune responses that protect the host against microbial invasion. Activation of these responses does not require prior exposure of the host to the specific virus.

The toll-like receptors (TLRs; the name reflects the similarity of these proteins to the *Drosophila* protein Toll) are important examples of pattern recognition receptors. They are located both on the cell surface and in endosomal vesicles, which allows these receptors to detect the presence of microbial
“triggers” (PAMPs) in the extracellular environment, in addition to those internalized into the cell following phagocytosis or receptor-mediated endocytosis. There are at least 10 mammalian TLRs, and different TLRs recognize different PAMPs. TLR3 is especially important for antiviral immunity, because its ligand is double-stranded RNA (dsRNA), which is produced in virus-infected cells. All TLRs have an extracellular portion that includes leucine- and cysteine-rich domains, and a conserved cytoplasmic portion that interacts with cellular signaling proteins. Other pathogen recognition/detection systems are also operational within the cell cytoplasm, and activation of the TLRs and/or any of the other sensors results in activation of common signaling pathways that involve cellular transcriptional factors, notably nuclear factor κB (NF-κB) that causes, depending on cell type: (1) expression of IFNs and inflammatory cytokines such as tissue necrosis factor and interleukins-1 and -12 (IL-1, IL-12); (2) activation of phagocytic cells and endothelial cells with increased production of inflammatory mediators and cell-surface expression of adhesion molecules; (3) production in phagocytic cells of microbial products such as nitric oxide. Collectively, these cellular responses that are triggered by activation of pattern recognition receptors are potent stimulators of inflammation and inhibitors of viral infection and replication.

Cytokines

Cytokines are messengers of the immune system that are responsible for the induction and regulation of both innate and adaptive immune responses. Specifically, cytokines are soluble mediators that facilitate communication between key cell populations, including the various subpopulations of lymphocytes, macrophages, dendritic cells, endothelial cells, and neutrophils. By way of general properties, cytokines are typically inducible glycoproteins that are transiently synthesized after appropriate stimulation of the cell that produces them. Individual cytokines usually are produced by more than one cell type and perform several, frequently divergent activities. There is much overlap (redundancy) in the activity of different cytokines; thus their interactions and activities are highly complex. Cytokines bind to specific receptors on the surface of target cells, with subsequent transcriptional activation of that cell. These activities can manifest as:

- autocrine effects on the same cell type that produces them
- paracrine effects on adjacent cells of different types
- endocrine effects, which are systemic effects on many cell types

Key cytokines involved in innate immune responses include the type I IFNs, tissue necrosis factor (TNF), IL-6, and the chemokines. Chemokines are a family of small proteins that are chemotactic for leukocytes, including, among many others, IL-8. Other cytokines such as IL-12 and type II IFN are critical to both innate and adaptive immune responses. The IFNs are especially critical to antiviral immunity and are discussed in detail in the following section.

Interferons

In 1957, Isaacs and Lindenmann reported that cells of the chorioallantoic membrane of embryonated hen’s eggs infected with influenza virus release into the medium a nonviral protein—“interferon” (IFN)—that protects uninfected cells against the same or unrelated viruses. It has since been determined that there are several types and subtypes of interferon (IFN), and that these proteins are key elements of antiviral resistance and are central to both innate and adaptive immune responses to viral infections.

There are three distinct types of IFN, designated as types I, II, and III, that each utilize different cellular receptors (Figure 4.2).

- **Type I interferon (type I IFN).** Type I IFNs include IFN-α, of which there are several types depending on species, and a single type of IFN-β; many cell types can...
produce these type I IFNs. Additional type I IFNs with specific functions include IFN-δ, ε, κ, and ω in pigs; τ in ruminants) interact with IFN-α, -β, -δ, and -ω receptors. Type I IFNs interact with IFNAR1 and IFNAR2; type II IFN-γ interacts with IFNAR1 (IFNAR) and IFNGR2; type III IFN-λ interacts with IFNAR1 and IFNGR1; type II IFN-γ interacts with IFNGR1 and IFNGR2. Type II IFN-λ is an antiparallel homodimer exhibiting a twofold axis of symmetry. It binds two IFNGR1 and IFNGR2 chains, assembling a complex that is stabilized by two IFNGR2 chains. These receptors are associated with two kinases from the JAK family: Janus (JAK) and tyrosine (TYK) for types I and III IFNs; JAK1 and JAK2 for type II IFN. All IFN receptor chains belong to the class 2 helical cytokine receptor family, which is defined by the structure of the extracellular domains of their members: approximately 200 amino acids structured in two subdomains of 100 amino acids (fibronectin type III modules), themselves structured by seven β-strands arranged in a β-sandwich. The 200 amino acid domains usually contain the ligand binding site. IFNAR2, IFNLIR1, IL10R2, IFNGR1, and IFNGR2 are classical representatives of this family, whereas IFNAR1 is atypical, as its extracellular domain is duplicated. GAS, IFN-γ-activated site; IRF9, IFN regulatory factor 9; ISGF3, IFN-stimulated gene factor 3 (refers to the STAT1–STAT2–IRF9 complex); ISRE, IFN-stimulated response element; P, phosphate, STAT1/2, signal transducers and activators of transcription 1/2. [From E. C. Borden, G. C. Sen, G. Uze, R. H. Silverman, R. M. Ransohoff, G. R. Foster, G. R. Stark. Interferons at age 50: past, current and future impact on biomedicine. Nat. Rev. Drug Discov. 6, 975–990 (2007), with permission.]

**Type II interferon (type II IFN).** There is a single form of type II IFN designated IFN-γ, which is a product of T cells and NK cells. Activated T cells are especially important sources of IFN-γ production, which is central to the expression of the cell-mediated immune aspects of adaptive immunity. Type II IFN binds the IFN-γ receptor (IFNGR), which is a tetramer composed of two heterodimers of IFNGR1 and IFNGR2, to activate a cell signaling pathway (involving JAK and STAT) to induce the cellular IFN-γ-activated site (GAS). This transcriptional activation induced by IFN-γ generates broad antimicrobial immunity in the treated cell, especially macrophages. IFN-γ is particularly important in conferring immunity to intracellular microorganisms other than viruses.

**Type III interferon (type III IFN).** Type III IFN represented by IFN-λ is only recently described and appears to represent an ancestral type I IFN, perhaps one with regulatory function.

In addition to their important antiviral activities, particularly those of type I IFN, the IFNs also stimulate...
adaptive immune responses, including enhanced cytotoxic T-lymphocyte-mediated cell lysis through increased expression of class I MHC on virus-infected cells. Similarly, IFN-γ promotes expression of class II MHC on macrophages, activates macrophages and NK cells, and modulates immunoglobulin synthesis by B lymphocytes. Type II IFN also exerts systemic effects including pyrexia and myalgia.

**Induction of IFN Production**

Induction of type I IFN involves activation via cellular pattern recognition receptors, which are non-specific sensors of viral infections that detect unique viral signatures (PAMPs), leading to transcription of numerous genes encoding proteins that are involved in innate and adaptive immune responses, including type I IFN. Importantly, these responses may be triggered by several redundant pathways, both cytoplasmic and extracytoplasmic (Figure 4.3). The TLRs are largely responsible for pathogen detection in extracytoplasmic compartments, and subsequent induction of production of type I IFN. The TLRs detect PAMPs and signal via cytoplasmic Toll/IL-1 receptor (TIR) domains to transcriptionally activate critical genes, including those encoding the type I IFNs. Different TLRs detect different PAMPs; thus TLR7 and TLR8 detect single-stranded RNA (ssRNA) and are important in type I IFN production in influenza and human immunodeficiency viral infections, TLR9 detects viral DNA, as in herpesvirus infection, and TLR3 detects dsRNA, which characteristically is produced during viral infections but is not present in normal cells. These receptors are predominantly located in the endosome, where they can readily detect viruses internalized after endocytosis, including viruses or their nucleic acid released from adjacent apoptotic or lysed cells. Induction of type I IFN transcription following activation of the extracytoplasmic path is dependent on the specific TLR that is activated; thus TLR3 utilizes a specific adapter designated TRIF (TIR-domain-containing adapter-inducing IFN-β) that mediates activation of: (1) IFN-β, (2) IRF3, and (3) activating protein 1 (AP1), leading to upregulation of IFN-β gene transcription. In contrast, activation of TLRs 7, 8, and 9 is mediated by the myeloid differentiation primary response protein 88 (MyD88) adapter molecule associated with the TIR domain, which results in activation of IRF7, NFκB, and AP1, which results in transcriptional activation of both the IFN-α and IFN-β genes (Figure 4.3). Especially high levels of expression of this latter pathway occur in dendritic cells, presumably because of...
their critical central role in both innate and adaptive immune responses.

Cytoplasmic pathways for pathogen sensing and type I IFN induction also can occur via TLR-independent signaling involving cytoplasmic RNA helicase proteins such as retinoic acid inducible gene (RIG-1) and melanoma differentiation-associated gene 5 (MDA5) (Figure 4.2). The cytoplasmic pathway includes mitochondrial antiviral signaling protein (MAVS; also referred to as IPS-1) and leads to activation of NFκB, AP-1, and IRF3, with resultant transcriptional activation of innate response genes including type 1 IFN. There are also TLR- and RIG-1-independent signaling pathways that provide further redundancy in the detection of microorganism triggers (PAMPs), which is so critical to a prompt antiviral response and, ultimately, to host survival.

**Action of Type I IFN**

Type I IFN produced and released from virus-infected cells (as described in the preceding section) exerts its effects on adjacent cells via receptor (IFNAR) binding and signaling that leads to induction of the IFN response element, with transcriptional activation of more than 300 IFN-stimulated genes (ISGs). Most of these ISGs encode cellular pattern recognition

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**FIGURE 4.4** The antiviral state. (A) Development of the antiviral state begins with the action of interferon on an uninfected cell. The result of the signal transduction cascade shown in Figure 4.2 is the induction of expression of up to 300 genes, of which three are shown here: RNase L, the 2′-5′ oligo(A) synthetase (2′-5′ OS), and the double-stranded DNA (dsRNA)-dependent protein kinase (PKR). These proteins are latent until they are activated by viral infection. PKR and 2′-5′ OS are activated by dsRNA that is produced during viral infection. Once activated, PKR autophosphorylates, and then phosphorylates eukaryotic initiation factor 2 (EIF2). The activated synthetase makes trimeric oligonucleotides, which in turn activate RNase L. (B) Phosphorylated EIF2 and activated RNase L are characteristic of the "antiviral state," in which a eukaryotic cell is refractory to infection by a wide variety of viruses. Phosphorylated EIF2 cannot serve to initiate translation of mRNA by ribosomes, and activated RNase L degrades mRNAs, both viral and cellular, so protein synthesis stops. Without protein synthesis, no virus replication can take place, but the inhibition of protein synthesis is transient and the cell may recover. [From Viruses and Human Disease, J. H. Strauss, E. G. Strauss, 2nd ed., p. 401. Copyright © Academic Press/Elsevier (2007), with permission.]
receptors or proteins that regulate either signaling pathways or transcription factors that amplify IFN production, whereas others promote an antiviral state via cytoskeletal remodeling, apoptosis, post-transcriptional events (mRNA editing, splicing, degradation), or post-translational modification (Figure 4.4). Those proteins proven to be critical to the induction of the IFN-induced antiviral state include:

- ISG15, which is a ubiquitin homolog that is not constitutively expressed in cells. Addition of ubiquitin to cellular proteins is key to regulation of the innate immune response, and ISG15 apparently can exert a similar function with more than 150 target proteins in IFN-stimulated cells. Activities of ISG15 can regulate all aspects of the IFN pathway, including induction, signaling, and action.

