STATE BIOTECHNOLOGICAL UNIVERSITY

FACULTY OF VETERINARY MEDICINE

DEPARTMENT OF EPIZOOTOLOGY AND MICRJBIOLOGY

VETERINARY VIROLOGY

PART 1. General Virology.

Methodological recommendations for students of 2-3 courses of the Faculty of Veterinary Medicine

For speciality 211 – "Veterinary Medicine" Faculty "Foreign students"

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The head of department Severin R.V.

Veterinary virology – 2022
Lecture 1. Introduction into virology.

A BRIEF HISTORY OF ANIMAL VIROLOGY

The origins of veterinary medicine are rooted in efforts to maintain the health of animals for food and fiber production, and animals essential for work-related activities. Control of animal disease outbreaks was not possible until the pioneering work of the late 19th century that linked microbes to specific diseases of plants and animals.

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Properties of Microorganisms and Toxins (comparison of the properties of bacteria and toxins)

Ivanovsky was sure it was a bacterium or toxin.

Properties	Bacteria	Toxins	Viruses
Microscopy	+	—	—
Growth on nonliving medium	+	—	—
Filterable	—	+	+
Juice infection	+	+	+
Juice infection (after dilution)	+	—	+

CHARACTERISTICS OF VIRUSES

Following the initial operational definition of a virus as a filterable agent, attempts were made to identify properties of viruses that made them distinct from other microorganisms. Even from the earliest times it was evident that the filterable agents could not be cultivated on artificial

media. However, all obligate intracellular parasites are not viruses (Table 1). Members of certain bacterial genera also are unable to replicate outside a host cell (eg, Ehrlichia, Anaplasma, Legionella, and Rickettsia). These “degenerate” bacteria lack key metabolic pathways, the products of which must be provided by the host cell.

Viruses lack all metabolic capabilities necessary to reproduce, including energy production and the processes necessary for protein synthesis. Viruses do not possess standard cellular organelles, such as mitochondria, chloroplasts, Golgi, and endoplasmic reticulum with associated ribosomes.

Outside the living cell, viruses are inert particles whereas, inside the cell, the virus utilizes the host cell processes to produce its proteins and nucleic acid to replicate itself. The outcome of an infection is the same—the production of more progeny viruses.

A second inviolate property of viruses is that they do not reproduce by binary fission. For viruses, the process of reproduction resembles an assembly line in which various parts of the virus come together from different parts to form new virus particles. Shortly after the virus attaches to a host cell, it enters the cell and the intact virus particle ceases to

exist. The viral genome then directs the production of new viral macromolecules, which results ultimately in the assembly and appearance of new progeny virus particles.
The period of time between the penetration of the virus particle into the host cell and the production of the first new virus particle is designated as the eclipse period. Uninterrupted, a single infectious particle can replicate within a single susceptible cell to produce thousands of progeny virus particles. In general, viruses contain only one type of nucleic acid that carries the information for replicating the virus.
Early studies defined viruses by their tiny size; however, "giant" viruses now have been identified that are physically larger than some mycoplasma, rickettsia, and chlamydia. The mimiviruses and pandoraviruses that infect amoeba are remarkable exceptions to existing rules: the mimivirus virion is approximately 0.75 μm (750 nm) in diameter. The pandoraviruses are even larger (up to 1 μm).
The discovery of these large viruses has revived the debate as to the origin of viruses. Furthermore, sequence data link mimiviruses to the nucleocytoplasmic large DNA viruses, specifically viruses in the families Poxviridae and Iridoviridae.

Chemical Composition of the Virion

The chemical composition of virus particles varies markedly between those of individual virus families. For the simplest of viruses such as parvoviruses (family Parvoviridae), the virion is composed of viral structural proteins and DNA, whereas in the case of picornaviruses (family Picornaviridae) it comprises viral proteins and RNA. The situation becomes more complex with the enveloped viruses such as members of the Herpesviridae and Paramyxoviridae families. For enveloped viruses, glycoproteins are the major type of protein present on the exterior of the membrane. The existence/presence of a lipid envelope provides an operational method with which to separate viruses into two distinct classes—those that are inactivated by organic solvents (enveloped) and those that are resistant (nonenveloped).

Viral Nucleic Acids in the Virion

Viruses exhibit remarkable variety with respect to genome composition and in strategies for the expression of their genes and for the replication of their genome. Viruses have perhaps exploited all possible means of nucleic acid replication for an entity at the subcellular level. The type and structural characteristics of the viral genomic nucleic acids are used to classify viruses. As viruses contain only one nucleic acid type with respect to transmitting genetic information, the virus world can simply be divided into RNA viruses and DNA viruses.
For RNA viruses, one major distinction is whether the virion RNA is of positive or of negative sense or polarity. Within the negative-strand group, there are single-strand whole-genome viruses (eg, Paramyxoviridae) and segmented genome viruses (eg, Orthomyxoviridae—six, seven, or eight segments; Bunyaviridae—three segments; Arenaviridae—two segments). The Retroviridae are considered diploid, in that the virion contains two whole-genomic positive-sense RNAs. Some RNA viruses possess genomes comprised of double-stranded RNA. The Birnaviridae have two segments and the Reoviridae have 10, 11, or 12 segments, depending on the genus of virus.
For the animal DNA viruses, the overall structure of the genomes is less complex, with either a single molecule of single-stranded (ss)DNA or a single molecule of double-stranded (ds)DNA.

Viral Proteins in the Virion

The genomes of animal viruses encode from as few as one protein to more than 100. Proteins that are present in virions (mature virus particles) are referred to as structural proteins, whereas proteins that are produced during the infection but are not incorporated into newly assembled virus particles are referred to as nonstructural proteins. Nonstructural proteins play essential roles in the virus replication process, such as regulating gene expression, replication of the genome, proteolytic processing of viral precursor proteins, facilitating the assembly of virus particles, or modification of the host innate response to infection. There is some ambiguity for enzymes that are essential for the initial stages of virus replication, such as the RNA polymerases for the negative-strand RNA viruses (Paramyxoviridae, Rhabdoviridae, etc.). As the first step in the replication cycle, once the nucleocapsid enters the cytoplasm the viral genome is transcribed, requiring that the polymerase is part of the mature virion. Whether the polymerase has a true structural role in the mature particle in addition to its transcription activity is less certain. Numerous other viral proteins that occur within the virions of complex viruses (Poxviridae, Herpesviridae, Asfarviridae) also appear to have no apparent structural role.

Virion proteins fall into two general classes: modified proteins and unmodified proteins. The capsids of the nonenveloped viruses are composed of proteins with few modifications, as their direct amino acid interactions are essential for the assembly of the protein shells. Proteolytic cleavage of precursor proteins in the nascent capsid is not uncommon in the final steps of assembly of the mature capsid proteins. Glycoproteins are predominantly found in those viruses that contain a viral membrane. These structural proteins can be either a type I integral membrane protein (amino terminus exterior) (eg, hemagglutinin (HA) of influenza virus) or type II (carboxyl terminus exterior) (eg, neuraminidase of influenza virus). Glycosylation patterns may differ even amongst viruses that mature in the same types of cells, because N- and O-linked glycosylation sites on the virion proteins vary among the virus families. The glycoproteins involved in virion assembly have a cytoplasmic tail that communicates with viral proteins on the inner surface of the membrane to initiate the maturation process for production of the infectious virus particle. Structural proteins in the infectious virus particle have a number of key functions: (1) to protect the genomic nucleic acid and associated enzymes from inactivation; (2) to provide receptor-binding sites for initiation of infection; and (3) to initiate or facilitate the penetration of the viral genome into the correct compartment of the cell for replication.

The virion—that is, the complete virus particle—of a simple virus consists of a single molecule of nucleic acid (DNA or RNA) surrounded by a morphologically distinct capsid composed of viral protein subunits (virus-encoded polypeptides). The protein subunits can self-assemble into multimer units (structural units), which may contain one or several polypeptide chains. Structures without the nucleic acid can be detected and are referred to as empty capsids. The meaning of the term nucleocapsid can be somewhat ambiguous. In a strict sense, a capsid with its nucleic acid is a nucleocapsid, but for simple viruses such as poliovirus, this structure is also the virion. For flaviviruses, the nucleocapsid (capsid 1 RNA) is enclosed in a lipid envelope and the nucleocapsid does not represent the complete virion. For paramyxoviruses, the nucleocapsid refers to a structure composed of a single

strand of RNA complexed to a viral protein that assembles in the form of an α helix. The nucleocapsid assembles into a complete virion by obtaining a lipid envelope from host cell membranes modified by the insertion of viral proteins.

Viral Membrane Lipids

For viruses that mature by budding through a cellular membrane, a major constituent of the virion is a phospholipid bilayer that forms the structural basis of the viral envelope. The maturation site for viruses can be the plasma membrane, nuclear membrane, Golgi, or the endoplasmic reticulum. For those viruses budding from the plasma membrane, cholesterol is a constituent of the viral membrane, whereas the envelopes of those viruses that bud from internal membranes lack cholesterol. The
budding process is not random, in that specific viral glycoprotein sequences direct developing particles to the proper location within the inner membrane surface. In polarized cells—cells with tight junctions, giving the cell a defined apical and basal surface—virus budding will be targeted to one surface over the other. For example, in MadinDarby canine kidney cells, influenza virus will bud on the apical surface, whereas vesicular stomatitis virus buds from the basal surface (see Fig. 2.13). The
transmembrane domain of viral glycoproteins targets specific regions of the cellular membrane for budding. For influenza virus, budding is associated with "lipid rafts," which are microdomains of the plasma membrane rich in sphingolipids and cholesterol.

VIRAL MORPHOLOGY

Early attempts to characterize viruses were hampered by the lack of appropriate technologies. A major advance in determining virus morphology was the development of negative-stain electron microscopy in 1958. In this procedure, electron-dense stains were used to coat virus particles and produce a negative image of the virus with enhanced resolution. Advances in determining virus morphology at the atomic level came from studies initially using X-ray crystallography and then combining this technique with other structural techniques such as electron cryomicroscopy (cryo-EM). In this process, samples are snap frozen and examined at temperatures of liquid nitrogen or liquid helium. Cryo-EM offered the advantage that the samples are not damaged or distorted in the process of analyzing the structure, as occurs with negative stain electron microscopy and X-ray crystallography.

For many viruses, this uniformity is met by having the symmetry of a type of polyhedron known as an icosahedron. X-ray crystallography can also be used to analyze subunits of a virus, such as was done for the HA protein of influenza virus.

Viruses come in a variety of shapes and sizes that depend on the shape, size, and number of their protein subunits and the nature of the interfaces between these subunits (Fig. 1.1). However, only two kinds of symmetry have been recognized in virus particles: icosahedral and helical. The symmetry found in isometric viruses is invariably that of an icosahedron; virions with icosahedron symmetry have 12 vertices (corners), 30 edges, and 20 faces, with each face an equilateral triangle (Parvoviruses represent one of the simplest capsid designs, being composed of 60 copies of the same protein subunit—three subunits per face of the icosahedron). In the simplest arrangement, the size of the protein subunit determines the volume of the capsid. With a single capsid protein of 60 copies, only a small genome can be accommodated within the capsid (canine parvovirus).

The nucleocapsid of several RNA viruses selfassembles as a cylindrical structure in which the protein
structural units are arranged as a helix, hence the term helical symmetry. In helically symmetrical nucleocapsids, the genomic RNA forms a spiral within the core of the nucleocapsid. Many of the plant viruses with helical nucleocapsids are rod-shaped, flexible, or rigid without an envelope. However, with animal viruses, the helical nucleocapsid is wound into a secondary coil and enclosed within a lipoprotein envelope (Rhabdoviridae).
There are viruses that do not conform to the simple rules of morphology. For example, members of the Poxviridae have "complex" symmetry. Similarly, there are highly pleomorphic viruses in which each virion has its own unique shape (eg, members of the Filoviridae).

VIRAL TAXONOMY

With the earliest recognition that infectious agents were associated with a given spectrum of clinical outcomes, it was natural for an agent to take on the name of the disease with which it was associated or the geographic location where it was found, as there was no other basis for assigning a name. Thus the agent that caused foot-andmouth disease in cattle becomes "foot-and-mouth disease virus," or an agent that caused a febrile disease in the Rift Valley of Africa became "Rift Valley fever virus." It is
not difficult at this time in history to see why this ad hoc method of naming infectious agents could lead to confusion and regulatory chaos, as different names may be given to the same virus. For example, hog cholera virus existed in North America whereas, in the rest of the world it was referred to as classical swine fever virus, not to be confused with African swine fever virus. Within the same
animal, one had infectious bovine rhinotracheitis (IBR) virus and infectious bovine pustular vulvovaginitis (IBPV) virus—both disease entities being caused by bovine herpesvirus 1. Even today, export certification documents may ask for tests to certify animals free of IBR virus and IBPV virus. This disease-linked nomenclature could not be changed until such time as the tools became available to define the physical and chemical nature of viruses. With negative-stain electron microscopy
as a readily available technology, the size and shape of viruses became a characteristic for defining them. This, along with the ability to define the type of nucleic acid in the virus particle, provided the beginnings of a more rational system of classifying and naming new viruses.
Even with a defined shape and a type of nucleic acid, there were still ambiguities in the classification systems that were being developed. Viruses that were transmitted by insect vectors were loosely defined as "arboviruses"— arthropod-borne viruses. However, there were viruses that "looked like" arboviruses (togaviruses—viruses with a symmetrical lipid membrane) and had the same nucleic
acid, but did not have an insect vector. These became "nonarthropod-borne" togaviruses. These ambiguities were increasingly resolved with access to the nucleotide sequences of these agents. Thus, for example, the "nonarbo" togaviruses became members of the genera Rubivirus,
Pestivirus, and family Arteriviridae. Whereas viruses initially were classified according to the diseases they caused, shared physical and chemical properties, and serologic cross-reactivity, the advent of
nucleic acid sequencing technologies developed in the molecular era allowed genetic

comparisons of different viruses to facilitate taxonomic classifications. In general, genetic relationships parallel those previously established by the older criteria. Virus sequencing also allows for phylogenetic comparisons to determine the evolutionary development and history of viral species. This is a powerful tool for defining viral ancestries. However, a major limitation to sequence-based classification is that inferences are compromised by the variable nature of viruses, especially for highly divergent RNA viruses. Despite this, phylogenies that analyze the most conserved motifs of viral RNA dependent RNA polymerase sequences have been used to generate higher-order classifications that define viral "supergroups" and establish family-level distinctions. For example, phylogenetic analyses of viruses including retroviruses and hepadnaviruses with reverse transcriptase activity have been more informative than polymerase sequences due to a higher degree of sequence conservation of reverse transcriptase genes. New methods presently being developed to circumvent inferences from
sequences will include comparing genome organization (eg, gene content and order) as well as protein secondary structure.

The International Committee on Taxonomy of Viruses (ICTV) was established in 1966 to establish, refine, and maintain a universal system of virus taxonomy. Given the uncertain origins of viruses, establishing the initial framework for this classification system was not without controversy. Subcommittees and study groups meet periodically to assess new data submitted from the research community to refine the classification system and to place new viruses in their most logical position in the taxonomy scheme. It was not until the Seventh Report of the ICTV (2000) that the concept of virus species as the lowest group in the viral taxa was accepted. The advent of nucleotide sequence
determination had a dramatic effect on all biological classification systems, and it has in many respects confirmed the major elements of the classification system. As the process of classification and defining nomenclature is an ongoing one because of the discovery of new viruses and
the generation of sequence data on historic virus isolates, it is impossible for a textbook to ever be truly "current." This textbook will use the information presented in the Ninth Report of the ICTV published in 2011, as updated by the ICTV online resource (http://www.ictvonline.org/index.asp).

The hierarchy of recognized viral taxa is: Order; Family; (Subfamily); Genus; Species. For example, human respiratory syncytial virus A2 would be found in this system as: Mononegavirales (order); Paramyxoviridae (family); Pneumovirinae (subfamily); Pneumovirus (genus); Human respiratory syncytial virus (species). The 2011 ICTV report lists 2284 species of virus and viroid distributed amongst 349 genera, 19 subfamilies, 87 families, and 6 orders. To be a member of the taxa higher than species, a virus must have all properties defining the classification. In contrast, species are considered a polythetic class, in which members have several properties in common but all
do not have to share a single defining property. For each genus, there has been designated a type species, which is a species that creates a link between the genus and the species. This designation is usually conferred on the species that necessitated the creation of the genus. The published
virology literature contains obvious inconsistencies with regard to whether the name of a specific virus is capitalized and/or written in italics: Bovine viral diarrhea virus versus bovine viral diarrhea virus, for example. In all cases dealing with taxonomy, the order, family, subfamily and genus names should be written in italics and capitalized. In

discussing a virus in the context of taxonomy at the species level, the name is written in italics and the first word is capitalized: for example, Canine distemper virus is a species in the genus Morbillivirus. However, when a virus is written about in terms of tangible properties such as its ability to cause disease, growth in certain cell lines, or its physical characteristics, the name is neither written in italics nor capitalized unless the name contains a proper noun; for example, one can grow canine distemper virus or West Nile virus in monkey cells.

There are instances when the abstract (taxonomy) and the concrete aspects of a virus are not clear in the context of the sentence. In this textbook we will attempt to use the ICTV conventions when clearly appropriate, but as this text deals mainly with the tangible aspects of viruses, most virus names will not be in italics. A basic question that has yet to be addressed is why we should bother with taxonomy at all. For some there seems to be a human need to place things into an ordered system. In characterizing an entity and defining a nomenclature, a basic understanding of the subject under study may be achieved. In a larger context, taxonomy provides a tool for comparing one virus with another or one virus family with another. It also enables one to assign biological properties to a new virus that is provisionally linked to a given family. For instance, if one has an electron micrographic image of a new virus that supports its identity as a coronavirus, then the discoverer can assume they have identified a single-stranded, positive-sense, nonsegmented RNA virus. Further, one can extrapolate that coronaviruses are mainly associated with enteric disease, but can also cause respiratory disease in "atypical" hosts after "species jumping." As a group, coronaviruses are difficult to culture in vitro, and may require the presence of a protease to enhance growth in tissue culture.

Conserved sequences—perhaps in the nucleocapsid—might provide a target for the development of a PCR test. Thus, identification of the morphology of an unknown virus can be useful, as the general properties of specific virus families can assist in the interpretation of individual clinical cases.

For example, confirming that an alphaherpesvirus was isolated from a particular case, or its presence identified by deep sequence analysis of clinical material, confers some basic knowledge about the virus without having explicitly to define the properties of the specific virus species responsible. However, current taxonomy of viruses is not without confusion. There is substantial variation in how viruses are classified currently within individual families; for example, viruses in the family Flaviviridae (genus Flavivirus) are still grouped according to their serological relationships, whereas viruses in the family Picornaviridae are increasingly subdivided into genera based on their genome sequences and organization. Furthermore, the designation of a "virus species" can include a variety of other "virus isolates" such that, despite their very different biological properties and host range (species tropism), feline panleukopenia virus and canine parvovirus 2 are both representatives of Carnivore protoparvovirus 1 (see Chapter 12: Parvoviridae). More detailed properties of the virus families that include significant pathogens of veterinary relevance will be found in specific chapters in Part II of this text.

Phylogenetic Comparison of Virus Sequences

Prior to the advent of molecular biology, viruses were classified according to their serological relationships. Sequencing technologies developed in the molecular era have allowed for genetic comparison of viruses, which generally match relationships that were previously serologically defined. Phylogenies are tree-like pictorial descriptions of the evolutionary history of a particular virus
species or family, where each branch tip represents a specific virus sequence; these "trees" are usually generated based on sequence comparison of the most conserved region of the viral genome (for representative examples, see Figs. 17.1, 18.1, and 21.2). Although inferences based on viral sequence data are compromised by the variable nature of viruses, especially for highly divergent RNA
viruses, phylogenetics has proven useful for generating higher-order classifications to define "supergroups" of viruses. Beyond its importance to virus taxonomy, the advent of molecular virology ushered in a new era for the study of virus evolution via phylogenetic comparisons.
This approach was used, amongst many examples, to determine that HIV originated from SIV that infect nonhuman primates. A phylodynamics approach can also be used to infer the origins, epidemiology, and dynamics of viruses during epidemics. By comparing gene sequences of viruses
and analysis of phylogenetic trees, valuable information can be derived regarding virus population growth and decline, the extent of population subdivision, and viral migration. These approaches prove especially useful for RNA viruses that change rapidly, allowing the resolution of phylogenetic relationships between samples obtained only days apart for example. They are also used to document dispersal of specific viruses, such as influenza viruses amongst birds and humans. In most cases, phylogenies only show evolutionary order and not the length of time between two sequences,
unless special molecular clock models are applied. For viruses that recombine or reassort, valid phylogenies also require analysis of multiple genomic regions.
Phylogenetic analyses can take many forms, although they invariably result in the generation of a phylogenetic tree. Neighbor joining tree-building algorithms calculate the genetic distance, measured via a matrix, between each pair of viral sequences being compared; the resulting topology minimizes the distance between nearest neighbors. This approach is rapid and therefore favored for generating a tentative tree or for choosing the best tree among multiple options, but since sequence data is
reduced to a distance matrix at the outset, if the matrix is incorrect a false tree may be produced. Maximum parsimony is a nonparametric statistical method for producing trees where branches are placed in the simplest way possible to support minimal evolutionary change.
Parsimony algorithms are best used when viruses share high genetic conservation and when the number of sequences being analyzed is low since the method is time-intensive. The approach is not always guaranteed to produce a true tree with high probability, especially when evolution is rapid. A third approach, maximum likelihood, which uses a parametric statistical model to provide
estimates for the parameters in the model and then determines the probability of observing the tree topology given the model, is even slower than parsimony, but is favored for confirming trees built using other algorithms, especially with small data sets since it is less affected by sampling error as compared to the other methods. Trees generated with all three algorithms rely on bootstrap

analyses, typically reported at branch nodes, to provide statistical support for their topologies. Bootstrap values of 95 or higher are statistically robust and denote that if the tree was rebuilt 100 times using the same method, the same relative positions of the sequences at the node would occur 95 out of 100 times.

VIRUS REPLICATION

In the previous chapter, viruses were defined as obligate intracellular parasites that are unable to direct any independent biosynthetic processes outside the host cell. It was further noted that the genetic complexity of viruses varies greatly between individual virus families, ranging from those
viruses that encode only a few proteins to others that encode several hundred proteins. Given this remarkable diversity, it is hardly surprising that the replication processes used by individual viruses would also be highly variable. However, all viruses must progress through the same general steps for
replication to occur. Specifically, all viruses must attach to a susceptible host cell, enter the cell, disassemble the virus particle (uncoating), replicate its own genetic material and express the associated proteins, assemble new virus particles, and escape from the infected cell (release). This chapter will outline the general processes involved in each of these steps.

Taxonomic Criteria

The most important criteria are: Host Organism(s): eukaryote; prokaryote; vertebrate, etc.
Particle Morphology: filamentous; isometric; naked; enveloped
Genome Type: RNA; DNA; ss- or ds-; circular; linear
- although a number of other criteria - such as disease symptoms, antigenicity, protein profile, host range, etc.
- are important in precise identification, consideration of the above three criteria - and in many cases, just morphology - are sufficient in most cases to allow identification of a virus down to familial if not generic level.

Genomic Replication Strategies of Viruses The old terms "eclipse phase" or "latent period" describe that part of a virus life cycle when no infectious virus can be extracted from cells which had just been exposed to infectious virions: a good illustration of the concept in terms of a virus assay experiment is shown here. What happens once a virus is uncoated, or partially uncoated, depends largely upon what sort of virus it is. The Baltimore Classification of viruses by their genome types and replication strategies makes it fairly easy to predict the broad sort of strategy that a virus with a given genome will employ in order to get replicated. This classification was originally devised by David Baltimore; it originally only had six categories, but the discovery of "DNA retroviruses" or PARARETROVIRUSES in the 1980s has necessitated a new Class VII.

Viruses Viruses may be defined as acellular organisms whose genomes consist of nucleic acid, and which obligately replicate inside host cells using host metabolic machinery and ribosomes to form a pool of components which assemble into particles called VIRIONS, which serve to protect the genome and to transfer it to other cells. They

are distinct from other so-called VIRUS-LIKE AGENTS such as VIROIDS and PLASMIDS and PRIONS

A virus is an infectious acellular entity composed of compatible genomic components derived from a pool of genetic elements.

The concept of a virus as an organism challenges the way we define life: viruses do not respire, nor do they display irritability; they do not move and nor do they grow, however, they do most certainly reproduce, and may adapt to new hosts. By older, more zoologically and botanically biased criteria, then, viruses are not living. However, this sort of argument results from a "top down" sort of definition, which has been modified over years to take account of smaller and smaller things (with fewer and fewer legs, or leaves), until it has met the ultimate "molechisms" or "organules" - that is to say, viruses - and has proved inadequate.

If one defines life from the bottom up - that is, from the simplest forms capable of displaying the most essential attributes of a living thing - one very quickly realises that the only real criterion for life is: The ability to replicate and that only systems that contain nucleic acids - in the natural world, at least - are capable of this phenomenon. This sort of reasoning has led to a new definition of organisms: "An organism is the unit element of a continuous lineage with an individual evolutionary history." The key words here are UNIT ELEMENT, and INDIVIDUAL: the thing that you see, now, as an organism is merely the current slice in a continuous lineage; the individual evolutionary history denotes the independence of the organism over time. Thus, mitochondria and chloroplasts and nuclei and chromosomes are not organisms, in that together they constitute a continuous lineage, but separately have no possibility of survival, despite their independence before they entered initially symbiotic, and then dependent associations. The concept of replication is contained within the concepts of individual viruses constituting continuous lineages, and having an evolutionary history. Thus, given this sort of lateral thinking, viruses become quite respectable as organisms: they most definitely replicate, their evolution can (within limits) be traced quite effectively, and they are independent in terms of not being limited to a single organism as host, or even necessarily to a single species, genus or phylum of host.

"Viruses are entities whose genomes are elements of nucleic acid that replicate inside living cells using the cellular synthetic machinery, and cause the synthesis of specialised elements [virions] that can transfer the genome to other cells". SE Luria, JE Darnell, D Baltimore and A Campbell (1978)

"Virus are submicroscopic, obligate intracellular parasites...[and] · Virus particles (virions) are formed from the assembly of pre-formed components; · Virus particles themselves do not "grow" or undergo division; · Viruses lack the genetic information which encodes apparatus necessary for the generation of metabolic energy or for protein synthesis (eg: ribosomes)". AJ Cann (1997). Principles of molecular virology, 2nd Edition. Academic Press, San Diego.

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until it has met the ultimate "molechisms" or "organules" - that is to say, viruses - and has proved inadequate.

Classical Properties of Living Organisms: · Reproduction · Nutrition · Respiration · Irritability · Movement · Growth · Excretion More modern definitions include the storage and replication of genetic information as nucleic acid, and the presence of or potential for, enzyme catalysis.

Other Autonomous or Semi-Autonomously Replicating Genomes. There are a number of types of genomes which have some sort of independence from cellular genomes: these include "retrons" or retrotransposable elements, bacterial and fungal (and eukaryotic organelle) plasmids, satellite nucleic acids and satellite viruses which depend on helper viruses for replication, and viroids. A new class of agents - PRIONS - appear to be "proteinaceous infectious agents" (see also here for an ICTV description, here for some local information and more links).

Plasmids Plasmids may share a number of properties with viral genomes - including modes of replication, as in ss circular DNA plasmids and viruses - but are not pathogenic to their host organisms, and are transferred by conjugation between cells rather than by free extracellular particles. Satellite Nucleic Acids Certain viruses have associated with them nucleic acids that are dispensable in that they are not part of the genome, which have no (or very little) sequence similarity with the viral genome, yet depend on the virus for replication, and are encapsidated by the virus. These are mainly associated with plant viruses and are generally ssRNA, both linear and circular - however, a circular ssDNA satellite of a plant geminivirus has recently been found. Satellite Viruses There are also viruses which depend for their replication on HELPER VIRUSES: a good example is tobacco necrosis satellite virus (sTNV), which has a small piece of ssRNA which codes only for a capsid protein, and depends for its replication on the presence of TNV. Another good example is the hepatitis delta agent with its circular ssRNA genome. The adeno-associated viruses (AAVs) are also satellite viruses dependent on the linear dsDNA adenoviruses for replication, but which have linear ssDNA genomes and appear to be degenerate or defective parvoviruses.

Viroids Viroids are small naked circular ssRNA genomes which appear rodlike under the EM, which are capable of causing diseases in plants. They code for nothing but their own structure, and are presumed to replicate by somehow interacting with host RNA polymerase, and to cause pathogenic effects by interfering with host DNA/RNA metabolism and/or transcription. A structurally similar disease agent in humans is the hepatitis B virus-dependent hepatitis delta agent, which additionally codes for a structural protein.

Retroid Elements and Retroviruses Retroviridae [ssRNA(+) viruses replicating via a longer-than-genome-length dsDNA intermediate], Hepadnaviridae, caulimoviruses and badnaviruses [family Caulimoviridae, gapped circular dsDNA viruses replicating via longer-than-genome-length RNA intermediates] all share the unlikely attribute of the use of an enzyme complex consisting of a RNA-dependent DNA polymerase/RNAse H in order to replicate. They share this attribute with several retrotransposons, which are eukaryotic transposable cellular elements with striking similarities with retroviruses [such as the yeast Ty element, the mammalian LINE-1 elements, and the Drosophila copia element]; and with retroposons, which are eukaryotic elements which transpose via RNA

intermediates, but share no obvious genomic similarity with any viruses other than the reverse transcriptase. Bacteria such as E coli also have reverse- transcribing transposons - known as retrons - but these are very different to any of the eukaryotic types while preserving similarities in certain of the essential reverse transcriptase sequence motifs. All of these elements are collectively known as RETROELEMENTS; the fact that the reverse transcriptases of all of them have some amino acid identity suggests a common evolutionary origin. Several reviewers have pointed out that just such an enzyme as reverse transcriptase would have been necessary for the transition from what is widely believed to have been an RNA world - that is, where all the extant organsisms had RNA genomes - to the present world in which all cellular organisms have DNA genomes.