- MxGTPase is a hydrolyzing enzyme that, like ISG15, is not constitutively expressed. The enzyme is located in the smooth endoplasmic reticulum, where it affects vesicle formation, specifically targeting the viral nucleocapsid in virus-infected cells to prevent virus maturation.

- The protein kinase (PKR) pathway is constitutively expressed at only a very low level, but is quickly upregulated by IFNAR signaling. In the presence of dsRNA, protein kinase phosphorylates elongation (translation) initiation factor eIF-2α and prevents recycling of cyclic nucleotides (GDP), which in turn halts protein synthesis. This IFN-induced pathway is especially important for inhibiting replication of reoviruses, adenoviruses, vaccinia and influenza viruses, amongst many others.

- The 2′-5′ oligoadenylate synthetase (OAS) pathway, like the PKR pathway, is constitutively expressed only at low level. After IFNAR stimulation and in the presence of dsRNA, this enzyme produces oligoadenylates with a distinctive 2′-5′ linkage, as contrasted with the normal 3′-5′ lineage. These 2′-5′ oligoadenylates in turn activate cellular RNase that degrades RNA, which cleaves viral messenger and genomic RNA. Picornaviruses are especially susceptible to inhibition by this pathway, as is West Nile virus.

- Many other pathways have been identified in IFN-treated cell cultures, but their individual significance remains to be unequivocally proven in knockout mice.

In summarized, type I IFN is produced after viral infection of many different types of cells, and the IFN released from these cells then induces an antiviral state in adjacent cells (autocrine or paracrine effect). The multiple antiviral pathways that are activated in IFN-treated cells are stringently regulated by requirement for the presence of cofactors such as dsRNA, meaning that these pathways can only be activated when the IFN-treated cell is subsequently infected with a virus. This stringent regulation is necessary because some of these antiviral defense mechanisms also compromise normal cellular functions.

**Apoptosis**

It was long thought that viruses killed cells by direct means such as usurping their cellular machinery or disrupting membrane integrity, ultimately leading to necrosis of the virus-infected cell. However, it is now clear that apoptosis is an important and common event during many viral infections. *Apoptosis* is the process of programmed cell death, which is essentially a mechanism of cell suicide that the host activates as a last resort to eliminate viral factories before progeny virus production is complete. There are two distinct cellular pathways that trigger apoptosis (Figure 4.5), both of which culminate in the activation of host-cell caspase enzymes that mediate death of the cell (the so-called executioner phase). Once activated, caspases are responsible for degradation of the cell’s own DNA and proteins. Cell membrane alterations in the doomed cell promote its recognition and removal by phagocytic cells. The two initiation pathways are:

1. **The Intrinsic (Mitochondrial) Pathway.** The mitochondrial pathway is activated as a result of increased permeability of mitochondrial membranes subsequent to cell injury, such as that associated with a viral infection. Severe injury alters the delicate balance between anti-apoptotic (e.g., Bcl-2) and pro-apoptotic (e.g., Bax) molecules in mitochondrial membranes and the cytosol, resulting in progressive leakage of mitochondrial proteins (such as cytochrome c) into the cytosol where these proteins activate cellular caspases.

2. **The Extrinsic (Death Receptor) Pathway.** The extrinsic pathway is activated by engagement of specific cell-surface receptors, which are members of the TNF receptor family (TNF, Fas, and others). Thus binding of the cytokine TNF to its cellular receptor can trigger apoptosis. Similarly, cytotoxic T lymphocytes that recognize virus-infected cells in an antigen-specific manner can bind the Fas receptor, activate the death domain, and trigger the executioner caspase pathway that then eliminates the cell before it becomes a functional virus factory.

In addition to death-receptor-mediated cytolyis, cytotoxic T lymphocytes and natural killer cells can initiate apoptosis of virus-infected target cell, utilizing preformed mediators such as perforin and granzyme that directly activate caspases in the target cell.

**Gene Silencing (Interfering RNA)**

Cells utilize small, interfering, RNA molecules (RNAi) to silence genes as a means of regulating normal developmental and physiological processes, and potentially to interfere with virus replication. The RNAi are produced from longer segments of either ssRNA or dsRNA, after their cleavage by an endoribonuclease (Dicer). Production of RNAi initiates formation of the RNA-silencing complex that includes an endonuclease (argonaute) that degrades those
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mRNAs with a sequence that is complementary to that of the RNAi (Figure 4.6). Cells can utilize this mechanism to disrupt virus replication through the production of RNAi that are complementary to specific viral genes; however, RNAi also may be produced during viral infections that specifically inhibit protective cellular antiviral pathways.

**Adaptive Immunity**

Adaptive immunity includes humoral and cellular components. Humoral immunity is mediated principally by antibodies released from B lymphocytes, whereas cellular immunity is mediated by T lymphocytes (Figure 4.7). In addition, dendritic cells, macrophages, NK cells, and cytokines are all critical to adaptive immune responses. Adaptive immunity is antigen specific, so that these responses take time (several days at least) to develop, and this type of immunity is mediated by lymphocytes that possess surface receptors that are specific to each pathogen. Adaptive immunity stimulates long-term memory after infection, meaning that protective immune responses can quickly be reactivated on re-exposure of the organism to the same pathogen.

**Cytokines** were described above in the section on innate immunity, but they are also critical to adaptive immune responses, emphasizing how innate and acquired immune responses are inter-related. Those cytokines that are especially important to adaptive immunity are principally produced by CD4+ T cells after antigenic stimulation, and they promote the proliferation, differentiation, and activation of lymphocytes. Important cytokines of adaptive immunity include interleukins IL-2, IL-4, IL-5, and IL-17 and IFN-γ (type II IFN).

**B lymphocytes** produce antibodies that are responsible for, amongst other activities, neutralization and clearance of cell-free viruses. Antibodies also mediate long-term protection against reinfection by many viruses. B lymphocytes express surface receptors that are specific to particular antigens: after exposure to antigen such as a viral infection, B cells develop into plasma cells that secrete antibodies with the same antigen specificity as that of the surface receptors of the B cells from which they were derived. Receptor diversity is generated by rearrangement of the genes encoding portions of individual immunoglobulin molecules. The acquired B cell response to infection involves immunoglobulin class switching and progressive specificity, known as **affinity maturation**.

A subclass of B lymphocytes, designated B1 cells, secrete broadly reactive immunoglobulin, known as natural antibody, without specific antigen stimulation. Thus natural antibodies provide linkage between the innate and acquired humoral responses and provide a first line of humoral defense.

**T lymphocytes** also possess antigen-specific surface receptors. Like B-cell antigen recognition, diversity and
**FIGURE 4.6** Mechanisms of RNA interference. Long double-stranded RNAs (dsRNAs) are cleaved by RNaseIII and subsequently processed by DICER into short interfering RNAs (siRNAs), which are short dsRNAs of 21–22 nts with 2-nt overhangs on the 3′ termini. siRNAs then enter the RNA-induced silencing complex (RISC), where the sense strand is selectively degraded. miRNAs are encoded as incompletely self-complementary hairpins in viral and cellular genomes. They are cleaved by Drosha in the nucleus and the pre-micro RNA (pre-miRNA) is exported to the cytoplasm by Exportin 5. There they are also processed by DICER. siRNAs silence genes by binding in the context of RISC to mRNAs that are exactly complementary, and causing mRNA degradation. miRNAs often contain some mismatches and generally exert their effects by inhibiting translation. siRNAs can also enter RNA-induced transcriptional silencing complexes (RITS), which recruit an enzyme to methylate DNA in chromatin, turning it into inactive heterochromatin. Both RITS and RISC contain argonaute proteins (Agos). miRNP, micro ribonucleoprotein. [From Viruses and Human Disease, J. H. Strauss, E. G. Strauss, 2nd ed., p. 403. Copyright © Academic Press/Elsevier (2007), with permission.]

**FIGURE 4.7** The principal classes of lymphocytes and their functions in adaptive immunity. [From Robbins & Cotran Pathologic Basis of Disease, V. Kumar, A. K. Abbas, N. Fausto, J. Aster, 8th ed., p. 185. Copyright © Saunders/Elsevier (2010), with permission.]
receptor specificity is generated by somatic rearrangement of the genes that encode the T cell receptor. There are two major classes of T cells; those with receptors that consist of a heterodimer of $\alpha$ and $\beta$ chains (so-called $\alpha\beta$ T cells), and those with a receptor composed of a heterodimer of $\gamma$ and $\delta$ chains ($\gamma\delta$ T cells). The $\alpha\beta$ T cells recognize small peptides expressed on the surface of cells in association with MHC antigens, which confers exquisite specificity to the cellular immune mechanisms mediated by these cells. In contrast, although $\gamma\delta$ T cells also recognize small peptides expressed on the cell surface, they do so without the requirement for MHC restriction. This lack of MHC restriction, coupled with the fact that $\gamma\delta$ T cells are especially abundant at mucosal surfaces that serve as portals of virus entry (like the mucosal lining of the gastrointestinal tract), suggests that these cells serve as sentinels to remove virus-infected cells. Thus $\gamma\delta$ T cells probably constitute a critical “bridge” between innate and adaptive antiviral immunity.

Cellular immunity (cell-mediated immunity) is mediated by effector lymphocytes and macrophages that specifically eliminate virus-infected cells. The lymphocytes that mediate cell lysis are cytotoxic T lymphocytes (CTLs) that typically express (CD8) on their cell surface (CD8+ CTLs), and which lyse cells that express viral antigen in the context of appropriate class I MHC molecules. Portions of immunogenic viral proteins produced in the cytosol of the infected cell are transported to the endoplasmic reticulum, where they associate with class I MHC molecules. This complex is directed to the cell surface, where the viral peptides can be recognized by antigen-specific cytotoxic T lymphocytes that then lyse the virus-infected cell by inducing apoptosis.

Dendritic cells are also key players in adaptive and innate immunity; they derive their names from their abundant fine cytoplasmic processes ("dendrites"). There are two major types of dendritic cells:

- **Interdigitating dendritic cells** are critical antigen-presenting cells that are located at portals of virus entry, such as the skin and within/beneath the mucosal epithelial surfaces lining the gastrointestinal, respiratory, and urogenital tracts. They are also present within the interstitium of virtually all tissues. These dendritic cells express surface pattern-recognition receptors that can quickly and generically respond to the presence of viral triggers (PAMPs) by the production and release of antiviral cytokines such as IFN. In addition, these cells migrate to the T cell regions of lymphoid tissues, where they can present antigen to T cells and, because they express high levels of stimulatory molecules such as MHC antigens, they are potent inducers of T cell activation.

- **Follicular dendritic cells** occur within germinal centers of lymphoid tissues such as lymph node and spleen. These cells efficiently capture (phagocytose) circulating antigens, which they then present to B lymphocytes that express the relevant surface receptor specificity, leading to B cell activation and development of humoral (antibody-mediated) immunity.

Macrophages are important bone-marrow-derived cells that are responsible for microbial phagocytosis and killing. They can be activated by cytokines into effector cells that mediate cellular immunity through their enhanced antimicrobial capacity. Macrophage activation is mediated by IFN-γ that is released by antigen-specific T cells, and by NK cells.

Major histocompatibility complex (MHC) antigens expressed on the surface of relevant cells are central to adaptive immunity. Major histocompatibility antigens are polymorphic proteins that make up the molecule of which it is to display portions of immunogenic proteins to antigen-specific T lymphocytes (Figure 4.8). Class I MHC antigens are expressed on the surface of all nucleated cells, and class I MHC molecules on the surface of virus-infected cells typically display
immunogenic proteins from the infecting virus that are recognized by antigen-specific cytotoxic T lymphocytes. Specifically, viral proteins produced in the cytoplasm of infected cells are degraded within proteasomes, and fragments of these proteins are then transported to the endoplasmic reticulum where they bind to newly synthesized class I MHC molecules. Association of $\beta_2$ microglobulin with the class I MHC and viral peptide complex forms a stable heterotrimer that is transported to the cell surface, where the viral antigen can be recognized by antigen-specific cytotoxic T lymphocytes. This T-cell-mediated killing is restricted to target cells that express the same class I MHC haplotype. Class II MHC antigen, in contrast, is expressed principally on antigen-presenting cells, namely B lymphocytes, macrophages, and dendritic cells. Class II MHC molecules display viral proteins at the cell surface that are recognized by antigen-specific CD4$^+$ T lymphocytes—that is, those with surface receptors that specifically recognize and bind to the displayed peptide. In this scenario, viral proteins are degraded into peptides within endocytic vesicles and these peptides then associate with class II MHC molecules that are synthesized within these same vesicles. The complex of class II MHC molecule and viral peptide is then transported to the cell surface for display and recognition by antigen-specific CD4$^+$ T lymphocytes.

Passive Immunity

Specific antibody alone is highly effective in preventing many viral infections. For example, artificial passive immunization (injection of antibodies) temporarily protects animals against infection with the viruses that cause canine distemper, feline panleukopenia, and porcine reproductive and respiratory syndrome, amongst many others. Furthermore, natural passive immunization—that is, the transfer of maternal antibody from dam to fetus or newborn—protects the newborn for the first few months of life against most of the infections that the dam has experienced.

Natural passive immunity is important for two major reasons: (1) it is essential for the protection of young animals, during the first weeks or months of life, from the myriad of microorganisms and viruses that are present in the environment into which animals are born; (2) maternally derived antibody interferes with active immunization of the newborn and must therefore be taken into account when designing vaccination schedules.

Maternal antibodies may be transmitted in the egg yolk in birds, across the placenta in primates and rodents, or via colostrum and/or milk in ungulates and other mammals. Different species of mammals differ strikingly in the predominant route of transfer of maternal antibodies, depending on the structure of the placenta of the species. In those species such as primates in which the maternal and fetal circulations are in relatively close apposition, antibody of the immunoglobulin (Ig) G (but not IgM) class is able to cross the placenta, and maternal immunity is transmitted mainly by this route. Some species with more complex placenta, such as mice, acquire maternal antibody through yolk-sac immunoglobulin receptors. In contrast, the complex placenta of most domestic animals serves as a barrier to maternal immunoglobulins; in these species, maternal immunity is transmitted to the newborn via colostrum and, to a much lesser extent, via milk. Different species differ in regard to the particular class or subclass of immunoglobulin that is transferred preferentially to the newborn in colostrum, but in most domestic animals it is mainly IgG. In cattle and sheep there is a selective transfer of IgG1 from the serum across the alveolar epithelium of the mammary gland during the last few weeks of pregnancy. Antibodies of the IgG1 class are important in protection against enteric infections as long as suckling continues.

The very substantial amounts of IgG present in colostrum are ingested and translocated in large intracytoplasmic vesicles by specialized cells present in the upper part of the small intestine to reach the circulation of the newborn. Small amounts of other antibodies (IgM, IgA) present in colostrum or milk may, in some species, also be translocated across the gut, but disappear quickly from the circulation of the young animal. The period after birth during which antibody, ingested as colostrum, is translocated is sharply defined and very brief (about 48 hours) in most domestic animals, but can be very prolonged in rodents; mice continue to acquire maternal IgG for up to 3 weeks.

In birds there is a selective transfer of IgG from the maternal circulation, so that IgG is concentrated in the egg yolk. IgG enters the vitelline circulation, and hence that of the chick, from day 12 of incubation. Some IgG is also transferred to the amniotic fluid and is swallowed by the chick. Close to the time of hatching, the yolk sac with the remaining maternal immunoglobulin is completely taken into the abdominal cavity and absorbed by the chick.

Maternal antibody in the blood stream of the newborn mammal or newly hatched chick is destroyed quite rapidly, with first-order kinetics. The half-life, which is somewhat longer than in adult animals, ranges from about 21 days in the cow and horse, to 8–9 days in the dog and cat, to only 2 days in the mouse. Of course, the newborn animal will be protected against infection with any particular virus only if the dam’s IgG contains specific antibodies, and protection may last much longer than one IgG half-life if the initial titer against that virus is high. Although the concentrations of IgA transferred via colostrum to the gut of the newborn animal are considerably lower than those of IgG, antibodies of this isotype are important in protecting the neonate against enteric viruses against which the dam has developed immunity. Moreover, there is evidence that even after intestinal translocation ceases, immunoglobulins present in ordinary milk—principally IgA but also IgG and IgM—may continue to provide some protective immunity against gut infections. Often the newborn encounters viruses while still partially protected. Under these circumstances the
virus replicates, but only to a limited extent, stimulating an immune response without causing significant disease, and the infected newborn thus acquires active immunity while partially protected by maternal immunity.

**Failure of Maternal Antibody Transfer**

The failure or partial failure of maternal antibody transfer is the most common immunodeficiency disease of livestock, and predisposes affected animals to infectious diseases, particularly enteric and respiratory diseases. Maternal immunization to ensure passive protection of newborn animals has become an important strategy in veterinary medical practice, in conjunction with sound management practices that ensure newborn animals quickly receive adequate amounts of colostrum.

**VIRAL MECHANISMS OF AVOIDANCE AND ESCAPE**

In the ongoing war and détente between virus and host, viruses have developed remarkably sophisticated mechanisms to avoid the various host protective responses. In addition to the many different strategies utilized by viruses to facilitate persistent infection (including growth in immune cells and/or in immunologically privileged sites, latency, integration, antigenic drift (see Chapter 3)), individual viruses have developed diverse and complex mechanisms of avoiding protective host innate and adaptive immune responses. Examples of these mechanisms include the following:

- Shutdown of host macromolecular synthesis
- Avoidance of CTL-mediated killing of virus-infected cells
- Prevention of NK-cell-mediated lysis of virus-infected cells
- Interference with apoptosis
- Counter defenses against cytokines
- Evasion of the antiviral state
- Virus-specific gene-silencing pathways

**Shutdown of Host Macromolecule Synthesis**

Many viruses, soon after infection, inhibit normal transcription and/or translation of cellular proteins, and rapidly subvert the machinery of the infected cell for production of progeny virions. This rapid shutdown of the host cell quickly impairs the innate immune response to the infecting virus, including the production of critical proteins such as class I MHC antigen and antiviral cytokines such as type I IFN. The result is that, without effective innate immune responses, the infecting virus can quickly replicate and disseminate before the host can develop an adaptive immune response. This strategy is widely used by RNA viruses, many of which have very rapid replication cycles.

**Avoidance of CTL-Mediated Killing of Virus-Infected Cells**

Cytotoxic T lymphocyte (CTL)-mediated killing of virus-infected cells requires the presentation of viral antigens on the surface of the infected cell in the context of the appropriate class I MHC molecule; thus viruses have developed different strategies to suppress the normal expression of class I MHC proteins so as to inhibit CTL-mediated lysis. These strategies include: (1) suppression of cellular production of class I MHC molecules by shutdown of host protein synthesis; (2) production of virus-encoded proteins that disrupt normal production of class I MHC proteins, or their transport from the endoplasmic reticulum to the Golgi apparatus or to the cell surface; (3) production of virus-encoded proteins that disrupt the function or viability of class I MHC molecules; (4) production of virus-encoded homologs of class I MHC molecules that can bind β2 microglobulin and viral peptides, but are otherwise dysfunctional in terms of mediating CTL activity.

**Prevention of NK-Cell-Mediated Lysis of Virus-Infected Cells**

In contrast to CTL-mediated lysis, which requires the presence of appropriate concentrations of class I MHC antigen on the surface of virus-infected cells, NK-cell-mediated cytolysis is promoted by reduced levels of class I MHC antigen on the cell surface. Also important to NK cell activity is the balance of inhibitory molecules (such as class I MHC antigen) and stimulatory molecules (such as heat-shock proteins) on the cell surface; thus some viruses selectively inhibit cellular production and expression of molecules that provide stimulatory signals for NK cell activity. Other viruses inhibit host-cell production of both stimulatory and inhibitory molecules, such that the infected cell is somewhat protected against both CTL- and NK-cell-mediated lysis.

**Interference with Apoptosis**

In addition to apoptosis induced by NK-cell or CTL-mediated cell lysis (as described in the preceding sections), viral infection alone can initiate apoptosis via either the extrinsic (death receptor) or intrinsic (mitochondrial) pathways (see pages 82/83). Apoptosis is especially deleterious to the relatively slow-growing DNA viruses, including poxviruses, herpesviruses, and adenoviruses, because apoptosis can result in death of cells infected with these viruses before maximal levels of virus replication have been completed. Thus these DNA viruses, in particular, have developed a remarkable variety of strategies to optimize their replication by inhibiting the various pathways that normally lead to apoptosis. The need for these viruses to prevent apoptosis to promote their own survival is reflected
by the fact that individual viruses may use a combination of strategies, including: (1) inhibition of the activity of executioner caspases that mediate cellular injury—notably by the serpins, which are protease inhibitors produced by poxviruses that bind to and block the proteolytic activity of caspases; (2) inhibition of the expression, activation, and signaling of death receptors, such as by production of viral receptor homologs that bind TNF so that it cannot initiate the extrinsic pathway, or molecules that specifically block the signaling cascade initiated by death receptor activation; (3) production of virus-encoded homologs of anti-apoptotic proteins such as Bcl-2; (4) production of proteins that sequester p53, which is a pro-apoptotic molecule that accumulates in cells infected with certain viruses; (5) other as yet poorly defined mechanisms of inhibition of apoptosis, apparently used by a myriad of viral proteins.

**Counter Defenses against Cytokines**

Cytokines are central to both innate and adaptive immune responses of animals to viral infections, thus viruses also have developed effective strategies to combat the activities of these important mediators of antiviral immunity. Certain viruses have acquired and modified cellular genes, creating viral genes that encode proteins that are homologs of cytokines or their receptors. Virus-encoded cytokine homologs can be functional (so-called virokines) and mimic the biological effect of the authentic molecule, or they can be non-functional and simply bind and block the specific cytokine receptor to neutralize that activity. Similarly, virus-encoded receptor homolog proteins typically bind to and neutralize the relevant cytokine. Other virus-encoded proteins interfere with dsRNA-activated pattern recognition receptor signaling pathways (such as TLR3 or RIG-I) that trigger production of type I IFN and other antiviral cytokines, or with the signaling pathways activated by the binding of IFN to its receptor (IFNAR). Collectively, these virus-encoded proteins can modulate the activities of a wide variety of critical cytokines such as IL-1, IL-6, IL-8, types I and II IFN, and TNF to the replicative benefit of the virus, by either inhibiting or promoting specific cytokine-mediated functions.