Viruses with RNA genomes which use RNA-dependent RNA polymerases for their replication may be the only remnants of that preDNA era; however, cellular elements and viruses which use reverse transcriptase may share a common origin as cell-derived "modules" coding for a reverse transcriptase, which evolved to become retrons and retroposons and retrotransposons. Addition of structural proteins may have allowed evolution of retroviruses. The evolution of the DNA retroviruses - Hepadnaviridae, caulimo- and badnaviruses - is more obscure; it appears as though these arose from retrotransposon-like sequences, but this probably occurred near the origin of of these types of element as they are so diverse in sequence and genome organisation.

It is believed that retrotransposons may contribute substantially to the evolution of their hosts. Evidence for this has been obtained by studying human LINE-1s (Long Interspersed Nuclear Elements) - a group of retrotransposable elements which make up approximately 15 % of the human genome. The vast majority of LINE-1s are no longer retrotransposition competent and it is believed that in humans only between 30 and 60 full length LINE-1s are currently active. There is strong evidence from sequences in the sequence databases to suggest that active LINE-1s play an important role in "exon shuffling" (belived to be the major mechanism of macro-evolution whereby entirely new genes are created by reshuffling the components of older genes). The most compelling evidence that LINE-1s do facilitate exon shuffling, however, is the experimental demonstration that they are not only able to move large amounts of non-LINE-1 exonic DNA but also insert this DNA into unrelated expressed genes to obtain chimeras which encode active hybrid gene products.

VIRIONS are virus particles: they are the INERT CARRIERS of the genome, and are ASSEMBLED inside cells, from virus-specified components: they do not GROW, and do not form by DIVISION. They may be regarded as the EXTRACELLULAR PHASE of the virus: they are exactly analogous to "spacecraft" in that they take viral genomes from cell to cell, and they protect the genome in inhospitable environments in which the virus cannot replicate.

Helical Nucleocapsids This is one of the SIMPLEST FORMS of viral capsid: the protein is "wound on" to the viral nucleic acid (generally ssRNA, though M13 and other filamentous phage virions contain circular ssDNA) in a simple HELIX, like a screw (see the diagram for tobacco mosaic virus, below).

In the case of TMV this is the entire virion: this is also the case for all RODLIKE and FILAMENTOUS virions where no membranes are involved. This includes all Tobamoviridae, Potyviridae, and Closteroviridae, but NOT Filoviridae, like Ebola virus (see here). In other cases, filamentous helical nucleocapsids may be enclosed within

matrix protein and a membrane studded with spike proteins: excellent examples of this are PARAMYXOVIRIDAE, images of which can be found here, at Linda Stannard's site.

Isometric Nucleocapsids These are built up according to simple structural principles, as amply outlined here, and in more detail here. Put simply, nearly all isometric virions are constructed around a BASIC ICOSAHEDRON, or solid with 20 equilateral trinagles for faces. It suffices to say that the "quasi-icosahedral" capsid is possibly Nature's most popular means of enclosing viral nucleic acids; they come in many sizes, from tiny T=1 structures (Nanoviruses, eg: banana bunchy top virus; 18 nm diameter) to huge structures such as those of Iridoviridae or Phycodnaviridae (over 200 nm diameter). A good example of a simple structure is illustrated below in the animated GIF: this shows cowpea chlorotic mottle (CCMV) virion surface structure (courtesy J-Y Sgro), which is composed of 180 copies of a single coat protein molecule.

The different colours in the picture represent different "positional states" of the capsid protein: subunits around 5-fold rotational axes of symmetry are BLUE, and cluster as PENTAMERS; subunits around 3-fold axes are RED and GREEN to reflect their different 2-fold symmetries; they cluster as HEXAMERS around "local 6-fold axes". Another recent example - that of turnip yellow mosaic virus (TYMV) - is given here. This has exactly the same basic structure, with a single type of coat protein subunit, only the pentamer-hexamer clustering is more pronounced A more complex capsid - that of the common-cold-causing Rhinovirus R16 (family: Picornaviridae), with 60 copies of 4 proteins in a T=3 structure - is shown below (animation modified from one by J-Y Sgro). This shows a capsid with a cutaway, to reveal internal structure. BLUE subunits around 5-fold axes are VP1; RED and GREEN are VP3 and VP2 respectively; YELLOW subunits (seen only internally) are VP4. The VP4 subunits are formed by autocatalytic cleavage of VP0 (into VP2 and VP4) upon binding of a "procapsid" with viral genomic ssRNA. See here for further details of picornaviruses, here for a scheme showing picornavirus assembly, and here for a scheme outlining polyprotein processing of picornaviruses, and here for material from the Leicester course.

Prion Diseases This document describes infectious agents which (almost certainly) do not have a nucleic acid genome. It seems that a protein alone is the infectious agent. The infectious agent has been called a prion. A prion has been defined as "small proteinaceous infectious particles which resist inactivation by procedures that modify nucleic acids". The discovery that proteins alone can transmit an infectious disease has come as a considerable surprise to the scientific community. Prion diseases are often called spongiform encephalopathies because of the post mortem appearance of the brain with large vacuoles in the cortex and cerebellum. Probably most mammalian species develop these diseases. Specific examples include: Scrapie: sheep TME (transmissible mink encephalopathy): mink CWD (chronic wasting disease): muledeer, elk BSE (bovine spongiform encephalopathy): cows Humans are also susceptible to several prion diseases: CJD: Creutzfeld-Jacob Disease GSS: Gerstmann-Straussler-Scheinker syndrome FFI: Fatal familial Insomnia Kuru Alpers Syndrome These original classifications were based on a clinical evaluation of a patients family history symptoms and are still widely used, however more recent and accurate molecular diagnosis of the disease is gradually taking the place of this classification. The incidence of sporadic CJD is about 1 per million per year. GSS occurs at about 2% of the rate of CJD. It is estimated that 1 in 10,000 people are infected with CJD at the time of death. These figures are likely to be underestimates since

prion diseases may be misdiagnosed as other neurological disorders. The diseases are characterised by loss of motor control, dementia, paralysis wasting and eventually death, typically following pneumonia. Fatal Familial Insomnia presents with an untreatable insomnia and dysautonomia. Details of pathogenesis are largely unknown. Visible end results at post-mortem are non-inflammatory lesions, vacuoles, amyloid protein deposits and astrogliosis.
GSS is distinct from CJD, it occurs typically in the 4th-5th decade, characterised by cerebellar ataxia and concomitant motor problems, dementia less common and disease course lasts several years to death. (Originally thought to be familial, but now known to occur sporadically as well). CJD typically occurs a decade later has cerebral involvement so dementia is more common and patient seldom survives a year (originally thought to be sporadic, but now known to be familial as well). FFI pathology is characterised by severe selective atrophy of the thalamus. Alpers syndrome is the name given to prion diseases in infants. Scrapie was the first example of this type of disease to be noticed and has been known about for many hundreds of years. There are two possible methods of transmission in sheep: 1. Infection of pasture with placental tissue carrying the agent followed by ingestion,or direct sheep-lamb transmission i.e. an acquired infection. 2. Parry showed considerable foresight by suggesting that it is not normally an infectious disease at all but a genetic disorder. He further suggested that selective breeding would get rid of the disease.

Humans might be infected by prions in 2 ways: 1. Acquired infection (diet and following medical procedures such as surgery, growth hormone injections, corneal transplants) i.e. infectious agent implicated. 2. Apparent hereditary mendelian transmission where it is an autosomal and dominant trait. This is not prima facie consistent with an infectious agent. This is one of the features that single out prion diseases for particular attention. They are both infectious and hereditary diseases (?see below). They are also sporadic, in the sense that there are also cases in which there is no known risk factor although it seems likely that infection was acquired in one of the two ways listed above. Kuru is the condition which first brought prion diseases to prominence in the 1950s. Found in geographically isolated tribes in the Fore highlands of New Guinea. Established that ingesting brain tissue of dead relatives for religious reasons was likely to be the route of transmission. They ground up the brain into a pale grey soup, heated it and ate it. Clinically, the disease resembles CJD. Other tribes in the vicinity with same religious habit did not develop the disease. It is speculated that at some point in the past a tribe member developed CJD, and as brain tissue is highly infectious this allowed the disease to spread. Afflicted tribes were encouraged not to ingest brain tissue and the incidence of disease rapidly declined and is now almost unknown.

Evidence suggests that a prion is a modified form of a normal cellular protein known as PrPc (for cellular), a normal host protein encoded by a single exon of a single copy gene. This protein is found predominantly on the surface of neurones attached by a glycoinositol phospholipid anchor, and is protease sensitive. Thought to be involved in synaptic function. The modified form of PrPc which may cause disease i.e. the prion is known as PrPsc (for scrapie) which is relatively resistant to proteases and accumulates in cytoplasmic vesicles of diseased individuals. It has been proposed that PrPsc when introduced into a normal cell causes the conversion of PrPc into PrPsc. Process is unknown but it could involve a chemical or conformational modification.

The Virus Life Cycle Viruses have a defined "life cycle" as do any other type of organisms; however, given that they are obligate intracellular parasites, this cycle revolves around: getting into a host cell replicating there, and getting out again. For eighteen years now I have taught this cycle under the heading "Entrance, Entertainment, and Exit*", as this is the best mnemonic I know to remind one of the process. Other courses tend to label these steps as (for example) Virus Entry Into Cells Replication of Viruses Assembly and Release of Virions

At least 9 different poxviruses cause disease in humans, but variola virus (VV) and vaccinia are the best known. VV strains are divided into variola major (25-30% fatalities) and variola minor (same symptoms but less than 1% death rate). "Variolation" = the administration of material from known smallpox cases (hopefully variola minor!!!) to protect recipients - practiced for at least 1000 years (Chinese) but risky - Jenner was nearly killed by variolation in 1756!

Affected cattle showed altered behaviour and a staggering gait, giving the disease its name in the press - 'mad cow disease'. On microscopic examination, the brains of affected cattle showed extensive spongiform degeneration. The initial explanation for the emergence of BSE in the UK was as follows: Since scrapie (a spongiform encepalopathy of sheep) is endemic in Britain, it was assumed that this was the source of the infectious agent in the feed. It was concluded that BSE is due to the use of contaminated foodstuffs.

ICTV Taxonomy

Viral taxa. The 7th lCTV Report formalized for the first time the concept of the virus species as the lowest taxon (group) in a branching hierarchy of viral taxa. As defined therein, “a virus species is a polythetic class of viruses that constitute a replicating lineage and occupy a particular ecological niche”. A “polythetic class” is one whose members have several properties in common, although they do not necessarily all share a single common defining property. In other words, the members of a virus species are defined collectively by a consensus group of properties. Virus species thus differ from the higher viral taxa, which are “universal” classes and as such are defined by properties that are necessary for membership.

- Viruses are real physical entities produced by biological evolution and genetics, whereas virus species and higher taxa are abstract concepts produced by rational thought and logic. The virus/species relationship thus represents the front line of the interface between biology and logic.
- Viruses (including virus isolates, strains, variants, types, sub-types, serotypes, etc.) should wherever possible be assigned as members of the appropriate virus species, although many viruses remain unassigned because they are inadequately characterized.
- All virus species must be represented by at least one virus isolate.
- Almost all virus species are members of recognized genera. A few species remain unassigned in their families although they have been clearly identified as new species.
- Some genera are members of recognized sub-families.
- All sub-families and most genera are members of recognized families. Some genera are not yet assigned to a family; in the future they may either join an existing family or constitute a new family with other unassigned genera.

Some families are members of the following recognized orders: Caudovirales, Nidovirales and The hierarchy of recognized viral taxa is therefore:
(Order)
Family
(Sub-family)
Genus
Species

- Only the aforementioned taxa are recognized by the ICTV. Other groupings (from clade to super-family), may communicate useful descriptive information in some circumstances but they have no formally recognized taxonomic meaning. Similarly, the term "quasi-species", although it captures an important concept, has no recognized taxonomic meaning.
The creation or elimination, (re)naming, and (re)assignment of a virus species, genus, (sub)family, or order are all taxonomic acts that require public scrutiny and debate, leading to formal approval by the full membership of the ICTV. In contrast, the naming of a virus isolate and its assignment to a pre-existing species are not considered taxonomic acts and therefore do not require formal ICTV approval. Instead they will typically be accomplished by publication of a paper describing the virus isolate in the peer-reviewed virology literature.
Descriptions of virus satellites, viroids and the agents of spongiform encephalopathies (prions) of humans and several animal and fungal species are included.

Virus taxonomy. The advent of nucleotide sequence determination has revolutionized biology and largely rationalized taxonomy, including that of viruses. The universal virus taxonomy provides a classification scheme that is supported by verifiable data and expert consensus. It is an indispensable framework both for further study of the currently recognized virus species and for the identification and characterization of newly emergent viruses, whether they result from natural, accidental, or deliberate dissemination. The current health of virus taxonomy is due to the efforts of hundreds of virologists from around the world, but more volunteers are always needed. Those interested in contributing their expertise are encouraged to contact the relevant Study Group Chair or any member of the ICTV Executive Committee.

Virus taxonomy. The advent of nucleotide sequence determination has revolutionized biology and largely rationalized taxonomy, including that of viruses. The universal virus taxonomy provides a classification scheme that is supported by verifiable data and expert consensus – ICTV (International…). It is an indispensable framework both for further study of the currently recognized virus species and for the identification and characterization of newly emergent viruses.

Phylum: Negamaviricota,
Order: Herpesvirales,
Family: Herpesviridae,
Subfamily: Alphaherpesvirinae,
Genus: Simplexvirus,
Species: Bovine alphaherpesvirus, Human alphaherpesvirus 1, Human alphaherpesvirus 2.

Primary isolate is a pure microbial or viral sample that has been obtained from an infected individual, rather than grown in a laboratory. In chemistry and bacteriology, the verb *isolate* means to obtain a pure chemical, bacteriological or viral sample. The noun 'isolate' refers to the sample itself.

According to the 'Bulletin of Experimental Treatments for AIDS, Year-End, 1999' glossary,[1] a primary isolate is "HIV taken from an infected individual, as opposed to that grown in a laboratory."

In biology, a **strain** is a low-level taxonomic rank used at the intraspecific level (within a species). Strains are often seen as inherently artificial concepts, characterized by a specific intent for genetic isolation.[1] This is most easily observed in microbiology where strains are derived from a single cell colony and are typically quarantined by the physical constraints of a Petri dish. Strains are also commonly referred to within virology, botany, and with rodents used in experimental studies.