**Evasion of the Antiviral State**

Predictably, viruses also have evolved elaborate strategies to circumvent the activity of important IFN-induced antiviral effector mechanisms such as the protein kinase (PKR) and 2′-5′ oligoadenylate synthetase (OAS) pathways. These include the production of virus-encoded proteins or RNA molecules (RNAi) that bind but do not activate critical enzymes (or genes encoding them) involved in these pathways, the production of non-functional enzyme homologs, and the stimulation of pathways that downregulate activity and function of these protective antiviral pathways. Other virus-encoded proteins sequester dsRNA, which is a critical co-factor for both PKR and OAS. Viruses in many different families of both DNA and RNA viruses have incorporated strategies for evading the host antiviral pathways, and additional examples undoubtedly will be identified in the future.

**Virus-Specific Gene Silencing Pathways**

Viruses have also developed counter defenses to cellular antiviral RNA interference pathways, either by the production of virus-encoded proteins or small interfering mRNA (siRNA) molecules that inhibit key steps of the cellular pathway depicted in Figure 4.4. Other viruses themselves produce RNAi molecules to silence key cellular genes involved in antiviral immunity.

**VACCINES AND VACCINATION AGAINST VIRAL DISEASES**

Vaccination is the most effective way of preventing viral diseases. Although deliberate exposure to virulent viruses such as smallpox (syn. variolation) was long recognized as an effective, albeit dangerous, method of prophylaxis, the concept of vaccination is considered to have been widely introduced by Edward Jenner in 1798 to protect humans against smallpox. Nearly a century later, the concept was shown by Louis Pasteur to have wider applications and, most notably, could be used to prevent rabies. With the advent of cell culture techniques in the 1950s, a second era of vaccination was introduced and many live-attenuated virus and inactivated-virus vaccines were developed. More recently, the field of vaccinology has witnessed the introduction of a number of novel “new generation” vaccines produced through various forms of recombinant DNA and related technologies. While live-attenuated and inactivated virus vaccines of the second era are still the “work horses” of veterinary practice, new generation vaccines are now complementing and, increasingly, replacing them.

There are some important differences between vaccination practices in humans and animals. Economic constraints are generally of less importance in human medicine than in veterinary medicine. There is also greater agreement about the safety and efficacy of vaccines in use in human medicine than there is with animal vaccines, and better mechanisms for reporting potential adverse consequences associated with the use of specific products. At the international level, the World Health Organization (WHO) exerts persuasive leadership for human vaccine usage, and maintains a number of programs that have no equivalents for animal vaccine usage by its sister agencies, the Food and Agriculture Organization and the Office International des Epizooties (OIE; syn. the World Organization for Animal Health). Furthermore, within countries, greater latitude is allowed in the manufacture and use of vaccines for veterinary diseases than is allowed by national regulatory authorities for human vaccines.
Before the recent advent of the new generation vaccines based on recombinant DNA technology, there were just two major strategies for the production of virus vaccines: one employing live-attenuated (syn. modified-live) virus strains and the other employing chemically inactivated (syn. killed) virus preparations. Live-attenuated virus vaccines replicate in the vaccine recipient and, in so doing, amplify the amount of antigen presented to the host’s immune system. There are important benefits in this approach, because the replication of vaccine virus mimics infection to the extent that the host immune response is more similar to that occurring after natural infection than is the case with inactivated or some subunit vaccines. When inactivated virus vaccines are produced, the chemical or physical treatment used to eliminate infectivity may be damaging enough to diminish the immunogenicity of the vaccine virus, especially the induction of virus-specific cell-mediated immune responses. As a result, inactivated vaccines often induce an immune response that is shorter in duration, narrower in antigenic spectrum, weaker in cell-mediated and mucosal immune responses, and possibly less effective in inducing sterilizing immunity. Nonetheless, very serviceable and safe inactivated vaccines are available and widely used.

The majority of vaccines in large-scale production for use in animals continue to include either live-attenuated or inactivated virus; however, new generation vaccines developed through recombinant DNA technologies offer significant improvements and potential advantages in terms of both their safety and their efficacy. A remarkable variety of such vaccines have recently been developed, an increasing number of which are now in commercial production.

Live-Attenuated Virus Vaccines

Live-attenuated virus vaccines, when they have been proven to be safe, have historically been the best of all vaccines. Several of them have been dramatically successful in reducing the incidence of important diseases of animals and humans. Most attenuated virus vaccines are injected intradermally, subcutaneously, or intramuscularly, but some are delivered orally, and a few by aerosol or to poultry in their drinking water. For these vaccines to be successful, the vaccine virus must replicate in the recipient, thereby eliciting a lasting immune response while causing little or no disease. In effect, a live-attenuated virus vaccine mimics a subclinical infection. The individual virus strain incorporated in a live-attenuated virus vaccine may be derived from any one of several sources.

Vaccines Produced from Naturally Occurring Attenuated Viruses

The original vaccine (vacca = cow), introduced by Jenner in 1798 for the control of human smallpox, utilized cowpox virus, a natural pathogen of the cow. This virus produced only a mild infection and lesions in humans, but, because it is antigenically related to smallpox virus, it conferred protection against the human disease. The same principle has been applied to other diseases—for example, the protection of chickens against Marek’s disease using a vaccine derived from a related herpesvirus of turkeys, and the protection of piglets against porcine rotavirus infection using a vaccine derived from a bovine rotavirus. Similarly, rabbits can be effectively protected against the pox viral disease, myxomatosis, with the naturally avirulent Shope rabbit fibroma virus.

Vaccines Produced by Attenuation of Viruses by Serial Passage in Cultured Cells

Most of the live-attenuated virus vaccines in common use today were derived empirically by serial passage of virulent “field” viruses (syn. “wild-type” viruses) in cultured cells. The cells may be of homologous or, more commonly, heterologous host origin. Typically, adaptation of virus to more vigorous growth in cultured cells is accompanied by progressive loss of virulence for the natural host. Loss of virulence may be demonstrated initially in a convenient laboratory model such as a mouse, before being confirmed by clinical trials in the species of interest. Because of the practical requirement that the vaccine must not be so attenuated that it fails to replicate satisfactorily in its natural host, it is sometimes necessary to compromise by using a virus strain that replicates sufficiently well that it may induce mild clinical signs in a few of the recipient (vaccinated) animals.

During repeated passage in cultured cells, viruses typically accumulate nucleotide substitutions in their genome, which in turn lead to attenuation. With the recent advent of high-throughput, whole-genome sequencing, the genetic basis of virulence and attenuation has been established with many viruses, which allows better prediction of vaccine efficacy and safety. Furthermore, it is increasingly clear that several genes can contribute to virulence and tropism of individual viruses, and do so in different ways. For example, in contrast to the severe, systemic infections associated with some wild-type or “field” viruses, live-attenuated vaccine strains of these same viruses administered by the respiratory route may replicate, for instance, only in the upper respiratory tract, or undergo only limited replication in the intestinal epithelium after oral administration.

Despite the outstanding success of empirically derived attenuated virus vaccines, there is a strong perceived need to replace what some veterinarians and veterinary scientists consider to be “genetic roulette” with rationally designed, specifically engineered vaccines. In these engineered live-attenuated vaccines, the mutations associated with attenuation of the parental virus are defined and predictable, as is the potential for reversion to virulence.
Antiviral Immunity and Prophylaxis

Vaccines Produced by Attenuation of Viruses by Serial Passage in Heterologous Hosts

Serial passage in a heterologous host was a historically important means of empirically attenuating viruses for use as vaccines. For example, rinderpest and classical swine fever (hog cholera) viruses were each adapted to grow in rabbits and, after serial passage, became sufficiently attenuated to be used as vaccines. Other viruses were passaged in embryonated hens’ eggs in similar fashion, although some such passaged viruses acquired novel and very undesirable properties. For example, live-attenuated bluetongue virus vaccines propagated in embryonated eggs can cross the placenta of ruminants vaccinated during pregnancy, with resultant fetal infection and developmental defects or loss. Similarly, embryonated-egg-propagated African horse sickness virus caused devastating consequences in humans infected after aerosol exposure to this vaccine virus.

Vaccines Produced by Attenuation of Viruses by Selection of Cold-Adapted Mutants and Reassortants

The observation that temperature-sensitive mutants (viruses that are unable to replicate satisfactorily at temperatures much higher than normal body temperature) generally display reduced virulence suggested that they might make satisfactory live-attenuated vaccines, although some viruses with temperature-sensitive mutations have displayed a disturbing tendency to revert toward virulence during replication in vaccinated animals. Attention accordingly moved to cold-adapted mutants, derived by adaptation of virus to grow at suboptimal temperatures. The rationale is that such mutant viruses would be safer vaccines for intranasal administration, in that they would replicate well at the lower temperature of the nasal cavity (about 33°C in most mammalian species), but not at the temperature of the more vulnerable lower respiratory tract and pulmonary airspaces. Cold-adapted influenza vaccines that contain mutations in most viral genes do not revert to virulence, and influenza vaccines based on such mutations are now licensed for human use; vaccines against equine influenza have been developed utilizing the same principle.

Non-Replicating Virus Vaccines

Vaccines Produced from Inactivated Whole Virions

Inactivated (syn. killed) virus vaccines are usually made from virulent virus; chemical or physical agents are used to destroy infectivity while maintaining immunogenicity. When prepared properly, such vaccines are remarkably safe, but they need to contain relatively large amounts of antigen to elicit an antibody response commensurate with that induced by a much smaller dose of live-attenuated virus vaccine. Normally, the primary vaccination course comprises two or three injections, and further (“booster”) doses may be required at regular intervals thereafter to maintain immunity. Killed vaccines usually must be formulated with chemical adjuvants to enhance the immune response, but these also can result in more adverse reactions to vaccination.

The most commonly used inactivating agents are formaldehyde, β-propiolactone, and ethylenimine. One of the advantages of β-propiolactone, which is used in the manufacture of rabies vaccines, and ethylenimine, which is used in the manufacture of foot-and-mouth disease vaccines, is that they are completely hydrolyzed, within hours, to nontoxic products. Because virions in the center of aggregates may be shielded from inactivation, it is important that aggregates be broken up before inactivation. In the past, failure to do this occasionally resulted in vaccine-associated disease outbreaks—for example, several foot-and-mouth disease outbreaks have been traced to this problem.

Vaccines Produced from Purified Native Viral Proteins

Lipid solvents such as sodium deoxycholate are used in the case of enveloped viruses, to solubilize the virion and release the components, including the glycoprotein spikes of the viral envelope. Differential centrifugation is used to semipurify these glycoproteins, which are then formulated for use as so-called split vaccines. Examples include vaccines against herpesviruses, influenza viruses, and coronaviruses.

Vaccines Produced by Recombinant DNA and Related Technologies

The relatively recent advent of molecular biology and its many associated technologies has facilitated the development of new vaccine strategies, each with inherent potential advantages and, in some instances, disadvantages as compared with those of the traditional vaccines. Such novel technologies have been used in the creation of new vaccines that already are in use and, given their substantial inherent potential advantages, it is anticipated that the availability and types of such products will only increase in the future.

Vaccines Produced by Attenuation of Viruses by Gene Deletion or Site-Directed Mutagenesis

The problem of the reversion to virulence of live-attenuated virus vaccines (i.e., a mutation by which the vaccine virus regains virulence) may be largely avoided by deliberate insertion of several attenuating mutations into key viral genes, or by completely deleting non-essential genes that contribute to virulence. Gene deletion is especially feasible
with the large DNA viruses that carry a significant number of genes that are not essential for replication, at least for replication in cultured cells. “Genetic surgery” is used to construct deletion mutants that are stable over many passages. Several herpesvirus vaccines have been constructed using this strategy; including a thymidine kinase (TK) deletion pseudorabies vaccine for swine that also includes a deletion of one of the glycoprotein genes (gE).

The deleted glycoprotein may be used as capture antigen in an enzyme-linked immunosorbent assay, so that vaccinated, uninfected pigs, which would test negative, can be distinguished from naturally infected pigs [the differentiation/discrimination of infected from vaccinated animals (DIVA) strategy], enabling eradication programs to be conducted in parallel with continued vaccination. A gE-deleted marker vaccine also is available for infectious bovine rhinotracheitis virus (bovine herpesvirus-1).

Site-directed mutagenesis facilitates the introduction of defined nucleotide substitutions into viral genes at will. As the particular genes that are influential in virulence and immunogenicity of individual viruses are increasingly defined, it is anticipated that existing empirically derived live-attenuated virus vaccines will be replaced by those engineered for attenuation through “customized” alteration of critical genes. The production of live-attenuated virus vaccines from molecular clones facilitates both the deliberate introduction of defined attenuating nucleotide substitutions into the vaccine virus, and consistent production of vaccine virus from a genetically defined “seed” virus. This strategy also potentially enables the use of differential serological tests to distinguish vaccinated and naturally infected animals (DIVA).

Subunit Vaccines Produced by Expression of Viral Proteins in Eukaryotic (Yeast, Mammalian, Insect), Bacterial, or Plant Cells

Eukaryotic expression vectors offer the potential for large-scale production of individual viral proteins that can be purified readily and formulated into vaccines. Once the critical viral protein conferring protection has been identified, its gene [or, in the case of an RNA virus, a complementary DNA (cDNA) copy of the gene] may be cloned into one of a wide choice of expression plasmids and expressed in any of several cell systems. Mammalian cells offer the advantage over cells from lower eukaryotes in that they are more likely to possess the machinery for correct post-translational processing and authentic maturation of complex viral proteins.

Useful eukaryotic expression systems include plant and yeast cells (Saccharomyces cerevisiae), insect cells (Spodoptera frugiperda), and various mammalian cells. Yeast offers the advantage that there is extensive experience with scale-up for industrial production; the first vaccine produced by expression of a cloned gene, human hepatitis B vaccine, was produced in yeast. Insect cells offer the advantage of simple technology derived from the silk industry: moth cell cultures (or caterpillars!) may be made to express very large amounts of viral proteins through infection with recombinant baculoviruses carrying the gene(s) of the virus of interest. The promoter for the gene encoding the baculovirus polyhedrin protein is so strong that the product of a viral gene of interest inserted within the baculovirus polyhedrin gene may comprise up to half of all the protein the infected cells make. For example, immunization of pigs with the capsid protein of porcine circovirus 2 expressed in insect cells from a recombinant baculovirus vector confers protective immunity against porcine-circovirus-associated diseases such as multisystemic wasting disease. Similarly, baculovirus-expressed E2 protein alone provides an effective recombinant subunit vaccine against classical swine fever virus.

Expression of protective viral antigens in plant cells can theoretically provide a very cost-effective and efficient method of vaccinating production animals. For example, plant cell lines have been developed that express the hemagglutinin and neuraminidase proteins of Newcastle disease virus for protective immunization of birds.

Vaccines Produced by Expression of Viral Proteins that Self-Assemble into Virus-like Particles

The expression of genes encoding the capsid proteins of viruses within certain families of non-enveloped icosahedral viruses leads to the self-assembly of the individual capsid proteins into virus-like particles (VLPs) that can be used as a vaccine. This strategy has been developed for various picornaviruses, caliciviruses, rotaviruses, and orbiviruses, and an effective VLP-vaccine has been developed recently against human genital papillomaviruses. The advantage of recombinant virus-like particles over traditional inactivated vaccines is that they are devoid of viral nucleic acid, and therefore completely safe. They may also be equated to an inactivated whole-virus vaccine, but without the potentially damaging loss of immunogenicity that can accompany chemical inactivation. However, the potential limitations of the strategy include production costs and low yields, stability of the VLP after production, and less effective immunity as compared with some existing vaccines.

Vaccines Utilizing Viruses as Vectors for Expression of Other (Heterologous) Viral Antigens

Recombinant DNA techniques allow foreign genes to be introduced into specific regions of the genome of either RNA or DNA viruses, and the product of the foreign gene is then carried into and expressed in the target cell. Specifically, the gene(s) encoding key protective antigens (those against which protective responses are generated in the host) of the virus causing a disease of interest are inserted into the genome of an avirulent virus (the recombinant vector). This modified avirulent virus is then administered either as
a live-attenuated virus vector or as a non-replicating (“suicide”) expression vector. Infected cells within the immunized host express the foreign protein, to which the animal will in turn mount an adaptive immune response (humoral and/or cellular). The approach is safe, because only one or two genes of the disease-causing virus typically are inserted into the expression vector, and because well-characterized viruses (such as existing live-attenuated vaccine viruses) can be used as the expression vector. Furthermore, animals vaccinated with such recombinant vaccines can be distinguished readily from infected animals (or those vaccinated with live-attenuated virus vaccines) using serological tests that detect antibodies to viral proteins that are not included in the vaccine construct (the so-called DIVA strategy).

**DNA Viruses as Vectors**

Individual genes encoding antigens from a variety of viruses have been incorporated into the genome of DNA viruses, especially vaccinia and several other poxviruses, adeno-viruses, herpesviruses, and adeno-associated viruses (which are parvoviruses).

Vaccination of animals with a significant number of different recombinant poxvirus-vectored vaccine constructs has effectively generated antibody and/or cell-mediated immune responses that confer strong protective immunity in the recipient animals against challenge infection with virulent strains of the heterologous viruses from which the genes were derived. For example, recombinant vaccinia virus vectored rabies vaccines incorporated into baits administered orally protect both foxes and raccoons against this zoonotic disease; this vaccine contains only the gene encoding the surface glycoprotein (G) of rabies virus. Similarly, the avian poxviruses have been increasingly used as expression vectors of heterologous genes in recombinant vaccine constructs. Fowlpox virus is a logical choice as a vector for avian vaccines but, perhaps surprisingly, fowlpox virus has also been shown to be a very useful expression vector in mammals: even though this virus, and the closely related canarypox virus, do not complete their replication cycle in mammalian cells, the inserted genes are expressed and induce strong cellular and humoral immune responses in inoculated animals. Because the large genome of poxviruses can accommodate at least a dozen foreign genes and still be packaged satisfactorily within the virion, it is theoretically possible to construct, as a vector, a single recombinant virus capable of protecting against several different viral diseases.

Recombinant poxvirus vectored vaccines that have been widely used to immunize mammals include vaccinia–rabies constructs used for the vaccination of foxes in Europe and raccoons and coyotes in the United States, and canarypox virus vectored vaccines to prevent influenza and West Nile disease in horses, distemper in dogs, ferrets and certain zoo animals/wildlife species, and feline leukemia and rabies in cats. Amongst many others, experimental recombinant canarypox virus vectored vaccines also have been successfully developed to prevent African horse sickness, bluetongue, Japanese encephalitis, and Nipah, and extensive trials have been carried out in humans with an experimental human immunodeficiency virus (HIV)–recombinant canarypox virus vaccine. Raccoonpox, capripox, and other poxviruses have also been successfully developed as recombinant expression vectors for potential use as vaccines in mammals. Rabbits can be effectively immunized against both myxomatosis (pox virus) and rabbit hemorrhagic disease (calicivirus) with a recombinant live-attenuated myxoma virus that expresses the VP60 gene of rabbit hemorrhagic disease virus. This combined vaccination strategy has the considerable advantage that rabbit hemorrhagic disease virus cannot be grown in cell culture, so that vaccination against rabbit hemorrhagic disease alone currently requires inactivation of virus collected from livers of virus-infected rabbits.

A number of DNA virus vectored vaccines have also been developed for use in poultry, including recombinant herpesvirus of turkey vectored vaccines against Newcastle disease virus, infectious laryngotracheitis virus, and infectious bursal disease virus; these vaccines include only genes encoding the protective antigens of the heterologous viruses, but they generate protective immunity in chickens against both Marek’s disease and the other respective disease (Newcastle disease, infectious laryngotracheitis, infectious bursal disease). Fowlpox virus vectored vaccines against Newcastle disease and H5 influenza viruses have also been developed, and the latter has been widely used in Mexico and Central America.

Chimeric DNA viruses also have been developed as vaccines in which the genes of a virulent virus are inserted into the genetic backbone of a related avirulent virus. For example, a chimeric circovirus vaccine used in swine includes a genetic backbone of porcine circovirus 1, which is avirulent (non-pathogenic) in swine, with the gene encoding the immunogenic capsid protein of pathogenic porcine circovirus 2. Antibodies to the capsid protein of porcine circovirus 2 confer immunity in vaccinated pigs. Like porcine circovirus 1, the chimeric virus replicates to high titer in cell culture, which makes vaccine production more efficient and cost effective.

It is anticipated that commercially available veterinary vaccines increasingly will utilize DNA viruses as expression vectors in the future, because of their inherent advantages in terms of safety and efficacy, and the ability in control programs to distinguish vaccinated animals from those exposed to infectious virus (DIVA).

**RNA Viruses as Vectors**

As with DNA virus vectored vaccines, RNA viruses, especially virus strains of proven safety, can be used as “genetic backbones” for insertion of critical immunogenic genes from other (heterologous) viruses. Chimeric RNA viruses utilize the replicative machinery of one virus for expression of the protective antigens of the heterologous virus. For example,
chimeric vaccines have been developed in which the genes encoding the envelope proteins of the traditional live-attenuated vaccine strain of yellow fever virus are replaced with corresponding genes of other flaviviruses such as Japanese encephalitis virus, West Nile virus, or dengue virus, or even with genes encoding critical immunogenic proteins of distinct viruses such as influenza. A chimeric vaccine based on yellow fever virus that includes the premembrane (preM) and envelope (E) proteins of West Nile virus was used for protective immunization of horses.

Positive-sense RNA viruses are especially convenient for use as molecular clones for the insertion of foreign genes because the genomic RNA of these viruses is itself infectious. Nevertheless, infectious clones also have been developed for negative-sense RNA viruses by including the replicase proteins at transfection. In poultry, a recombinant Newcastle disease virus vaccine that expresses the H5 gene of influenza virus has been developed and widely used in China for protective immunization of birds against both Newcastle disease and H5 avian influenza. Additional negative-sense RNA viruses such as rhabdoviruses are also being evaluated as potential gene vectors, along with positive-sense RNA viruses such as Nidoviruses (coronaviruses, arteriviruses).

Recombinant replicon particles offer a similar but slightly different strategy that has been developed with certain RNA viruses, including flaviviruses and alphaviruses such as Venezuelan equine encephalitis, Semliki Forest, and Sindbis viruses. Recombinant alphavirus replicon particles are created exclusively from the structural proteins of the donor alphavirus, but the genomic RNA contained in these particles is chimeric, in that the genes encoding the structural proteins of the replicon alphavirus are replaced by those from the heterologous virus. As an example, replicon particles derived from the vaccine strain of Venezuelan equine encephalitis virus that co-express the GPs and M envelope proteins of equine arteritis virus induce virus-neutralizing antibody and protective immunity in immunized horses; neither infectious Venezuelan equine encephalitis virus nor equine arteritis virus is produced in immunized horses, as the replicon genome includes only the non-structural proteins of Venezuelan equine encephalitis virus and the structural protein genes of equine arteritis virus.