A **Variant** is a virus or worm based on an earlier virus or worm with one or more minor changes. A virus or worm that gains notoriety may eventually have hundreds of variants. Extremely simple viruses such as Vienna may be used as a template for more complex code and therefore have a lot of variants. Script worms and macro viruses such as Laroux, Melissa, Triplicate and Spyki, often have many variants because their code travels with them. Other extremely simple and prolific worms such as Slammer and Witty may have few to no variants because they cause too much disruption to be profitable and/or using them to send a political message would ensure the message is drowned out by the worm's/virus's destructiveness.

Creation of Variants

The author of the original virus or worm may make changes to their original creation. Often, a self-spreading program will contain bugs that inhibit its spreading or destruction ability. Even if the coder is ethical and only sends their code to antivirus vendors, s/he will want to fix the code. Sometimes a coder creates a virus or worm deliberately with a bug that prevents it from being destructive or spreading, but as a second thought creates a bug-free version.

Many first-time virus/worm coders will either disassemble a spreading program or find its source code in some other way in order to get an idea of the kind of coding necessary to create their own self-spreading program at a later date. They may reassemble or compile the source code. Even if no changes were consciously made to the code, the original code may be altered slightly during disassembly, assembly or compilation, producing a mostly similar program to the original, but with a few features that make it distinct from the original. Script kiddies are known to (and may get their name from) take someone else's code, make a few small changes to it and call it their own, giving rise to many variants.

Etymology

The word is from the Latin neuter *vīrus* referring to poison.

Virulent, from Latin *virulentus* (poisonous). A meaning of "agent that causes infectious disease" is first recorded in 1728, before the discovery of viruses by Dmitri Ivanovsky in 1892.

The English plural is *viruses*, whereas the Latin word is a mass noun, which has no classically attested plural.

The adjective *viral* dates to 1948.

The term *virion* (plural *virions*), which dates from 1959, is also used to refer to a single, stable infective viral particle that is released from the cell and is fully capable of infecting other cells of the same type.

A **serotype** or **serovar** is a distinct variation within a species of bacteria or virus or among immune cells of different individuals. These microorganisms, viruses, or cells are classified together based on their cell surface antigens, allowing the epidemiologic classification of organisms to the sub-species level.[1][2][3] A group of serovars with common antigens is called a **serogroup** or sometimes *serocomplex*.

Serotyping often plays an essential role in determining species and subspecies. The *Salmonella* genus of bacteria, for example, has been determined to have over 2600 serotypes, including *Salmonella enterica* serovar Typhimurium, *S. enterica* serovar Typhi, and *S. enterica* serovar Dublin.[2] *Vibrio cholerae*, the species of bacteria that causes cholera, has over 200 serotypes, based on cell antigens. Only two of them have been observed to produce the potent enterotoxin that results in cholera: O1 and O139.

Veterinary virology – 2018
Lecture 2. VIRAL REPLICATION.

VIRUS REPLICATION

In the previous chapter, viruses were defined as obligate intracellular parasites that are unable to direct any independent biosynthetic processes outside the host cell. It was further noted that the genetic complexity of viruses varies greatly between individual virus families, ranging from those
viruses that encode only a few proteins to others that encode several hundred proteins. Given this remarkable diversity, it is hardly surprising that the replication processes used by individual viruses would also be highly variable. However, all viruses must progress through the same general steps for
replication to occur. Specifically, all viruses must attach to a susceptible host cell, enter the cell, disassemble the virus particle (uncoating), replicate its own genetic material and express the associated proteins, assemble new virus particles, and escape from the infected cell (release). This chapter will outline the general processes involved in each of these steps.

GROWTH OF VIRUSES

Before the development of in vitro cell culture techniques, viruses had to be propagated in their natural host. For bacterial viruses (bacteriophages), this was a relatively simple process. Consequently, scientists were able to develop laboratory-based research methods to study bacteriophages long before they were able to conduct comparable studies with plant or animal viruses. For animal viruses,
samples from affected animals were collected and used to infect other animals, initially of the same species. When consistent results were obtained, attempts were usually made to determine whether other species might also be susceptible. These types of experiments were performed in an effort to determine the host range of any presumed viral agent. Although progress was made in defining the
biological properties of viruses, this manner of propagation had obvious major drawbacks, especially with viruses affecting large animals. A most serious issue was the infection status of the recipient animals. For example, an undetected infectious agent in a sheep could alter the clinical signs observed after inoculation of that sheep with the test agent, and samples collected from this individual might now include several infectious agents, potentially confounding future experiments.
In an attempt to avoid this type of contamination problem, animals that were to be used in research studies were raised under more defined conditions. As new infectious agents were discovered and tests developed for their detection, the research animals became more “clean” and the concept of the “specific pathogenfree” (SPF) animal was born. It is noteworthy; however, that animals that were thought to be specific pathogen free could be infected with pathogens that were still undefined or undeclared. For example, pneumonia virus of mice (mouse pneumovirus) was discovered when “uninfected” control animals inoculated with lung extracts from other control animals died during experimental influenza virus infection studies. Many early virological and immunological studies were compromised by using rodents unknowingly infected with mouse hepatitis virus, lactate

dehydrogenase-elevating virus, or other agents. Although live animals are no longer commonly used for routine virus isolation/propagation, animals are used still extensively for assessing viral properties such as virulence, pathogenesis, and immunogenicity.

The search for culture systems suitable for the propagation and study of viruses led to the discovery, in 1931, that vaccinia virus and herpes simplex virus could be grown on the chorioallantoic membrane of embryonated chicken eggs, as was already known for fowlpox virus, a pathogen of birds. It was soon determined that viruses in many families of animal viruses can be grown in embryonated eggs, probably because of the wide variety of cell and tissue types present in the developing embryo and its environment. Consequently, the embryonated chicken egg became a standard culturing system for routine isolation and propagation of avian viruses and select mammalian viruses. In some cases, embryonated eggs entirely replaced research animals for the growth of virus stocks, and if the viral infection resulted in the death of the embryo, this system could also be used to quantify (titrate) the amount of virus in a virus stock or specimen (as described in greater detail later in this chapter). The egg system, which is labor-intensive and expensive, has largely been replaced by vertebrate cell culture-based systems; however, it is still widely used for the isolation and growth of influenza viruses and many avian viruses. Various in vitro cell culture systems have been utilized since artificial medium was developed to maintain cell viability outside the source animal. These include organ cultures, explant cultures, primary cell cultures, and cell lines.

An organ culture consists of an intact organ, which maintains the cellular diversity and the three-dimensional structure of the tissue. Organ cultures are utilized for short-term experiments. Explant cultures consist of portions (eg, a slice or fragment) of an organ or tissue. Although explant cultures lack the complexity of the intact organ, their cellular components exist in a state that more closely models the in vivo environment than do cells propagated as primary cell cultures or cell lines. The creation of primary cell cultures utilizes proteases such as trypsin or collagenase to disassociate individual cells of a given tissue such as fetal kidney or lung. The individual cells are then permitted to attach to a cell culture matrix on which they will divide for a limited number of cell divisions. The limited lifespan of most primary cells requires continual production of the cells from new tissue sources, which can lead to variable cell quality between batches. This problem was largely overcome with the generation of immortalized cell lines, which in theory are capable of unlimited cell divisions. Initially, the generation of immortalized cell lines (transformation) was an empirical process with a low probability of success but it is now possible to immortalize virtually any cell type, so the number of cell lines representing different species is increasing rapidly.

The advent of in vitro animal cell culture brought research studies on animal viruses in line with those involving bacteriophage, and enhanced the quality and reliability of diagnostic testing. The ability to isolate and propagate animal viruses in cultured cells also made it possible to identify viruses as the etiologic agents of specific diseases through the successful application of Koch's postulates. Fulfilment of Koch's postulates requires that the infectious agent be isolated in pure culture; an achievement that was not possible for viruses prior to the development of cell culture systems.

Replacement of living animals with cell culture systems decreased, but did not entirely eliminate, the problems associated with the presence of adventitious viruses. For example,

early batches of the modified live poliovirus vaccine were contaminated with SV40 virus, a simian polyomavirus originating from the primary monkey kidney cultures used for vaccine production.
Similarly, interpretation of the results of some early studies on newly described parainfluenza viruses is complicated because of virus contamination of the cell cultures used for virus isolation. Contamination of ruminant cell cultures with bovine viral diarrhea virus has been an especially insidious and widespread problem. Some contaminated cell cultures and lines were probably derived from infected fetal bovine tissue, but far more commonly, cells became infected through exposure to fetal bovine serum contaminated with bovine viral diarrhea virus. Fetal bovine serum became a standard supplement for cell culture medium in the early 1970s. The fact that many ruminant cell lines became infected from contaminated serum has compromised much research pertaining to ruminant virology and immunology, confounded diagnostic testing for bovine viral diarrhea virus and caused substantial economic losses as a result of contaminated vaccines. The extent of the problem was not fully defined until the late 1980s when high quality diagnostic reagents became available.
As with experimental animals, problems with contaminating viral infections of cell cultures were only defined when the existence of the relevant infectious agent became known. Standard protocols for the use of serum in biological production systems now require irradiation of the serum to inactivate all viruses, known or unknown. With current technology allowing amplification and detection of virtually all nucleic acid species in cells, coupled with rapid sequencing of these products, a complete
profile of cell cultures for contaminating organisms is now feasible. Recognition of Viral Growth in Culture Prior to the development of cell culture systems, identifying the presence of a viral agent in a plant or animal host was dependent upon the recognition of signs not found in an unaffected (control) host, death being the most extreme outcome and easiest to determine. Similarly, the presence
of a replicating virus in cultured cells can be detected by identifying specific cellular characteristics that arise as a consequence of virus infection. In broad terms, any observable cellular characteristic that is present in virusinfected cells and which is absent in uninfected cells maintained under identical growth conditions is referred to as a cytopathic effect (CPE). Virus-induced forms of CPE are generally observed through microscopic examination of the test culture system (Fig. 2.1). The most common forms of CPE observed in cultured cells are cell lysis and significant changes in cell morphology. Examples of morphological changes include the rounding, clumping, shrinkage, and detachment of individual cells from the cell culture matrix.
Virus-induced fusion of neighboring cells represents another form of CPE. For example, cells infected with avian reovirus commonly fuse to form multinucleated cells or syncytia (Fig. 2.1A). Many members of the family Paramyxoviridae can cause this type of morphological change in cultured cells, but the extent of syncytium formation is cell type dependent. The type of cytopathology noted in culture can be characteristic for a given class of virus. For example, alphaherpesviruses produce distinct
cytopathology characterized by rounded cells, with or without small syncytia, which spreads very rapidly through a susceptible cell culture (Fig. 2.1B). Cells infected with some types of viruses acquire the ability to bind (adsorb) red blood cells (syn., erythrocytes) on their surface; a property referred to as hemadsorption. For example, cells infected with bovine parainfluenza virus 3 adsorb chicken red blood cells to the plasma

membrane (Fig. 2.1D). Binding of red blood cells to the surface of the infected cell is actually mediated by viral glycoproteins that are expressed on the cell surface and which bind to receptors on the red blood cells.

Consequently, hemadsorption only occurs with viruses that bud from the plasma membrane, and may be specific for red blood cells of a given animal species. Viruses that induce hemadsorption also show the ability to hemagglutinate red blood cells in cell-free medium. As discussed later in the chapter, this property can be used as the basis for quantifying the amount of virus within a sample. The same viral proteins that permit hemadsorption are also responsible for the hemagglutination reaction. There are, however, viruses that can themselves hemagglutinate red blood cells but not cause hemadsorption to cells infected with the same virus (eg, adenoviruses and alphaviruses).

Another type of morphological change commonly observed in virus-infected cells is the formation of inclusion bodies. Inclusion bodies are intracellular abnormalities, commonly new structures, which arise as a direct consequence of virus infection. Inclusion bodies can be observed with a light microscope after fixation and treatment with cytological stains, but, as with hemadsorption, not all viruses will produce obvious inclusion bodies. The type of virus infecting a cell can be inferred by the location and shape of the inclusions. For example, cells infected with herpesviruses, adenoviruses, and parvoviruses can have intranuclear inclusions, whereas cytoplasmic inclusions are characteristic of infections with poxviruses, orbiviruses, and paramyxoviruses. The composition of the inclusions will vary with the virus type. The cytoplasmic Negri bodies identified in rabies virus-infected cells are composed of aggregates of nucleocapsids, whereas the intranuclear inclusions that occur in adenovirus-infected cells consist of crystalline arrays of mature virus particles. Cytological stains are rarely used to identify cells infected with specific viruses, but are mainly used as a screening test to assess the presence of any virus.

In the absence of a metagenomic screening procedures, detection of viruses that produce no cytopathology (CPE), do not induce hemadsorption or hemagglutinate, or produce no definable inclusions, is accomplished using virus-specific tests. For example, this is the case in screening bovine cells for the presence of noncytopathic bovine viral diarrhea virus.

The most commonly used tests in this type of situation are immunologically based assays such as the fluorescent antibody assay (immunofluorescence assay, IFA) or immunohistochemical staining assay. The quality of these assays is dependent on the specificity of the antibodies that are used. With the development of monoclonal antibodies and monospecific antisera, this issue has been largely resolved. Other virus-specific tests are based on the detection of virusspecific nucleic acid in the infected cells. Initially, assays of this sort relied on the use of nucleic acid probes capable of hybridizing in a sequence-specific manner with the target nucleic acid. Hybridizationbased assays have largely been replaced by those based on polymerase chain reaction (PCR) because of their enhanced sensitivity and ease of performance.

VIRUS REPLICATION

A fundamental characteristic that separates viruses from other replicating entities is the manner in which new virus particles are synthesized. Unlike eukaryotic and prokaryotic cells, which increase their numbers through the processes of mitosis and binary fission, respectively, new virus particles are assembled de novo from the various structural

components that are synthesized during the
virus infection. The earliest recognition of this unique replication pattern came from studies using bacteriophage. The outline of the experimental proof of concept was relatively simple: (1) add a chloroform-resistant phage to a culture of bacteria for several minutes; (2) rinse the bacteria to remove nonattached phage; (3) incubate the culture and remove samples at various periods of time; (4) treat
sampled bacterial cultures with chloroform to stop growth; (5) quantify the amount of phage at each of the time periods. The result of this type of experiment is what we now refer to as a one-step growth curve, which in principle, can be performed with any virus that can be propagated in cell culture. The remarkable finding of this type of study was that infectious virus "disappeared" from the infected cultures for a variable period of time, depending on the virushost-cell system. This is referred to as the eclipse period, and represents the period of time that begins with cell entry/uncoating and ends
with the appearance of newly formed infectious virus particles. Following the end of the eclipse period there is an essentially exponential increase in production of infectious virus particles until the host cell is unable to maintain its metabolic integrity. Depending on the type of virus, there may be sudden release of virus particles following lysis of the host cell, as exemplified by T-even bacteriophages, or a prolonged release of virus particles via sustained budding of virus particles at a cell membrane site, such as with influenza A virus.