For influenza viruses and other viruses with segmented genomes, the principle of chimeric viruses was well established before the advent of recombinant DNA technology. Reassortant viruses were produced by homologous reassortment (segment swapping) by co-cultivation of an existing vaccine strain virus with the new isolate. Viruses with the desirable growth properties of the vaccine virus but with the immunogenic properties of the recent isolate were selected, cloned, and used as vaccine. For example, inactivated chimeric H5N3 influenza virus has been developed as a vaccine for use in poultry.

**Vaccines Utilizing Viral DNA (“DNA Vaccines”)**

The discovery, in the early 1990s, that viral DNA itself can be used for protective immunization offered a potentially revolutionary new approach to vaccination. Specifically, a plasmid construct that included the β-galactosidase gene expressed the enzyme for up to 60 days after it was inoculated into mouse skeletal muscle. From this early observation, there has been an explosion of interest in the development of DNA vaccines and this methodology has been utilized experimentally for a wide range of potential applications. The first commercially available DNA vaccine was developed to protect salmon against infectious hemopoietic necrosis virus, and a DNA-based vaccine to prevent West Nile disease in horses is now available. However, commercial utilization of this strategy in veterinary vaccines has otherwise been slow.

With hindsight, the discovery that DNA itself could confer protective immunity was perhaps not that surprising. In 1960, it was shown that cutaneous inoculation of DNA from Shope papillomavirus induced papillomas at the site of inoculation in rabbit skin. Subsequently, it was shown for many viruses that genomic viral DNA, RNA, or cDNA of viral RNA, could complete the full replicative cycle following transfection into cells. The strategy of DNA vaccines is to construct recombinant plasmids that contain genes encoding key viral antigens. The DNA insert in the plasmid, on injection, transfects cells and the expressed protein elicits an immune response that in turn simulates a response to the respective viral infection. DNA vaccines usually consist of an E. coli plasmid with a strong promoter with broad cell specificity, such as the human cytomegalovirus immediate early promoter. The plasmid is amplified, commonly in E. coli, purified, and then simply injected into the host. Intramuscular immunization is most effective. Significant improvement in response to vaccination has been achieved by coating the plasmid DNA onto microparticles—commonly gold particles 1–3 μm in diameter—and injecting them by “bombardment,” using a helium-gas-driven gun-like apparatus (the “gene gun”).

Theoretical advantages of DNA vaccines include purity, physiochemical stability, simplicity, a relatively low cost of production, distribution, and delivery, potential for inclusion of several antigens in a single plasmid, and expression of antigens in their native form (thereby facilitating processing and presentation to the immune system). Repeated injection may be given without interference, and DNA immunization can induce immunity in the presence of maternal antibodies. However, DNA vaccination is yet to be widely used, because the practical application of the technology is considerably more challenging in humans and animals than it is in laboratory animals. Unsubstantiated concerns have also been raised regarding the fate and potential side-effects of the foreign, genetically engineered DNA and, for animals that will enter the human food chain, the costs of proving safety are likely to be significant.
**Other Potential Vaccine Strategies**

**Vaccines Utilizing Bacteria as Vectors for Expression of Viral Antigens**

Viral proteins (or immunogenic regions thereof) can be expressed on the surface of engineered bacteria that infect the host directly. The general approach is to insert the DNA encoding a protective viral antigen into a region of the genome of a bacterium, or one of its plasmids, which encodes a prominent surface protein. Provided that the added viral protein does not seriously interfere with the transport, stability, or function of the bacterial protein, the bacterium can multiply and present the viral epitope to the immune system of the host. Enteric bacteria that multiply naturally in the gut are the ideal expression vectors for presenting protective epitopes of virulent enteric viruses to the gut-associated lymphoid tissue, and attenuated strains of *E. coli*, *Salmonella* spp., and *Mycobacterium* spp. are being evaluated for immunization against enteric pathogens, including viruses, and/or for the preferential stimulation of mucosal immunity.

**Synthetic Peptide Vaccines**

With the increased ability to locate and define critical epitopes on viral proteins, it is also possible to synthesize peptides chemically that correspond to these antigenic determinants. Appropriately designed synthetic peptides can elicit neutralizing antibodies against many viruses, including foot-and-mouth disease virus and rabies virus, but in general this approach has been disappointing, probably because of the conformational nature of many critical epitopes included in the authentic protein. Specifically, conformational epitopes are not composed of linear arrays of contiguous amino acids, but rather are assembled from amino acids that, while separated in the primary sequence, are brought into close apposition by the folding of the polypeptide chain(s). An effective antigenic stimulus requires that the three-dimensional shape that an epitope has in the native protein molecule or virus particle be maintained in a vaccine. Because short synthetic peptides lack any tertiary or quaternary conformation, most antibodies raised against them are incapable of binding to virions, hence neutralizing antibody titers may be orders of magnitude lower than those induced by inactivated whole-virus vaccines or purified intact proteins. In contrast, the epitopes recognized by T lymphocytes are short linear peptides (bound to MHC protein). Some of these T cell epitopes are conserved between strains of virus and therefore elicit a cross-reactive T cell response.

**Vaccines Utilizing Anti-Idiotypic Antibodies**

The antigen-binding site of the antibody produced by each B cell contains a unique amino acid sequence known as its idiootype or idiotypic determinant. Because anti-idiotypic antibody is capable of binding to the same idioype as binds the combining epitope on the original antigen, the anti-idiotypic antibody mimics the conformation of that epitope. Thus the anti-idiotypic antibody raised against a neutralizing monoclonal antibody to a particular virus can conceivably be used as a vaccine. It remains uncertain whether this points the way to a practical vaccine strategy, but there are situations, probably in human rather than veterinary medicine, in which such vaccines, if efficacious, would have advantages over orthodox vaccines, primarily because of their safety.

**Methods for Enhancing Immunogenicity of Virus Vaccines**

The immunogenicity of inactivated vaccines, especially that of purified protein vaccines and synthetic peptides, usually needs to be enhanced; this may be achieved by mixing the antigen with an adjuvant, incorporation of the antigen in liposomes, or incorporation of the antigen in an immunostimulating complex. Similar approaches are also used to enhance the immunogenicity of recombinant vaccines, and the immunogenicity of these vaccines can be potentially even further enhanced through incorporation of immunopotentiating agents into or along with the expression vector. There is a considerable research effort currently focused on strategies for more efficient and effective antigen delivery for vaccination.

Adjuvants are formulations that, when mixed with vaccines, potentiate the immune response, humoral and/or cellular, so that a lesser quantity of antigen and/or fewer doses will suffice. Adjuvants differ greatly in their chemistry and in their modes of action, but they typically can prolong the process of antigen degradation and release and/or enhance the immunogenicity of the vaccine by recruiting and activating key immune cells (macrophages, lymphocytes, and dendritic cells) at the site of antigen deposition. Alum and mineral oils have been used extensively in veterinary vaccines, but many others have been developed or are currently under investigation, some of which remain proprietary. Among many examples, synthetic biodegradable polymers such as polyphosphazene can serve as potent adjuvants, especially when used with microfabricated needles for intradermal inoculation of antigen. Immunomodulatory approaches to enhance the immunogenicity of vaccines also continue to be investigated—specifically, molecules that can enhance critical innate and adaptive immune responses or inhibit suppressors thereof.

Liposomes consist of artificial lipid membrane spheres into which viral proteins can be incorporated. When purified viral envelope proteins are used, the resulting “virosomes” (or “immunosomes”) somewhat resemble the original envelope of the virion. This not only enables a reconstitution of viral envelope-like structures lacking nucleic acid and other viral components, but also allows the incorporation
of non-pyrogenic lipids with adjuvant activity. When viral envelope glycoproteins or synthetic peptides are mixed with cholesterol plus a glycoside known as Quil A, spherical cage-like structures 40 nm in diameter are formed. Several veterinary vaccines include this “immunostimulating complex adjuvant (ISCOM)” technology.

The recognition of the innate immune system as defined by pattern recognition receptor (PRR) mediated stimulation of transcription of cytokines and regulatory proteins established the link between innate and adaptive immunity. Attempts to enhance the adaptive immune response by utilizing the innate immune system have taken several different approaches. TLR-9 recognizes DNA molecules with methylation patterns not routinely found in eukaryotic cells. Cytosine guanine oligonucleotides (CpG ODNs) have been developed to activate the TLR-9 pathway in conjunction with various antigens and DNA vaccines. Although enhanced immune responses have been noted in mouse models, positive responses may be linked to a given species and to the sequence and size of the CpG ODN. CpG ODNs did not accelerate an immune response to a foot-and-mouth-disease virus vaccine, but positive responses were noted in chickens immunized with a killed influenza virus vaccine and CpG ODNs. Enhanced production of cytokines induced by the innate immune response can be achieved by expressing the cytokines in a viral expression vector along with the antigen of interest. Alternatively, a DNA vaccine expressing a viral antigen can be given along with a DNA molecule coding for a given cytokine. Numerous studies have shown enhanced immune responses when cytokines are used to augment the response naturally induced by an immunization process.

Given the recent development and increasing commercial production of new vaccine types and adjuvants, be they natural or artificial, it anticipated that vaccine formulations and their methods of delivery will change quickly in the coming years.

Factors Affecting Vaccine Efficacy and Safety

In much of the world, vaccines are made under a broad set of guidelines, termed Good Manufacturing Practices. Correctly prepared and tested, all vaccines should be safe in immunocompetent animals. As a minimum standard, licensing authorities insist on rigorous safety tests for residual infectious virus in inactivated virus vaccines. There are other safety problems that are inherent to live-attenuated virus vaccines and, potentially, new generation recombinant virus vaccines.

The objective of vaccination is to protect against disease and, ideally, to prevent infection and virus transmission within the population at risk. If infection with wild-type virus occurs as immunity wanes after vaccination, the infection is likely to be subclinical, but it will boost immunity. For enzootic viruses, this is a frequent occurrence in farm animals, cats and dogs in shelters, and birds in crowded pens.

The efficacy of live-attenuated virus vaccines delivered by either the mouth or nose is critically dependent on subsequent replication of the inoculated virus in the intestinal or respiratory tract, respectively. Interference can occur between the vaccine virus and enteric or respiratory viruses, incidentally infecting the animal at the time of vaccination. In the past, interference occurred also between different attenuated viruses contained in certain vaccine formulations; for example, it has been proposed by some that canine parvovirus infection may be immunosuppressive to such an extent that it interferes with the response of dogs to vaccination against canine distemper.

IgA is the most important class of immunoglobulin relevant to the prevention of infection of mucosal surfaces, such as those of the intestinal, respiratory, genitourinary, and ocular epithelia. One of the inherent advantages of orally administered live-attenuated virus vaccines is that they often induce prolonged synthesis of local IgA antibody, which confers relatively transient immunity to those respiratory and enteric viruses the pathogenic effects of which are manifested mainly at the site of entry. In contrast, IgG mediates long-term, often life-long, immunity to reinfection against most viruses that reach their target organ(s) via systemic (viremic) spread. Thus the principal objective of vaccination is to mimic natural infection—that is, to elicit a high titer of neutralizing antibodies of the appropriate class, IgG and/or IgA, directed against the relevant epitopes on the virion in the hope of preventing infection.

Special difficulties also attend vaccination against viruses known to establish persistent infections, such as herpesviruses and retroviruses: a vaccine must be remarkably effective if it is to prevent, not only the primary disease, but also the establishment of life-long latency. Live-attenuated virus vaccines are generally more effective in eliciting cell-mediated immunity than inactivated ones; however, they also carry some risk of themselves establishing persistent infections in the immunized host.

Adverse Effects from Live-Attenuated Virus Vaccines

Underattenuation

Some live-attenuated virus vaccines may cause clinical signs in some vaccinated animals—in effect, a mild case of the disease. For example, some early canine parvovirus vaccines that had undergone relatively few cell culture passages produced an unacceptably high incidence of disease. However, attempts to attenuate virulence further by additional passages in cultured cells may lead to a decline in the ability of the virus to replicate in the vaccinated animal, with a corresponding loss of immunogenicity.
Such side-effects are typically minimal with current animal virus vaccines, and do not constitute a significant disincentive to vaccination. However, it is important that live-attenuated virus vaccines are used only in the species for which they were produced; for example, canine distemper vaccines cause fatalities in some members of the family Mustelidae, such as the black footed ferret, so that recombinant or inactivated whole-virus vaccines must be used.

**Genetic Instability**

Some vaccine virus strains may revert toward virulence during replication in the recipient or in contact animals to which the vaccine virus has spread. Ideally, live-attenuated vaccine viruses are incapable of such spread, but in those that do there may be an accumulation of back mutations that gradually can result in restoration of virulence. The principal example of this phenomenon is the very rare reversion to virulence of Sabin poliovirus type 3 oral vaccine in humans, which eventually led to its replacement by the safer, although not necessarily more efficacious, non-replicating vaccine. Temperature-sensitive mutants of bovine viral diarrhea virus have also proven to be genetically unstable.

**Heat Lability**

Live-attenuated virus vaccines are vulnerable to inactivation by high ambient temperatures, a particular problem in the tropics, where maintenance of the “cold chain” from manufacturer to the point of administration to animals in remote, hot, rural areas can be challenging. To some extent the problem has been alleviated by the addition of stabilizing agents to the vaccines, selection of vaccine strains that are inherently more heat stable, and by packaging them in freeze-dried form for reconstitution immediately before administration. Simple portable refrigerators for use in vehicles and temporary field laboratories are invaluable.

**Presence of Contaminating Viruses**

Because vaccine viruses are grown in animals or in cells derived from them, there is always a possibility that a vaccine will be contaminated with another virus from that animal or from the medium used for culturing its cells. An early example, which led to restrictions on international trade in vaccines and sera that are still in effect, was the introduction into the United States in 1908 of foot-and-mouth disease virus as a contaminant of smallpox vaccine produced in calves. Similarly, the use of embryonated eggs to produce vaccines for use in chickens may pose problems (e.g., the contamination of Marek’s disease vaccine with reticuloendotheliosis virus). Another important source of viral contaminants is fetal bovine serum, used universally in cell cultures; all batches must be screened for contamination with bovine viral diarrhea virus in particular. Likewise, porcine parvovirus is a common contaminant of crude preparations of trypsin prepared from pig pancreases, which is used commonly in the preparation of animal cell cultures. The risk of contaminating viruses is greatest with live-attenuated virus vaccines, but may also occur with inactivated whole-virus vaccines, as some viruses are more resistant to inactivation than others; the prion agents are notoriously resistant to traditional methods of sterilization, for example.

**Adverse Effects in Pregnant Animals**

Attenuated virus vaccines are not generally recommended for use in pregnant animals, because they may be abortigenic or teratogenic. For example, live-attenuated infectious bovine rhinotracheitis vaccines can be abortigenic, and the live-attenuated feline panleukopenia, classical swine fever, bovine viral diarrhea, Rift Valley fever, and bluetongue vaccines are all teratogenic if they cross the placenta to infect the fetus at critical stages of gestation. These adverse effects are usually the result of primary immunization of a non-immune pregnant animal at a susceptible stage of gestation, so that it may be preferable to immunize pregnant animals with inactivated vaccines, or to immunize the dam with a live-attenuated vaccine before mating. Contaminating viruses in vaccines sometimes go unnoticed until used in pregnant animals; for example, the discovery that bluetongue virus contamination of canine vaccines caused abortion and death in pregnant bitches was most unexpected.

**Adverse Effects from Non-Replicating Vaccines**

Some inactivated whole-virus vaccines have been found to potentiate disease. The earliest observations were made with inactivated vaccines for measles and human respiratory syncytial virus, in which immunized individuals developed more severe disease than did those that remained unvaccinated before infection. Similar events have occurred in veterinary medicine, including the enhanced occurrence of feline infectious peritonitis in cats immunized with a recombinant vaccinia virus that expressed the feline coronavirus E2 protein before challenge infection. Despite the production of neutralizing antibodies after immunization, the kittens were not protected and died quickly of feline infectious peritonitis after challenge. There are numerous instances of disease induced by incomplete inactivation of non-replicating vaccines, and others wherein contaminating viruses survived the inactivation process.

**Vaccination Frequency and Inoculation Site Reactions**

Beyond the schedule of primary vaccination, there is little agreement and much current debate as to how often animals
need to be revaccinated. For most vaccines, there is comparatively little definitive information available on the duration of immunity. For example, it is well recognized that immunity after vaccination with live-attenuated canine distemper vaccine is of long duration, perhaps lifelong. However, the duration of immunity to other viruses or components in a combined vaccine may not be of such long duration. In companion-animal practice, the cost of vaccination, relative to other costs, is small when clients visit their veterinarian, so it has been argued that, if revaccination does no harm, it may be considered a justified component of the routine annual “check-up” in which a wide spectrum of healthcare needs may be addressed. In many countries, annual revaccination has become a cornerstone of broad-based companion-animal preventive healthcare programs, although the rationale for this approach is conjectural at best.

This concept of annual vaccination was further disturbed in the mid-1990s by reports of highly aggressive subcutaneous fibrosarcomas in cats at sites of vaccination (often behind the shoulder). All the factors responsible for these vaccine-associated cancers remain to be thoroughly proven; however, a contaminating virus within the vaccines themselves is not responsible, and the prevailing suspicion is that irritation induced by the vaccine constituents is responsible. Regardless, this phenomenon rekindled the debate of frequency of revaccination in companion animals, leading to new recommendations on the preferred vaccination site, vaccination interval (extended from 1 to 3 years for some vaccines), and systems for reporting adverse responses.

Vaccination Policy and Schedules

The available range of vaccines, often in multivalent formulations and with somewhat different recommendations from each manufacturer regarding vaccination schedules, means that the practicing veterinarian needs to educate her/himself constantly about vaccine choice and usage. Multivalent vaccine formulations confer major practical advantages by reducing the number of visits the owner must make to the veterinarian. Also, multivalent vaccines allow more extensive use of vaccines against agents of secondary importance. Unlike the situation in human medicine, however, where there is general agreement on vaccine formulations and schedules for vaccination against all the common viral diseases of childhood, there is no such consensus in veterinary medicine. Furthermore, unlike the situation in human medicine in which there are few vaccine manufacturers, there are many veterinary vaccine manufacturers, each promoting their own products. The reader is referred to the specific resources on vaccination schedules specific for each animal species provided at the end of this section, but some general considerations for vaccination are described here.

Optimal Age for Vaccination

The risk of most viral diseases is greatest in young animals. Most vaccines are therefore given during the first 6 months of life. Maternal antibody, whether transferred transplacentally in primates or, as in domestic animals and birds, in the colostrum or via the yolk sac, inhibits the immune response of the newborn or newly hatched to vaccines. Optimally, vaccination should be delayed until the titer of maternal antibody in the young animal has declined to near zero. However, any delay in vaccine administration may leave the animal defenseless during the resulting “window of susceptibility.” This is potentially life threatening in crowded, highly contaminated environments or where there is intense activity of arthropod vectors. There are a number of approaches to handling this problem in different animal species, but none is fully satisfactory. The problem is complicated further because young animals do not necessarily respond to vaccines in the same way as older animals do. In horses, for example, antibody responses to inactivated influenza vaccines are poor until recipients become yearlings.

Because the titer of passively acquired antibody in the circulation of newborn animals after receiving colostrum is proportional to that in the dam’s blood, and because the rate of its subsequent clearance in different animal species is known, it is possible to estimate, for any given maternal antibody titer, the age at which no measurable antibody remains in the offspring. This can be plotted as a nomograph, from which the optimal age of vaccination against any particular disease can be read. The method is seldom used, but might be considered for exceptionally valuable animals in a “high-risk” environment.

In practice, relatively few vaccine failures are encountered if one simply follows the instructions from the vaccine manufacturers, who have used averaged data on maternal antibody levels and rate of IgG decay in that animal species to estimate an optimal age for vaccination. It is recommended commonly, even in the case of live-attenuated virus vaccines, that a number of doses of vaccine be administered, say at monthly intervals, to cover the window of susceptibility in animals with particularly high maternal antibody titers. This precaution is even more relevant to multivalent vaccine formulations, because of the differences in levels of maternal antibody against each virus.

Dam Vaccination

The aim of vaccination is generally thought of as the protection of the vaccinee. This is usually so, but in the case of certain vaccines [e.g., those for equine herpes (abortion) virus-1, rotavirus infection in cattle, parvovirus infection in swine, infectious bursal disease of chickens] the objective is to protect the vaccinee’s offspring either in utero (e.g., equine abortion) or as a neonate/hatchling. This is achieved...
by vaccination of the dam. For neonates/hatchlings, the level of maternal antibody transferred in the colostrum and milk or in the egg ensures that the offspring have a protective level of antibody during the critical early days. Because many attenuated virus vaccines are abortigenic or teratogenic, inactivated vaccines are recommended for dam vaccination.

**Available and Recommended Vaccines**

The types of vaccines available for each viral disease (or the lack of any satisfactory vaccine) are discussed in each chapter of Part II of this book. There is clearly enormous geographic variation in the requirements for individual vaccines, particularly for highly regulated diseases such as foot-and-mouth disease. There are also different requirements appropriate to various types of livestock husbandry (e.g., for dairy cattle, beef cows, and their calves on range, or cattle in feedlots and in poultry for breeders, commercial egg layers, and broilers). Similarly, vaccination schedules for dogs, cats, horses, pet birds, and other species such as rabbits should reflect science-based criteria in addition to individual risk. Thus the reader is referred to specialty organizations that publish guidelines for the vaccination of, for example: horses [the American Association of Equine Practitioners (http://www.aaep.org/vaccination_guidelines.htm)], cats [the American Association of Feline Practitioners (http://www.catvets.com/professionals/guidelines/publications/?Id=176)], and dogs [the American Animal Hospital Association (http://secure.aahanet.org/eweb/dynamicpage.aspx?site=resource&webede=CanineVaccineGuidelines)]. Relatively few vaccines are widely available for use in pet birds, but those that are include vaccines for polyoma virus, Pacheco’s disease virus, canarypox and, in enzootic areas, West Nile virus.

For some species, including production animals, protection against viral infections and diseases is by exclusion. Laboratory rodents, for example, are maintained in various types of microbial barrier environments. Rarely, laboratory mice at high risk for ectromelia virus infection during outbreaks in highly valuable mouse populations may be individually vaccinated with the IHD-T strain of vaccinia virus.