The one-step growth curve can be used to divide the virus replication cycle into its component parts, which include attachment, the eclipse period (entry, uncoating, replication of component parts, virion assembly), and release of virus particles. Although the replication cycles of all conventional viruses follow these same general steps, the details of each step can vary widely depending on the specific virus. Therefore, the kinetics of the onestep growth curve differs with the unique properties of the
specific virushost-cell system used. To ensure that all steps of the virus replication cycle are temporally synchronized, it is important that the infection be initiated with enough virus particles to simultaneously infect all cells in the culture. This is achieved by using a high multiplicity of infection [typically 10 plaque forming units (pfu) of virus/cell]. A discussion focusing on the individual steps of the general virus replication cycle now follows. This discussion includes expanded descriptions and details of complete replication cycles for model viruses representing four major groups [positive strand RNA viruses (picornavirus), negative strand RNA viruses (rhabdovirus), retroviruses, and DNA viruses (adenovirus)]. More comprehensive discussions covering the specific details of individual virus families are found in Part II of this book.

Attachment

The critical first step in the virus replication cycle is the attachment of the virus particle to a host cell. Attachment requires specific interactions between components of the virus particle (eg, capsid proteins or envelope glycoproteins) and components of the host cell (eg, a glycoprotein
or carbohydrate moiety). This process can be conceptually simple whereby attachment can involve interactions between a single component of the virus with a single component of the cell. For example, binding of influenza A virus to a host cell requires only an interaction between the viral hemagglutinin (HA) glycoprotein and a sialic acid residue on the cell surface. Alternatively, attachment-related interactions can be complex and involve

sequential interactions between multiple components of both the virus and the cell. Examples of this type of cell binding are described below in the expanded discussions of the adenovirus and retrovirus replication cycles. Many host proteins are not widely expressed but instead are expressed in a cell- or tissue-specific manner.

Therefore, receptor usage plays an important role in defining the tissue/organ specificity (tropism) of a virus. In turn, the tissue and organ specificity of a virus largely defines its pathogenic potential and the nature of the disease it causes. Similarly, cellular components (eg, proteins, carbohydrate structures, etc.) can differ markedly between organisms, thus, receptor usage also influences the types of organisms (host species) that a virus can infect (host range). Virus particles interact with cell-surface molecules which are referred to as receptors, coreceptors, attachment factors, or entry factors depending on the role(s) that they play in the attachment and entry processes. Frequently,

the term "viral receptor" is used to describe these cellsurface molecules, which is something of a misnomer, as cells certainly do not maintain receptors for the purpose of binding viruses. Rather, viruses have evolved to use host cell molecules that perform functions related to normal cellular processes. Initial contact of a virus particle with the cell surface often involves short-distance electrostatic interactions with charged molecules such as heparin sulfate proteoglycans. This initial contact may simply help to concentrate virus on the surface of the cell, which facilitates the establishment of more specific interactions with other receptor-like molecules. The affinity of binding

between an individual virus component and its cellular ligand may be low; however, the virus surface possesses many receptor binding sites, thus the affinity of binding between the virus and the host cell is enhanced by the establishment of multiple virus/receptor interactions.

Although viruses require at least one receptor to be expressed on the surface of the host cell, some viruses must also engage an intracellular receptor(s) in order to initiate a productive infection. These intracellular interactions do not play a role in attachment to the cell but instead are required for the final stages of the entry/uncoating process; and therefore, will be discussed in more detail below.

The identification of host cell factor(s) that serve as receptors for virus attachment is important for understanding the molecular details of specific virus replication cycles, and also has practical implications as this knowledge can inform the design of antiviral drugs. In recent years, numerous host cell components capable of functioning as receptors/entry factors for viruses have been identified. These include ligand-binding receptors (eg, chemokine receptors, transferrin receptor 1), signaling

molecules (eg, CD4), cell adhesion/signaling receptors (eg, intercellular adhesion molecule-1, ICAM-1), enzymes, integrins, and glycoconjugates with various carbohydrate linkages, sialic acid being a common terminal residue (Table 2.1). As shown in Table 2.1, different viruses may use the same receptor/entry factor (eg, Coxsackievirus and some adenoviruses), which results in these viruses having a shared or overlapping cell/tissue tropism. The number and identity of host cell molecules that play a part in the initial interactions of virus with host cells will certainly increase as new viruses are identified and as existing viruses are better characterized.

The process of identifying receptors/entry factors is more complicated than initially imagined, as viruses within a given family may use different receptors. Furthermore, different strains of the same virus can utilize different receptors and adaptation of a virus to growth in

cell culture can change receptor usage of the virus. For example, wild-type strains of foot and mouth disease virus bind to integrins in vivo, but cell culture-passaged strains of the virus can use heparan sulfate. This change in receptor specificity alters the pathogenicity of the virus, clearly
indicating that receptor usage influences the disease process. Some viruses with a broad host range, such as arthropod-borne viruses and some of the alphaherpesviruses, are thought to use several different host-specific receptors, which accounts for their ability to grow in cells
from many hosts. Alternatively, a virus can use a common receptor that is expressed in multiple host species. For example, Sindbis virus was recently shown to utilize a protein called natural resistance-associated macrophage protein (NRAMP) as a receptor in insect cells, and to use
the mammalian homolog (NRAMP2) for binding to cultured mammalian cells and in the tissues of mice. Two additional issues related to the virus/cell attachment process are notable. A model was recently proposed in which cell receptors that normally function in the recognition and clearance of apoptotic cells are used for cell attachment/entry by dengue virus and perhaps by related
flaviviruses. Flaviviruses bud through the membrane of the endoplasmic reticulum, and consequently, are thought to incorporate phosphatidylserine (PtdSer) into the outer leaflet of the viral envelope. PtdSer is also enriched on the outer leaflet of the plasma membrane of cells undergoing apoptosis due to lipid reshuffling, and is bound directly or indirectly by members of the TIM and TAM
families of transmembrane receptor proteins, respectively.
TIM and TAM proteins are expressed by a number of cell types including macrophages and dendritic cells, which are normal targets of dengue virus infection. Under normal circumstances the binding between the TIM/TAM proteins on myeloid cells and the PtdSer on apoptotic cells leads to the uptake and clearance of the apoptotic cell. By incorporating PtdSer into the viral envelop, dengue virus is thought to mimic an apoptotic cell, enabling the virus to be bound and internalized by cells expressing
the TIM/TAM proteins. This mechanism does not appear to be unique to flaviviruses as a similar model has beenproposed for vaccinia virus (family Poxviridae). A second somewhat indirect mechanism of cell binding/entry is also best exemplified by dengue virus. This mechanism is
referred to as antibody-dependent enhancement of infection, which occurs when the virus particle is bound by nonneutralizing IgG antibodies that in turn are bound by activating Fcγ receptors expressed on the surface of mononuclear phagocytic cells (eg, macrophages). This interaction leads to internalization of the antibody/virus complex and eventual release of infectious virus into the
cytoplasm of the phagocytic cell. Foot and mouth disease virus and feline coronavirus can also infect cells through this antibody-mediated enhancement mechanism in vitro, but its importance in the natural infection process is conjectural.

Entry and Uncoating

The binding of a virus to a receptor on a host cell represents the first step in the replication cycle; however it will not result in a productive infection unless this event leads
to entry of the virus into the cell with subsequent uncoating of the virus particle and release the viral genome into the proper intracellular compartment (cytoplasm or nucleus

depending on the virus). Though the plasma membrane is only about 7 nm thick it serves as an effective physical barrier that blocks the free passage of viruses into the cell. However, viruses have evolved a range of strategies for breaching this barrier and gaining access to the cell interior. Depending on the specific
virus, uncoating of the virus particle occurs after the particle has entered the cell or concurrently with the cell entry process. The virus particle is metastable, which means that its structure is generally stable enough to move as a physical entity from cell to cell or from one host to another, but it is primed to undergo structural rearrangements and to disassemble when exposed to the proper biological stimuli. As detailed below, the biological stimuli that induce the entry and/or uncoating processes for different viruses include, but are not limited to, binding to specific host cell proteins, proteolysis by host cell enzymes, and exposure to acidic pH. This section of the chapter will focus on general mechanisms of virus entry into host cells, and will include multiple examples of specific virus/host interactions that initiate the uncoating process.

A bound virus particle (or sometimes the virus genome alone) enters a host cell by one of two general
mechanisms: (1) direct entry across the plasma membrane or (2) entry into the cell within a membrane-bound vesicle. As described below, viruses that enter the cell within membrane-bound vesicles must still pass through a limiting membrane to gain access to the cytosol. In both of these mechanisms the receptor molecule(s) assists in the entry process, and the nature of the receptor can determine the mechanism by which the virus enters the host cell. Mechanisms of direct entry across the plasma membrane will be addressed first. Cell entry by the picornaviruses will be used as an example of direct entry by a nonenveloped virus, and the mechanism presented is
based on the current model for direct entry of poliovirus.

Poliovirus attaches to a host cell by binding to the poliovirus receptor (PVR, CD155). Noncovalent interactions established between the poliovirus receptor and proteins that form the capsid induce significant changes in the capsid structure. Most significantly, a protein located inside
the capsid (VP4) is expelled from the virus particle, and capsid protein VP1 undergoes conformational changes that cause the hydrophobic N-terminus of the protein to translocate from the capsid interior to the capsid surface where it is inserted into the plasma membrane of the host cell. The N-terminal sequences of multiple VP1 proteins are thought to associate and form a pore in the plasma
membrane through which the genomic RNA is released into the cytoplasm of the host cell (Fig. 2.5).

All enveloped viruses must mediate the process of membrane fusion to enter their host cell. For enveloped viruses that achieve direct entry at the cell surface, fusion occurs between the virus envelope and the plasma membrane, and this process occurs under neutral pH conditions
(pH-independent entry). This mode of cell entry is characteristic of paramyxoviruses (eg, Newcastle disease virus and measles virus) and some (eg, human immunodeficiency virus, HIV) but not all retroviruses.

The initial stages of the entry process for these viruses are conceptually similar to those of poliovirus in that binding of the virus to an appropriate receptor molecule stimulates conformational changes in a viral protein that in turn facilitates passage of the viral genome into the cell. In this case, conformational changes occur within a spike-

associated glycoprotein that transitions
from a native conformation (prefusion conformation) into an alternate conformation (postfusion conformation) that is capable of mediating fusion between the viral envelope and the plasma membrane. In the case of Newcastle disease virus, binding of host cell receptors is performed by the
hemagglutinin neuraminidase (HN) glycoproteins, which form homotetrameric spikes that project from the virion surface (Fig. 2.6). Receptor binding stimulates conformational changes in HN, which in turn destabilize and induce conformational changes in a neighboring protein called the fusion (F) protein. F proteins consist of disulfide-liked heterodimers (F1/F2), which assemble into homotrimeric
spikes. The N-terminal sequences of F1 are highly hydrophobic and are referred to as a "fusion peptide." Prior to receptor binding, the F proteins assume their prefusion conformation, in which the fusion peptide sequences are sequestered from the hydrophilic environment that surrounds the virus. Following receptor binding by HN, conformational changes are induced in the F proteins that
result in the projection of the fusion peptides towards the host cell where they insert themselves into the lipid bilayer of the plasma membrane. Continued conformational changes in the F proteins draw the plasma membrane towards the virus envelope. When the two membranes make contact, mixing of their lipids occurs and eventually the membranes are fused together to form a fusion pore. As
the fusion pore grows in size the viral envelope becomes fully incorporated into the plasma membrane of the cell and the genome of the virus is released into the cytoplasm of the host cell. In both of the virus systems just described (poliovirus and Newcastle disease virus), the entry and uncoating processes occur simultaneously and are only initiated after the virus particle has bound to a biologicallyrelevant receptor protein.
Natural receptor/ligand interactions at the cell surface often initiate signaling pathways and cellular processes that lead to the internalization of the receptor/ligand complex into a membrane-bound vesicle. Many viruses have evolved strategies to exploit these same signaling pathways and cellular processes to gain entry into the host cell. Endocytosis is the general mechanism whereby
extracellular materials are internalized in membranebound vesicles (Fig. 2.7). One form of endocytosis is translocate from the capsid interior to the capsid surface where it is inserted into the plasma membrane of the host cell. The N-terminal sequences of multiple VP1 proteins are thought to associate and form a pore in the plasma membrane through which the genomic RNA is released
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extracellular materials are internalized in membranebound vesicles (Fig. 2.7). One form of endocytosis is the early endosomal pathway. Endosome contents will subsequently be delivered to late endosomes and eventually to endolysosomes. As the endosome vesicles progress through the pathway their interior pH becomes increasingly acidic and the composition of resident cell proteins
changes. For some viruses, the acidic pH within the endosome serves as the stimulus for structural changes in the virus particle that facilitate exit from the endosome and uncoating of the virion (pH-dependent entry). This process has been studied in detail using influenza A virus.
Attachment of influenza A virus to a host cell is mediated by the viral hemagglutinin (HA) spike, a homotrimeric structure composed of three disulfide-linked HA1/HA2 heterodimers. The HA spike binds to sialic acid residues on the cell surface and bound virus particles are then taken into the cell within endosomes. The decreasing pH within the endosome induces profound conformational
changes in HA that cause the N-terminal sequences of HA2, which function as a fusion peptide, to extend outward from the virion and insert into the endosome membrane. Much

like the F proteins of Newcastle disease virus, the HA proteins continue to refold, drawing the virus envelope and the endosome membrane together and eventually causing them to fuse. Fusion between the two
membranes results in the release of the virus genome into the cytoplasm. The genome of influenza A virus consists of eight nucleocapsids (negative sense RNAs complexed throughout their length by NP protein) that are associated with each other and with multiple copies of the M1 protein. The M1 protein appears to aggregate the eight nucleocapsids through noncovalent protein:protein interactions. As the endosome becomes acidified, hydrogen ions are transported through a virion-associated ion channel (M2 ion channel) to acidify the virion interior. The drop in intraparticle pH dissociates M1 from the complex which allows the nucleocapsid aggregate to disassemble into individual nucleocapsids that are small enough to be imported into the nucleus through a nuclear pore. Thus, in the case of influenza A virus, exposure to acidic pH stimulates two separate uncoating processes (ie, membrane
fusion and release of individual nucleocapsids).
As predicted, infection of cells by those viruses that enter via clathrin-mediated endocytosis is inhibited by compounds that prevent endosome acidification (eg, bafilomycin A1, chloroquine, NH4Cl). Some viruses that enter the cell through clathrin-mediated endocytosis require biological stimuli beyond exposure to acidic pH in order to escape from the endosome compartment. Ebola virus mediates
membrane fusion at the late endosome or endosome/lysosome stage of the pathway. Membrane fusion is mediated by the GP spike glycoprotein that consists of GP1/GP2 heterodimers. The GP protein does not become fully primed for membrane fusion until it is cleaved by two host cell proteases (cathepsin L and cathepsin B), which the virus does not encounter until it reaches the late
endosome/lysosome. In addition, primed GP is not stimulated to perform the conformational changes required for membrane fusion until it has bound to an internal receptor called Niemann-Pick C1 (NPC1), which is a resident protein of the late endosomelysosome membrane. The ability of Lassa virus (family Arenaviridae) to exit the endosome also depends on binding to an internal receptor.
A second major endocytosis pathway that is exploited by viruses for entry into host cells is the caveosome system (Fig. 2.7). In this pathway, viruses bound at the cell surface enter small membrane invaginations called caveolae. Caveolae are coated on their cytoplasmic side by caveolin proteins. Similar to the endosome system, the invaginations can be bound to cargo molecules and pinch off the plasma membrane to form vesicles called caveosomes. Unlike the endosomal system, the caveosomes maintain a neutral pH within the vesicle. However, there appears to be a pathway for
caveosomes to enter the endosomal system, which would allow pH activation of some viruses. Alternatively, caveosomes can be delivered to the endoplasmic reticulum. Virus entry through the caveosome system has been studied extensively using SV40 virus (family Polyomaviridae). As a general rule, enveloped viruses do not use the caveosome system; this may be a function of particle size, as the vesicles formed by the endosomal system are larger and can accommodate the
generally larger size of virions that possess lipid envelopes.
In many cases, the entry of the virus particle, nucleocapsid, or genomic nucleic acid into the cytoplasm is not the final step in the initiation of the replication process for the virus. Commonly, the initial steps of the virus entry and uncoating processes do not result in

release of the genome in a form that can initiate replication-related processes (eg, translation, transcription, or replication). Furthermore, these early events often do not place the viral genome in the proper cellular compartment for replication. Again, cellular processes are involved in stimulating additional uncoating processes, and in the transport of the viral units to the required location. For example, Semliki forest virus (family Togaviridae) enters the cell by clathrin-mediated endocytosis and fusion of the virus envelope with the endosome membrane releases the icosahedral nucleocapsid into the cytoplasm. The capsid structure is then disassembled following binding of the capsid proteins by the cell's 60S ribosomal subunits.