Commercially raised rabbits, as well as pet rabbits, are often vaccinated against myxoma virus and rabbit hemorrhagic disease virus, where these agents are highly prevalent, such as in Europe. These rabbit diseases also illustrate the political context of veterinary vaccination: vaccines may not be available in some countries, such as the United States, because vaccination may obscure surveillance for natural outbreaks of disease.

**Vaccination of Poultry and Fish**

In the United States alone, the annual production of poultry birds exceeds $22 billion. All commercially produced birds are vaccinated against several different viral diseases, although there is variation in the types of vaccines used in different countries. The strategy for vaccination of poultry against viral diseases is no different than that for mammals, but the cost of each vaccine dose is tiny; much of this economy of scale is linked to low-cost delivery systems (aerosol and drinking water). Further economies have been achieved by the introduction of in-ovo immunization of 18-day-old embryonated eggs; an instrument (called an Inovoject), capable of immunizing 40,000 eggs per hour, is used. The most frequently used vaccines are against Marek’s disease; formerly inoculated individually into 1-day-old chicks, these are now delivered in this way. By 2009, more than 95% of meat chickens (broilers) in the United States were vaccinated by this method.

Vaccination is used to prevent infectious hemopoietic necrosis and infectious pancreatic necrosis in fish. Vaccines to these diseases include DNA and subunit protein vaccines that are administered either by injection or orally. The objective of vaccination in fish is the same as in mammals; indeed, the phylogenetic origins of the vertebrate immune system can be traced to the first jawed vertebrates, including bony fish (teleosts). Antiviral immunity, although less understood in fish as compared with mammals or birds, involves both innate and acquired response mechanisms. Specifically, cellular and humoral innate responses involve equivalent cell types, signaling molecules, and soluble factors as are found in mammals. These include phagocytes equipped with pattern recognition receptors (PRRs) such as TLRs that lead to pro-inflammatory responses and interferon induction; induction of type 1-like interferons is essential for antiviral innate immune responses in fish, and their production is stimulated by dsRNA and signaling pathway in a manner analogous to that in mammals. Increasing evidence demonstrates that the innate immune response induces an antiviral state in addition to priming adaptive immunity. Similarly, adaptive responses involving T and B lymphocytes and specific immunoglobulin production are critical for antiviral immunity in fish.

The structure of the T cell receptor complex (αβγδ) has remained virtually constant throughout the evolution of jawed vertebrates, including teleosts, whereas the organization and usage of the B cell receptors in fish varies from that of other vertebrates, as fish possess two distinct B-cell lineages (sIgM+ or sIgY+ζ+)—both of which are important for antiviral immunity and affinity maturation of immunoglobulins—and a less pronounced memory response is typical of the adaptive response in fish as compared with mammals or birds. As fish are poikilotherms, the magnitude of the immune response in most fish is profoundly influenced by water temperature, which may play a causal role in seasonal viral disease patterns in both captive and wild fish populations.
OTHER STRATEGIES FOR ANTIVIRAL PROPHYLAXIS AND TREATMENT

Passive Immunization

It is possible to confer short-term protection against specific viral disease by the subcutaneous administration of an appropriate antibody, such as immune serum, immunoglobulin, or a monoclonal antibody. Homologous immunoglobulin is preferred, because heterologous protein may provoke a hypersensitivity response, as well as being more rapidly cleared by the recipient. Pooled normal immunoglobulin contains sufficiently high concentrations of antibody against all the common viruses that cause systemic disease in the respective species. Higher titers occur in convalescent serum from donor animals that have recovered from infection or have been hyperimmunized by repeated vaccinations; such hyperimmune globulin is the preferred product if available commercially.

A more common practice is to vaccinate (preferably using an inactivated virus vaccine) the pregnant dam or female bird approximately 3 weeks before anticipated parturition or egg laying. This provides the offspring with passive (maternal) immunity via antibodies present in the egg (in birds) or in colostrum and milk (in many wild and domestic mammals). This is particularly important for diseases in which the major impact occurs during the first few weeks of life, when active immunization of the newborn cannot be accomplished early enough. Furthermore, this strategy avoids the use of live-attenuated vaccines that may themselves be pathogenic to neonates.

Chemotherapy of Viral Diseases

If this had been a book about bacterial diseases of domestic animals, there would have been a large section on antimicrobial chemotherapy. However, the antibiotics that have been so effective against bacterial diseases have few counterparts in our armamentarium against viral diseases. The reason is that viruses are intimately dependent on the metabolic pathways of their host cell for their replication, hence most agents that interfere with virus replication are toxic to the cell. In recent years, however, and spurred in large part by investigation of devastating human viral diseases such as acquired immunodeficiency syndrome, influenza, and B-hepatitis, increased knowledge of the biochemistry of virus replication has led to a more rational approach in the search for antiviral chemotherapeutic agents, and a number of such compounds have now become a standard part of the armamentarium against particular human viruses. Antiviral chemotherapeutic agents are not in common use in veterinary practice, partly because of their very high cost, but some of the antiviral drugs used in human medicine have already also been utilized in veterinary medicine. Accordingly, it is appropriate to outline briefly some potential developments in this field.

Several steps in the virus replication cycle represent potential targets for selective antiviral drug attack. Theoretically, all virus-encoded enzymes are vulnerable, as are all processes (enzymatic or non-enzymatic) that are more essential to the replication of the virus than to the survival of the cell. Table 4.1 sets out the most vulnerable steps and provides examples of antiviral drugs that display activity, indicating some that have already been licensed for use in humans.

A logical approach to the development of new antiviral drugs is to isolate or synthesize substances that might be predicted to serve as inhibitors of a known virus-encoded enzyme such as a transcriptase, replicase, or protease. Analogs of this prototype drug are then synthesized with a view to enhancing activity and/or selectivity. A further refinement of this approach is well illustrated by the nucleoside analog, acycloguanosine (aciclovir)—an inhibitor of herpesvirus DNA polymerase. Aciclovir is in fact an inactive prodrug that requires another herpesvirus-coded enzyme, thymidine kinase, to phosphorylate it to its active form. Because this viral enzyme occurs only in infected cells, aciclovir is non-toxic for uninfected cells, but very effective in herpesvirus-infected cells. Aciclovir and related analogs (e.g., valacyclovir, ganciclovir) are now available for treatment of herpesvirus infections in humans, and they have also been used on a limited scale in veterinary medicine, such as for treatment of feline herpesvirus 1 induced corneal ulcers and equine herpesvirus-1 induced

| TABLE 4.1 Possible Targets for Antiviral Chemotherapy in Veterinary Medicine |
|-----------------------------|-----------------------------|
| Target                      | Prototype Drug |
| Attachment of virion to cell receptor | Receptor analogs |
| Uncoating                   | Rimantadine<sup>a</sup> |
| Primary transcription from viral genome | Transcriptase inhibitors |
| Reverse transcription       | Zidovudine—AZT<sup>a</sup> |
| Regulation of transcription | Lentivirus tat inhibitors |
| Processing of RNA transcripts | Ribavirin<sup>a</sup> |
| Translation of viral RNA into protein | Interferons<sup>a</sup> |
| Post-translational cleavage of proteins | Protease inhibitors |
| Replication of viral DNA genome | Acycloguanosine (Aciclovir<sup>a</sup>) |
| Replication of viral RNA genome | Replicase inhibitors |

<sup>a</sup>Licensed for human use.
encephalomyelitis. They have also been used in humans exposed to the zoonotic herpes virus of macaques, herpes simiae (B virus) that may have catastrophic consequences in infected humans.

Drugs also have been developed to treat influenza virus infections in people and, potentially, animals. For example, oseltamivir phosphate (Tamiflu) is a prodrug that, after its metabolism in the liver, releases an active metabolite that inhibits neuraminidase, the virus-encoded enzyme that releases budding virions from the surface of infected cells and cleaves the virus receptor so that released virions do not bind to already infected cells. Inhibition of neuraminidase, therefore, slows virus spread, giving the immune system the opportunity to “catch up” and mediate virus clearance.

Ribavirin is also a prodrug that is metabolized to purine RNA metabolites that interfere with the RNA metabolism that is required for virus replication. This drug has been used in the treatment of human respiratory syncytial virus and hepatitis C virus infections.

X-ray crystallography has opened a major new approach in the search for antiviral drugs. Now that the three-dimensional structure of many viruses is known, it has been possible to characterize receptor-binding sites on capsid proteins at the atomic level of resolution. Complexes of viral proteins with bound cellular receptors can be crystallized and examined directly. For example, for some rhinoviruses, receptor-binding sites on virions are in “canyons”—that is, clefts in the capsid surface. Drugs have been found that fit into these clefts, thereby preventing virus attachment to the host cell. Further information is provided by mapping the position of the particular amino acid residues that form these clefts, thereby allowing the design of drugs that better fit and better interfere with the viral infection process. This approach also lends itself to the development of drugs that block virus penetration of the host cell or uncoating of virus once inside the cell. If any of these strategies are successful in human medicine, adaptation to veterinary usage may follow.

**VIRUSES AS VECTORS FOR GENE THERAPY**

In addition to their central role as pathogens, viruses also have contributed much to the current understanding of both cellular and molecular biology. Individual viruses, or components thereof, have been exploited as molecular tools, and viruses also offer a novel and useful system for the expression of heterologous genes. Specifically, with the advent of cloning and genetic manipulation, foreign genes can readily be inserted into the genome of many viruses so that they can be used as expression vectors. These viral gene vectors include those that deliver the gene of interest without replicating in the host (“suicide” vectors) and those that do replicate in the host, with or without integration into the genome.

The use of both DNA and RNA viruses as recombinant vaccine vectors was described earlier in this chapter, but this same strategy also can potentially be exploited for therapeutic use. Viral-vector gene therapy strategies offer a novel and especially attractive approach to the correction of specific genetic disorders, particularly those with a defined missing or dysfunctional gene. Correction of such disorders requires the long-term expression of the specific protein that is absent or dysfunctional; thus viruses with the capability of safely and stably inserting the target gene into the genome of the affected individual are a logical choice as vectors for this purpose. To this end, a variety of viruses have been evaluated as potential gene vectors, including retroviruses because of their inherent ability to integrate into the host genome, poxviruses, adenoviruses, adeno-associated viruses (which are paroviruses), herpesviruses, and various positive- and negative-sense RNA viruses.

Adeno-associated viruses have received much recent attention as potential vectors for gene therapy. They are small DNA viruses (family Paroviridae, genus Dependovirus) that can infect both dividing and non-dividing cells, and they can insert their genome into that of the host cell. Furthermore, integration of the viral genome of adeno-associated viruses occurs at specific sites within the host genome, as opposed to that of retroviruses, insertion of which is typically random and potentially mutagenic. Adeno-associated viruses are considered to be avirulent (non-pathogenic), and the capacity for integration is readily abolished by genetic manipulation. Recombinant adeno-associated viruses that express appropriate proteins have been evaluated for the correction of a variety of human genetic disorders, including hemophilia and muscular dystrophy.

The strategy of targeted gene delivery is also potentially applicable for therapeutic intervention by the delivery of molecules with the capacity to modulate disease processes, especially chronic diseases with an immune-mediated pathogenesis that might be susceptible to regional expression of immunomodulatory molecules.

Another potential application of targeted gene delivery using recombinant viruses is to control the reproduction of wildlife and feral species, including those species considered to be pests, by targeted delivery of immunogenic proteins critical for reproductive activity.