Disassembly of the capsid releases the viral positive strand RNA, which is then available to be translated into the viral proteins that will orchestrate downstream replication processes. Similarly, the initial steps of cell entry by adenovirus (described in more detail below) deliver a modified virus particle to the cytoplasm, but replication of the adenovirus DNA occurs in the nucleus. Therefore, translocation of viral components from the cytoplasm to the nucleus is a required step in infection by almost all DNA viruses (poxviruses are a notable exception). For most of the longer translocation needs, the microtubule transport system is used, and movement of the virus particle is often facilitated by molecular motors such as dynein or kinesin. Actin filaments can also be utilized for more localized movements. For the DNA viruses and RNA viruses such as influenza virus that utilize the nucleus for their replication site, nuclear localization signals exist on key viral proteins that interact with soluble cellular proteins of the nuclear import system. These proteins link the viral units to the nuclear pore complex, either permitting translocation of the viral unit into the nucleus (parvoviruses) or inducing the transport of the nucleic acid into the nucleus (adenoviruses, herpesviruses). The replication cycle of individual virus families are described in more detail in Part II of this book.

Viral Protein and Nucleic Acid Synthesis

Up to this point in the replication process, the virus particle has been somewhat passive as no biosynthetic activity directed by the viral genome has occurred. The preliminary steps of the infection process have placed the viral genome in position to take active control of the replication cycle and to remodel the cell to assist in the production of progeny virus particles. The details of the next phases of the replication cycle, which include the expression of viral proteins and replication of the viral genome, differ markedly between viruses, and play a major role in determining the evolutionary relationships between viruses and in the placement of viruses into proper taxonomic groupings. Examples of four different replication strategies will be described in succeeding pages in order to emphasize specific aspects of virus replication and to demonstrate the diversity of replication strategies.

Representative Examples of Virus Replication. Strategies.

Picornaviruses

The family Picornaviridae includes a number of important pathogens of animals and humans, for example poliovirus, hepatitis A virus, and foot and mouth disease virus (see Chapter 26: Picornaviridae). Picornaviruses are small, relatively simple, nonenveloped

viruses. The virus particle has an icosahedral symmetry and consists of a protein capsid and a genome comprised of a single strand of positive sense RNA. The genomic RNA contains a small virus-encoded protein (VPg) covalently bound to its 5' end and a genetically encoded 3' poly A tail. The picornavirus entry process
differs depending on the specific virus. The capsid of some picornaviruses (eg, poliovirus) undergoes conformational changes at the plasma membrane in response to receptor binding, and these changes are thought to create a transmembrane protein pore through which the virus genome is extruded from the virion into the cytoplasm of the host cell (as described above, Fig. 2.5). Other picornaviruses, such as foot and mouth disease virus, enter cells via receptormediated endocytosis and release their genome into the cytoplasm following conformational changes induced by the acidification of the endosome.
Regardless of the mechanism used, the entry process results in release of the genomic RNA into the cytoplasm of the host cell where it will be used as a template for protein synthesis and for the replication of new viral genomes as depicted in Fig. 2.8.
Shortly after the genomic RNA is released into the cytoplasm, the VPg protein is removed from the RNA by a cellular enzyme that normally functions in the repair of cellular DNA. Following the removal of VPg, the RNA associates with the cellular translational system and is used as a template for synthesis of the viral proteins. However, unlike most host-cell mRNAs, the picornavirus genomic RNA lacks a standard 5' cap structure which is normally required to initiate the assembly of a ribosome
onto the mRNA template (cap-dependent translation).
Therefore, picornaviruses have had to evolve a mechanism for assembling host cell ribosomes onto viral mRNAs in the absence of a 5' cap (cap-independent translation). This function is provided by RNA sequences located near the 5' end of the genomic RNA itself. In picornaviruses, the AUG codon that is used to initiate translation is located an unusually long distance from the 5' end of the RNA (743 nt in the case of poliovirus). The long nontranslated region between the 5' end and the AUG start site assumes multiple secondary and tertiary structures due to extensive intramolecular base pairing.
The majority of this region is referred to as the internal ribosome entry site (IRES) based on its ability to interact with cellular components of the translational machinery and to assemble ribosomes internally on the RNA a short distance upstream of the start codon. As virus replication proceeds, translation of cellular proteins decreases markedly as ribosomes are assembled almost exclusively
onto viral mRNAs. The restriction of cellular protein synthesis is due to the cleavage and subsequent inactivation of the translation initiation factor eIF4G by a virus-encoded protease (designated L protease or 2A protease depending on the virus). eIF4G is required for cap-dependent translation and in its absence ribosomes are not assembled on capped mRNA.
Translation of viral mRNA is not affected by cleavage of eIF4G as these mRNAs lack a cap and ribosomes are assembled internally on viral mRNAs by the IRES. The selective inhibition of cellular translation reduces competition for ribosomes and reduces the ability of the cells to produce an array of antiviral molecules such as type I interferons that are made in response to the viral infection (see Chapter 4: Antiviral Immunity and Virus Vaccines).

The genomic RNA of picornaviruses includes only a single open reading frame that is translated into a single large polyprotein that is subsequently cleaved by virusencoded

proteases (which are embedded within the polyprotein) into the individual structural and nonstructural proteins of the virus. Intermediate cleavage products are designated P1, P2, and P3 (Fig. 2.8). Proteins that are used to assemble the capsid (VP1, VP2, VP3, and VP4) are ultimately derived from P1. Proteins required for genome replication and interference with host cell processes are ultimately derived from P2 and P3. The input genomic RNA will be translated repeatedly to generate virus proteins but eventually it will be used as a template for replication. Replication of the picornavirus RNA is performed in close association with remodeled cellular membranes and requires most of the proteins derived from the P2 and P3 precursor proteins as well as several cellular proteins. The host cell does not provide an RNAdependent RNA polymerase (RdRp) enzyme capable of replicating the viral RNA genome; and therefore, picornaviruses (and nearly all other RNA viruses) have evolved their own RdRp enzyme for this purpose. Picornaviruses encode an RdRp enzyme called 3Dpol, which is derived from the P3 precursor protein. 3Dpol is a primerdependent polymerase and the primer that is used in the replication process is the VPg protein itself.

A tyrosine residue within VPg donates the hydroxyl group onto which two uridine nucleosides are added by 3Dpol to form VPgUU2OH. The addition of the uridine nucleosides is templated by two adenosine nucleosides located in the non-base paired region of a RNA stem loop structure located internally on the genomic RNA. This stem loop structure is referred to as the cis-acting replication element (CRE). The actual sequence and internal location of CRE varies among different picornaviruses but all contain two or more adjacent adenosine residues within their loop structure which serve as the template for the uridylylation of VPg. Following its synthesis, the VPgUU2OH primer is translocated to the terminal sequences of the 3' poly A tract where it is hybridized to the RNA through A:U base pairing. The VPgUU2OH primer is then extended by 3Dpol to form a full-length complementary negative strand. The negative strand terminates in at least two adenosine residues, which facilitates base pairing with another VPgUU2OH primer and the synthesis of positive strand RNAs. Many of the newly synthesized positive strand RNAs will be used as mRNAs following the enzymatic removal of VPg. Other positive strand RNAs will retain VPg and be packaged into progeny virions.

The capsid structure of picornaviruses consists of multiple copies of a structural subunit called the protomer. The protomer of most picornaviruses contains single copies of the structural proteins VP1, VP3, VP0 (a precursor to VP2 and VP4), each of which is derived from P1. Sixty protomers associate through noncovalent interactions to form the icosahedral capsid. The exact mechanism by which the RNA genome is incorporated into the developing capsid remains unclear, but two primary models have been proposed. The first model proposes that individual protomers assemble on a genomic RNA and incorporation of the genome occurs coincident with the capsid assembly process. The second model proposes that protomers interact in the absence of RNA to form empty capsid structures into which the genomic RNA is then somehow inserted. In both models, the final step of capsid maturation involves cleavage of VP0 into VP2 and VP4 by what is believed to be an autoproteolytic process.

The ratelimiting process for particle maturation appears to be the availability of VPg-containing RNA. All steps of picornavirus virion assembly occur intracellularly, and late in infection crystalline arrays of virus particles form in the cytoplasm of infected cells. Ultimately, these virus particles are released from the cell en mass following dissolution of the cell structure.

The replication cycle of picornaviruses illustrates several properties that are common to many positive strand RNA-based viruses. First, the RNA genome is infectious, meaning that the genomic RNA itself is capable of initiating a productive infection when introduced into a host cell in the absence of any viral proteins. Second, the positive sense genomic RNA is able to associate with ribosomes and serve as a template for the production of viral proteins which then perform the processes of replicating the viral RNA and of manipulating critical host-cell metabolic and defense-related processes. Third, viral proteins can be synthesized as larger precursor proteins (polyproteins) that are subsequently resolved into the individual structural and nonstructural proteins by virus-encoded proteases. Finally, these viruses often induce the remodeling of cellular membrane structures that provide sites for viral RNA synthesis.

Rhabdoviruses

Vesicular stomatitis virus is the prototypical member of the Rhabdoviridae family (see Chapter 18: Rhabdoviridae), and the following description of rhabdovirus replication is based on the replication cycle of this virus (Fig. 2.9). Rhabdoviruses are enveloped viruses that have a distinctive bullet-shaped morphology. The rhabdovirus genome consists of a single strand of negative sense RNA. Unlike the genomic RNA of picornaviruses, the genomic RNA of rhabdoviruses is not naked, but instead exists as a nucleocapsid consisting of an RNA complexed throughout its length with repeating copies of the nucleocapsid (N) protein (1 N protein:9 nt of RNA). Infection of a host cell is initiated by attachment of the virus glycoproteins (G) to receptors expressed on the plasma membrane, and cell entry via receptor-mediated endocytosis. Decreasing pH within the endosomal vesicle induces conformational changes in the G proteins, which in turn mediate fusion of the viral envelope with the endosomal membrane. Membrane fusion results in the release of the helical nucleocapsid into the cytoplasm.

In contrast to picornaviruses, the genomic RNA of rhabdoviruses cannot serve as a template for protein synthesis. Consequently, the first biosynthetic process initiated following release of the nucleocapsid is transcription of the genomic RNA into translatable mRNAs. Positive strand RNA viruses such as the picornaviruses do not package their RdRp enzyme as a structural component of the virus particle as their genome can be readily translated to produce the enzyme components soon after entry into the cytoplasm. In contrast, negative strand RNA viruses such as the rhabdoviruses do package their RdRp enzyme within the virus particle because the synthesis of viral proteins cannot proceed until the viral genome has been transcribed into mRNAs, and no host cell enzyme capable of performing this function is available in the cytoplasm.

The rhabdovirus RdRp enzyme is a multisubunit complex consisting of the large (L) protein, which possesses the catalytic activity of the complex, and the phosphoprotein (P) which functions as an essential, but noncatalytic cofactor. The RdRp complex enters the cytoplasm as a component of the nucleocapsid. The genetic organization of the genomic RNA is highly conserved among the different rhabdoviruses. The 3'-terminal sequences encode for a short nontranslated RNA ("leader"), followed by the coding sequences for 5 genes in the order of N, P, matrix (M), G, and L, and concludes with the 5'-terminal sequences that encode a short, nontranslated "trailer" RNA. Each of these sequences is separated from adjacent sequences by a short, highly conserved intergenic region that plays an important role in transcription as explained below. The ability of the RdRp enzyme to utilize the
genomic RNA as a template for transcription or replication is dependent upon two critical parameters. First, the RdRp complex can only access the genomic RNA via the highly conserved 3'-terminal sequences, thus the transcription and replication processes only initiate at this site. Second, the RdRp can only access and utilize viral RNA that is complexed with N protein; naked RNA cannot serve as a functional template for any viral process mediated by the RdRp.

Transcription of the viral nucleocapsid results in the synthesis of a series of capped, polyadenylated monocistronic mRNAs, which is achieved as follows. Transcription is initiated at the 3' end and continues until the RdRp enzyme enters the first intergenic region. Each intergenic region contains a sequence that signals the end of transcription of the upstream gene and a sequence that signals the start of transcription of the downstream gene. After the RdRp encounters the first intergenic region it stops transcribing and releases the short leader RNA. The RdRp then scans to the next transcription start signal and begins transcription of the first gene (N gene). In addition to functioning as an RdRp, the L protein has capping activity and will synthesize a methylated 5' cap structure on the nacent mRNA. When the RdRp encounters the next intergenic region it will pause over a short poly-uridine tract and through a process involving iterative slippage, will use these residues repetitively to synthesize a long poly-adenosine sequence before releasing the mRNA (now containing both a methylated 5' cap and a poly A tail).

This process will be repeated until individual mRNAs representing each viral gene have been transcribed. Reinitiation of transcription following release of an mRNA is an error prone process and some RdRp complexes detach from the template before successfully reinitiating transcription of the downstream gene. RdRp complexes that do detach from the template are unable to re-access the template at an internal site and must reinitiate transcription at the 3' end of negative strand. This situation results in transcriptional attenuation, in which the gene nearest the 3' end of the genome (N gene) is transcribed at the highest level, and transcription of downstream genes decreases progressively with transcription of the L gene (5' terminal gene) occurring at the lowest level.

Replication of the viral genome requires a full-length positive sense RNA that can serve as template for synthesis of the negative sense genomic RNA. Each of the viral mRNAs is of subgenomic length, thus none of these RNAs can serve this purpose. As protein synthesis progresses, a full-length plus strand (antigenome) of viral RNA is produced and this RNA

is used in the process of genome replication. The switch from transcription of monocistronic mRNAs to synthesis of genome-length positive strands appears to occur once the cytoplasmic concentration of N protein reaches a critical threshold level. Viral mRNAs are devoid of protein, but the fulllength plus and minus strand RNAs are bound throughout their length by repeating copies of N protein. The N protein is an RNA binding protein and is maintained in a soluble, RNA-free form through an association with dimers of the P protein. Once a sufficient level of N protein is achieved, N protein is transferred from the soluble N/P2 complexes onto the nascent leader RNAs as soon as they are synthesized by the RdRp.

Additional N proteins will continue to bind to the RNA as it is synthesized. The presence of N protein on the nascent RNA has a profound effect on RdRp function which under these conditions is unaffected by the regulatory signals of the intergenic regions and continues to synthesize a full-length positive strand. The full-length positive strand RNA (in complex with N protein) in turn serves as the template for synthesis of full-length negative strand RNAs. The newly synthesized negative strand RNAs can serve as templates for more mRNA (secondary transcription), as templates for replication, or as genomes for incorporation into progeny virions.

Maturation of rhabdoviruses occurs by budding of newly forming virions through the plasma membrane of the host cell. This process requires specific interactions between components of the three major structural elements of the virus particle; specifically, the G proteincontaining envelope, the matrix, and the nucleocapsid. Budding of virus particles occurs through regions of the plasma membrane that contain a high concentration of G protein. The G proteins are synthesized in association with the rough endoplasmic reticulum and are transported to the plasma membrane through the exocytotic pathway where they become concentrated in so-called membrane microdomains. The M protein is initially synthesized as a soluble monomer but as the infection proceeds many copies of the M protein localize to the cytoplasmic side of the plasma membrane where they too assemble into M-rich membrane microdomains. Nucleocapsids interact with the M proteins in the microdomains through noncovalent interactions between N and M proteins. In the case of vesicular stomatitis virus, the M protein appears to be primarily responsible for driving the actual budding process, but this is thought to be enhanced by interactions between M proteins and the cytoplasmic portion of the G proteins.

Though not detailed here, the budding process also requires functions provided by cellular proteins. In simple terms, virion budding involves the association of the internal components of the virus (nucleocapsid and matrix) with the G-rich membrane microdomains, evagination of the plasma membrane at these sites, and eventual membrane scission. Rhabdoviruses produce RNA molecules that are functional ligands for several different cellular pattern recognition receptors [PRRs, eg, retinoic acid-inducible gene 1 (RIG-I), melanoma differentiation-associated protein 5 (MDA5), and toll-like receptor 7 (TLR7)], and their recognition can stimulate a type I interferon response by the host cell (see Chapter 4: Antiviral Immunity and Virus Vaccines). For example, the leader RNA that is produced during the transcription process and the full-length genome and antigenome RNAs possess a 5’ triphosphate, and these uncapped RNAs serve as ligands

for RIG-I. In addition, viral RNA of positive or negative sense can be bound by TLR7 following delivery of viral products to the endosome as occurs during autophagy.

Rhabdoviruses are sensitive to the antiviral effects of type I interferons; however, like the picornaviruses, rhabdoviruses have evolved strategies for inhibiting this innate antiviral defense system of the host cell. Inhibition of the interferon system by vesicular stomatitis virus is mediated by the M protein which limits the synthesis of type I interferon and the products of interferon stimulated genes (ISGs) by globally suppressing the transcription of host cell genes and by inhibiting the export of cellular mRNAs out of the nucleus. Rabies virus has evolved an alternative strategy for interfering with the interferon response that is mediated by the P protein. The P protein interferes with the RIG-I signaling pathway which prevents activation of the type I interferon genes. In addition, rabies virus P protein inhibits the nuclear localization of phosphorylated STAT 1 and STAT 2 proteins, which limits activation of ISGs and subsequent establishment of the antiviral state [by proteins such as protein kinase R (PKR) and 2'5' oligoadenylate synthetase (OAS) as described in detail in Chapter 4, Antiviral Immunity and Virus Vaccines].

Retroviruses

The Retroviridae family includes pathogens of both humans and animals (see Chapter 14: Retroviridae). The retrovirus particle is enveloped and contains envelopeassociated glycoprotein spikes. The spike is a multiprotein structure that consists of transmembrane (TM) subunits
and surface (SU) subunits. The TM and SU subunits associate with one another to form heterodimers. Three identical TM/SU heterodimers then assemble to form the functional trimetric spike. The interior structures of the virion include a matrix that underlies the envelope and is constructed from repeating copies of the matrix protein (MA), a capsid that is constructed from repeating copies of the capsid protein (CA), and two RNA-based nucleocapsids. The RNA components of the nucleocapsids are identical and consist of single stranded, positive sense RNA (retroviruses are diploid for every virus gene). Each RNA is capped at its 5' end, contains a poly A tail and is complexed throughout its length by multiple copies of the nucleocapsid protein (NC). Although retroviruses are technically positive strand RNA viruses, their replication cycle is markedly different from that of other positive
strand RNA viruses such as the picornaviruses that were discussed earlier. The following description of the retrovirus replication cycle, and depicted in Fig. 2.10, is based on that of a simple retrovirus, and some details will not apply to all members of the Retroviridae family.

Infection of a cell by a retrovirus begins with virus binding to receptors (and to coreceptors in some
instances) on the host cell. In general, receptor binding is mediated by the SU component of the spike. Most retroviruses appear to enter the host cell at the plasma membrane and no change in pH is required to initiate or complete this process. However, some retroviruses appear to enter host cells via receptor-mediated endocytosis in a manner similar to that described for the rhabdoviruses.
For retroviruses that enter the cell at the plasma membrane, receptor binding stimulates conformational changes in the SU subunit, which in turn induce conformational changes in

the TM subunit that then mediates fusion between the virus envelope and the plasma membrane.

Membrane fusion causes the loss of the envelope, disassembly of the matrix, and release of the virus core. The core consists of the capsid, the nucleocapsids and two viral enzymes [reverse transcriptase (RT) and integrase (IN)] that are required early in the infection process. Although the details of the next step in the infection process are not entirely understood, evidence suggests that the core undergoes structural changes, probably mediated by cellular proteins, and these structural changes
are required to initiate the process of reverse transcription by the core-associated RT enzyme. RT is a multifunctional enzyme that possesses RNA-dependent DNA polymerase (RdDp) activity. RT is a primer-dependent polymerase and uses the viral single stranded RNA as a template to synthesize a linear, complementary double stranded DNA (cDNA) product. The primer used to initiate the synthesis of DNA is a cell-derived tRNA that is base paired to a primer binding site on the RNA. This tRNA was acquired from the cell that generated the virus particle and it enters the newly infected cell already
bound to the viral RNA. The molecular details of the reverse transcription process are not presented here, but three important outcomes of the process should be noted.

First, the viral RNA is degraded in the process, thus the viral gene segments, which are fully capable of functioning as mRNA, are never translated into proteins. Second, the process causes the duplication and transposition of specific viral sequences which together result in the formation of repeated sequences at the termini of the cDNA. These direct repeats are referred to as the long terminal repeats (LTRs) and they perform important replicationrelated functions as described below. Third, upon completion of the reverse transcription process the cDNA product exists as a component of a nucleoprotein complex called the preintegration complex (PIC). The complex also contains the virus protein (eg, the IN enzyme) and cellular proteins that are now poised to mediate the integration of the cDNA into a chromosome of the host cell if access to cellular DNA can be achieved.

Most retroviruses are not able to transport the PIC into the nucleus, and therefore, these viruses can only integrate their cDNA into a chromosome of an actively dividing cell as cell division involves the temporary dissolution of the nuclear membrane. Retroviruses belonging to the genera Lentivirus and Spumavirus have evolved mechanisms for transporting their PIC into the nucleus that make it
possible for these viruses to integrate their cDNA into chromosomes of nondividing cells. The integration reaction is initiated by IN which cleaves host cell DNA at the site selected for integration, and the process is dependent on interactions between IN and the LTR sequences of the
cDNA. The final steps of the integration process are performed by cell-derived DNA repair enzymes. Integration of the cDNA into a host cell chromosome is essentially random and does not require specific host DNA sequences, but integration generally occurs within regions of a chromosome that are transcriptionally active, and consequently, more readily accessible. The integrated
viral cDNA is referred to as the provirus, and establishment of the provirus must be achieved before the expression of viral genes can occur.

The DNA sequences that control transcription of the viral genes are located within the LTRs. These sequences are similar to those that regulate the expression of cellular genes (eg, TATA box, binding sites for cellular transcription factors, etc.). Therefore, the LTR sequences are accessible to the transcription machinery of the host cell and viral transcripts are synthesized by cellular RNA pol
II, capped by cellular capping enzymes, and polyadenylated by the cellular poly A polymerase enzyme. Transcription is initiated within the LTR that is positioned upstream of the viral genes and continues through the entire provirus sequence. A polyadenylation signal encoded by the downstream LTR is utilized for the addition of a poly A tail. Due to their sequence similarity, both LTRs are capable of initiating transcription; however, transcription activity initiated by the upstream LTR
typically interferes with the initiation of transcription from the downstream LTR. This phenomenon, which is referred to as promoter occlusion, normally prevents the downstream LTR from initiating transcription of downstream cellular sequences.

The single viral mRNA contains the sequence of all viral genes in the order of gag (encoding MA, CA, NC proteins, and the protease (PR) enzyme), pol (encoding the RT and IN enzymes), and env (encoding the glycoprotein precursor of SU and TM). In some retroviruses, the PR enzyme in
encoded in the pol region. Depending on the particular retrovirus, the open reading frames that encode Gag, Pol and Env can be in frame with one another or out of frame, and the open reading frames that encode Gag and Pol can be continuous or overlapping. The capped and polyadenylated
transcripts produced by the simple retroviruses experience one of two alternative fates. If the transcript is exported from the nucleus without being spliced it will serve as a transcript for the synthesis of the Gag polyprotein (encoding only MANCCAPR) and/or a larger GagPol polyprotein (encoding MANCCAPRRTIN). The Gag open reading frame terminates with a stop codon; and therefore, the majority of ribosomes that translate the unspliced transcript will only synthesize the Gag polyprotein.

However, a small percentage of translating ribosomes will synthesize the larger GagPol polyprotein using one of two mechanisms. If the Gag and Pol open reading frames are continuous and in frame then the GagPol polyprotein can be produced if the translating ribosome reads through the Gag stop codon. This process, in which the ribosome treats the stop codon as a sense codon, is referred to as stop codon suppression. If the Gag and Pol open reading frames are overlapping and out of frame, then the GagPol polyprotein can be produced if the translating ribosome shifts from its original reading frame (the Gag reading frame) into the Pol reading frame. The ribosomal frame shift is facilitated
by sequences within gag and occurs just upstream of the Pol open reading frame. Typically, the Gag and GagPol polyproteins are not resolved into their individual protein components within the cell, but instead only undergo proteolytic processing after they have been incorporated into progeny virions during the virus assembly process. This process will be described in more detail below.

Alternatively, the transcript can be spliced prior to being exported to the cytoplasm. Splicing removes an intron that includes the sequences encoding Gag and Pol; and

therefore, spliced transcripts only retain the Env open reading frame and can only be translated into the Env glycoprotein that serves as the precursor to SU and TM. Translation of this transcript occurs in association with the endoplasmic reticulum and the Env glycoproteins are processed and routed to the cell surface using the endoplasmic reticulum/Golgi apparatus protein export system. The Env glycoprotein is cleaved into its SU and TM components by the host cell enzymes called furin (or by a furin-like enzyme) during its transits through the trans-Golgi or after its arrival at the cell surface. Splicing of viral transcripts is not unique to the retroviruses. For example, splicing of viral transcripts occurs during the replication of influenza A virus (Family Orthomyxoviridae), Borna disease virus (Family Bornaviridae), and of most DNA viruses.

In addition to functioning as mRNA for the production of the Gag and GagPol polyproteins, the unspliced transcript can also be incorporated into progeny virions as genomic RNA. These full-length RNAs possess a packaging signal located within the Gag sequence. Packaging signals of two RNAs interact with one another, facilitating the formation of RNA:RNA dimers. The spliced viral mRNAs lack this sequence and are unable to participate in dimer formation. Similar to the rhabdoviruses, formation of the retrovirus particle generally requires specific and coordinated interactions between components of the three major structural elements of the virus particle. With respect to retroviruses these structural elements include the spike-modified membrane microdomains, the Gag and GagPol polyproteins, and the dimeric RNAs. Key interactions responsible for virion assembly and budding include those that take place between the MA-component of the polyproteins and the cytoplasmic tails of TM (and with the membrane), and those that take place between the NC-component of the polyproteins and the dimeric RNA.

These interactions help to drive the budding process by which immature virions are formed. The newly budded immature virions contain the Gag and GagPol polyproteins and lack defined internal structures such as a matrix, capsid, or nucleocapsids. To this point in the process, the PR enzyme has been inactive; however, soon after formation of the immature virion, the PR enzyme is activated and proceeds to process the Gag and GagPol polyproteins into their individual constituent proteins. Once released, these proteins then assemble into the matrix, capsid, and nucleocapsid structures that are characteristic of the mature, infectious virus particle. Proteolytic processing of the GagPol polyproteins also releases RT and IN which are now available to perform the early replication events required to infect the next cell.

Adenoviruses

Adenoviruses belong to the family Adenoviridae (see Chapter 10: Adenoviridae). Unlike picornaviruses, rhabdoviruses and retroviruses, the adenovirus genome consists of DNA. The adenovirus particle is nonenveloped and consists of an icosahedral capsid that is constructed from hexon (trimers of protein II) and penton (pentamers of protein III) subunits. Prominent structures called fibers (trimers of protein IV) are associated with the penton subunits and project outward from each of the icosahedron’s 12 vertices. The adenovirus particle also has numerous proteins located internally; some of which are in contact with

the penton and hexon subunits, and others that are associated with the DNA genome. The genomic DNA consists of 3036 kbp of linear dsDNA, contains terminal inverted repeat sequences that play an important role it the DNA replication process, and is covalently bound to a virus-encoded protein (terminal protein) at each 5' end.

The following description of the adenovirus replication cycle, and the representation of the process that is depicted in Fig. 2.11, is based on that of human adenovirus 2. The initial interaction of adenovirus with a host cell is mediated by the fibers that bind to a host cell protein called the coxsackievirus and adenovirus receptor (CAR). High-affinity binding between the fiber and this receptor allows the penton base proteins to make contact with cellular integrins, whose normal function is to bind the host cell to components of the extracellular matrix. This binding initiates the process of clathrin-mediated entry with subsequent internalization of the virion into clathrincoated pits, and initiates the first steps of virion uncoating. The interactions that occur between the fibers and CARs and between pentons and integrins, and perhaps other factors that are not yet adequately characterized, induce substantial changes in the capsid structure. These changes include the shedding of the fibers and externalization of a lytic factor (protein VI) from the virion interior into the endosome lumen. Protein VI mediates disruption/fragmentation of the endosomal membrane which allows the modified capsid to enter the cytoplasm.

After release from the endosome the virions associate with the molecular motor dynein which then transports them along microtubules to a nuclear pore. At the nuclear pore the capsid establishes interactions with a number of host cell proteins, including the nuclear pore filament protein Nup214, kinesin-1, and histones, which further destabilize the virion structure and result in the release of the viral DNA into the nucleus. Gene expression programs of most DNA viruses are temporally regulated with specific genes being expressed at different times. The expression of adenovirus genes occurs in three phases, which are referred to as immediate early, early, and late. The adenovirus genes are arranged in sets called transcription units. Each transcription unit is controlled by a single promoter that is used by the transcriptional machinery of the host cell, and polyadenylation signals that define the 3' ends of the viral transcripts.

Each transcription unit directs the synthesis of a single primary RNA; however, alternative splicing yields a population of mRNAs that encode multiple different proteins. The first transcription unit to become transcriptionally active is E1A which encodes the immediate early proteins. The E1A primary transcripts are alternatively spliced to form transcripts that encode a family of E1A proteins. The major E1A proteins (289R and 243R) perform several critical functions that are required during the initial stages of the infection. First the E1A proteins interfere with the type I interferon response as will be discussed below. Second, they induce the host cell to enter the S phase of the cell cycle by directly interacting with the retinoblastoma (Rb) tumor suppressor protein.

Adenoviruses typically infect terminally differentiated cells that are not actively dividing.

By inducing the host cell to enter the S phase the virus creates a cellular environment that is more conducive to replication of the viral DNA. The major E1A proteins also activate the transcription units for the early genes, as well as activate some cellular promoters. Collectively, the proteins expressed from the early genes perform three major functions; including the inhibition of apoptosis, replication of viral DNA, and inhibition of host immune defenses. Two proteins expressed from the E1B transcription unit (E1B-19K and E1B-55K) inhibit apoptosis, which is a normal cellular response to unscheduled entry into the S phase (as induced by E1A) and to cellular stress induced by virus infection. The E1B-19K protein is a homolog of the antiapoptotic cellular protein Bcl-2.

Like Bcl-2, E1B-19K binds to the pro-apoptotic protein Bax and inhibits its ability to mediate mitochondrial release of cytochrome C, which is a potent inducer of the intrinsic apoptosis pathway (see Chapter 3: Pathogenesis of Viral Infections and Diseases). The E1B-55K protein induces the rapid turnover of the tumor suppressor protein called p53 which becomes stabilized in the infected cell as a consequence of E1Amediated inactivation of Rb. Under normal conditions, stabilized p53 activates transcription of cellular genes that cause cell cycle arrest (eg, p21) and of genes such as Bax, which promote apoptosis.

Three proteins expressed from the E2 transcription unit cooperate to replicate the viral DNA. The precise details of the genome replication process will not be addressed here, but the general functions of these three proteins will be described. One of these proteins is the DNA polymerase that catalyzes the replication of the DNA. The second protein is the preterminal protein (Pre-TP) which serves as a primer for DNA replication in much the same way as VPg served as a protein primer for replication of the picornavirus RNA genome, except that no cis-acting replication-like element is required. At the conclusion of the DNA replication process a Pre-TP remains covalently attached to each 5’ end of the DNA. Later in the replication process as DNA genomes are incorporated into newly assembling virions, Pre-TP is cleaved by a virus-encoded protease into a smaller form called the terminal protein (TP). The third E2 protein is the DNA binding protein (DBP) which binds to the single stranded DNA that is displaced from the dsDNA template during the replication process. After being displaced and bound throughout its length by DBP, the ssDNA serves as a template for the synthesis of a genome-length dsDNA.

The final transcription unit to become active is that which controls expression of the late genes. The late genes encode the major structural proteins of the virus and nonstructural proteins that function in the virus assembly process. Late gene expression does not begin until after the onset of DNA replication and it is enhanced by a virus-encoded protein called IVa2. Transcription of the late genes is controlled by the major late promoter which defines the 5’ end of all late mRNAs. The 3’ end of late transcripts is determined by any one of 5 different polyadenylation signals that are present within the primary transcript. The use of alternative polyadenylation signals leads to the production of a nested set of five different transcripts, some of which retain one or more internal polyadenylation sites. The polyadenylated transcripts are then alternatively

spliced into multiple unique transcripts, each of which is then translated into a different late protein. The late proteins are then transported to the nucleus where they participate in the virion assembly process. Unlike the picornaviruses which possess a simple icosahedral structure, the adenovirus virion is a large and complex structure. Simple self-assembly models cannot account for this degree of complexity. Accordingly, viral proteins have been identified that act as chaperones for moving structural proteins to maturation sites and others
that act as scaffolds for assembling the virion subunits. A virus-encoded protease that requires DNA as a cofactor to prevent premature proteolysis participates in the maturation process by degrading scaffold proteins and cleaving precursor proteins.

Late in infection, inclusion bodies composed of large crystalline arrays of newly assembled virions appear in the nucleus of the host cell. Release of progeny virions occurs following lysis of the host cell.
As with other viruses, adenovirus infections are detected by microbial pattern recognition receptors
(PRRs) of the host cell and infection initiates a type I interferon response (see Chapter 4: Antiviral Immunity and Virus Vaccines). However, adenoviruses actively limit the effectiveness of the response in several ways.

First, the E1A proteins inhibit the activity of a cellular protein complex (hBRe1) that preferentially activates transcription of interferon stimulated genes (ISGs). Consequently, this activity of E1A greatly reduces the intracellular levels of the effector proteins that normally contribute to the interferon-induced antiviral state. Adenoviruses also express high levels of noncoding virusassociated RNAs that inhibit the activity of PKR, an ISG product that inhibits virus replication by globally suppressing protein synthesis within the cell. The majority of the virus-associated RNA exists in dsRNA form due to extensive intramolecular sequence complementarity. These virus-associated RNAs bind to the dsRNA binding site of inactive PKR but this interaction does not activate the enzyme, and PKR that has been bound by virusassociated RNA is unable to bind other dsRNAs and, therefore, remains in an inactive form. Late in infection, adenoviruses selectively inhibit the synthesis of host-cell proteins by preventing the export of cellular mRNAs from the nucleus and by blocking translation of host-cell mRNAs through modifications of key translation initiation factors. The late mRNAs encoded by the virus are
exempt from these effects due to a unique sequence called the tri-partite leader that is present at the 5’ end of all adenovirus late mRNAs.

Assembly and Release Near the end of the replication cycle the newly synthesized structural proteins and genomic molecules are assembled in a step-wise manner into new virus particles. Depending on the virus, the release of newly assembled virus particles from the host cell occurs as a separate step
following the assembly process, or occurs concurrently with the assembly process. Similar to other aspects of the replication cycle, details of the virus assembly and release processes differ significantly between viruses. Based on the obvious structural differences between viruses that possess an envelope and those that lack an envelope, and on the profound effect that envelope acquisition has upon the assembly and release processes, this section

of the chapter will discuss the assembly and release of nonenveloped viruses and enveloped viruses separately.

The external capsid structure of virtually all nonenveloped animal viruses consists of an icosahedron, and these exist in variable levels of complexity. For the structurally simple icosashedral viruses such as those belonging to the Parvoviridae, Polyomaviridae, Papillomaviridae, and Picornaviridae families, the structural proteins spontaneously associate into the repeating structural subunit of the capsid called the protomer. A defined number of protomers are then used to assemble the mature icosahedral capsid. The protomers of some nonenveloped viruses are able to assemble into capsids in the absence of the genomic molecule (eg, canine parvovirus and human papilloma virus); for other viruses the genome appears to serve as the nucleation site for protomer assembly and capsids only form in the presence of a genomic molecule (eg, SV40, Polyomaviridae). The structure of some icosahedral viruses is more complex and does not consist of just a single type of repeating subunit. For example, the external structure of the adenovirus particle is assembled from individual hexamer subunits, pentamer subunits and trimeric fibers.

Assembly of the adenovirus particle requires chaperone proteins that facilitate the proper assembly and folding of other structural components of the virus particle, and scaffold proteins that serve as temporary components of intermediate structures that are generated during the assembly process. The proteins that serve as chaperones and/or scaffolds can be displaced during the assembly process and may not remain as a structural component of the mature virion. In addition, cleavage of some virion-associated proteins is required to convert the intermediate structures into the mature, infectious form of the virus particle. Nonenveloped viruses that contain an RNA genome replicate and complete their assembly process in the cytoplasm of the host cell. Nonenveloped viruses that contain a DNA genome typically replicate and complete their assembly process in the nucleus. Most nonenveloped viruses are released only when the cell lyses, thus for these viruses the processes of virus assembly and virus release are separate and sequential events.

Enveloped viruses acquire their envelope as the internal structures of the virus (eg, nucleocapsid(s) and matrix components) bud through a cellular membrane. Depending on the virus, budding can occur at the plasma membrane, through the membranes of the endoplasmic reticulum or Golgi apparatus, or through the inner membrane of the nucleus. For most enveloped viruses budding occurs at regions of the membrane in which cellular glycoproteins have for the most part been displaced by the glycoproteins of the virus (Fig. 2.12). This ensures that the viral glycoproteins are incorporated into the virion during the budding process. However, displacement of host cell glycoproteins is not an absolute requirement and some viruses (eg, human immunodeficiency and vesicular stomatitis viruses) readily incorporate host cell glycoproteins into virus particles during budding. The viral glycoproteins typically associate into oligomers (usually homotrimers or homotetramers) to form the spike (peplomer) structures.

Viral glycoproteins typically consist of a hydrophilic domain projecting outward from the

membrane, a hydrophobic transmembrane domain, and a short hydrophilic domain projecting into the cytoplasm (or virion interior). In general, the icosahedral nucleocapsids (eg, togviruses and flaviviruses) and helical nucleocapsids (eg, orthomyxoviruses and rhabdoviruses) of an enveloped virus assemble prior to budding. These preformed nucleocapsids then localize to the appropriate cellular membrane and participate in the budding process. In the relatively rare case of an enveloped virus with icosahedral symmetry (eg, togaviruses), each nucleocapsid protein (C protein) interacts directly with the cytoplasmic domain of a single membrane glycoprotein (E2), and these interactions help drive the budding process. For viruses that possess helical nucleocapsids, the budding process tends to be
driven by interactions between the matrix proteins and the cell membrane and/or the surface glycoproteins, and between matrix proteins and the proteins of the nucleocapsid. For some viruses, the energy and forces that are required to induce curvature of the membrane and eventual membrane scission is provided by protein:protein and protein:lipid interactions mediated by virus constituents
alone. However, many enveloped viruses utilize proteins of the cellular endosomal sorting complexes required for transport (ESCRT) system to assist in the budding process. One of the normal functions of the ESCRT proteins is to catalyze the budding of membrane-bound vesicles into the endosome to form multivesicular bodies.
This process is physiologically similar to the budding of a virus with respect to the process being initiated on the cytoplasmic side of a membrane and the product of membrane scission (a vesicle or a virion) being formed on the extracytoplasmic side of the membrane. Depending on which proteins of the ESCRT system are involved, these proteins assist in the virus budding process itself, and/or
in the final step of membrane scission which is required to release and fully envelop the virus. Viruses that acquire their envelope from an internal membrane enter the secretory pathway of the cell and are transported in exocytotic vesicles to the cell surface where they are released upon fusion of the vesicle with the plasma membrane (exocytosis) (Fig. 2.12). For these viruses the processes of virus
assembly and virus release are separable and occur in sequence. Viruses that bud through the plasma membrane are released directly into the extracellular environment, thus, for these viruses the processes of virus assembly and release occur simultaneously and are essentially inseparable.

Many glycoproteins encoded by enveloped viruses are synthesized as precursor proteins than are subsequently processed by site-specific proteolysis before or after being incorporated into the mature virion. This is particularly common for glycoproteins that mediate the process of membrane fusion during cell entry. Cleavage of the precursor is most commonly performed by the cellular enzyme called furin (or a furin-like protease), which is an ubiquitously expressed endoprotease that resides in the
trans-Golgi compartment and at the cell surface. Furin cleaves its substrates on the carboxyl side of a
BXBB sequence motif (B represents Arg or Lys and X represents a nonspecified residue). The precursor of the hemagglutinin glycoprotein of influenza A virus (HA0) is not cleaved by furin, but instead is cleaved into the functional subunits of the HA spike (HA1 and HA2) after the newly budded virion has been released from the host cell.

HA0 is cleaved by trypsin-like enzymes present in secretions of the respiratory tract of humans and the gastrointestinal tract of the avian host. This finding was a major discovery in the early 1970s that allowed the routine propagation of influenza virus viruses in cell culture (in which trypsin is added to the growth medium). In general, cleavage of the precursor converts a protein that is not functional for membrane fusion, into its fusion-competent form. Cleavage of the precursor primes the virus to perform the entry and uncoating processes in response to proper stimuli (as discussed earlier), and is generally required to produce an infectious virus particle. Influenza virus also depends on a second enzymatic activity in order to be efficiently released from the host cell and to prevent aggregation of virus particles. The HA spike of influenza A virus binds sialic acid and uses this carbohydrate moiety as a receptor for attaching to host cells. However, proteins that comprise the HA and neuraminidase (NA) spikes also possess sialic acid, thus newly released virions are inclined to bind to the host cell from which they budded, and to neighboring virions. The enzymatic activity of the NA spike inhibits these nonproductive binding events by cleaving sialic acid from the cell surface and from the virion itself. The drug called oseltamivir (Tamiflu) inhibits the enzymatic activity of NA, which causes virion aggregation and restricts cell to cell spread.

It should be noted that for most viruses (eg, togaviruses, herpesviruses, and retroviruses) incorporation of genomic molecules into the capsid or nucleocapsid structure is highly selective and is dependent of the presence of a highly conserved sequence called a packaging signal that is only present in the appropriate genomic RNA or DNA molecules. In contrast, other viruses (eg, rhabdoviruses and parvoviruses) are not highly selective with respect to the nucleic acid that is packaged and viral nucleic acids of both polarity (genomic and antigenomic) are packaged into virions.
Unlike most other cell types in the body, epithelial cells display polarity, which means that they possess an apical surface that interfaces with the external environment (eg, lumen of respiratory tract or gastrointestinal tract) and a basolateral surface that interfaces with underlying cells. These surfaces are chemically and physiologically distinct. Viruses that are shed to the exterior (eg, influenza A virus) tend to bud from the apical plasma membrane, whereas other viruses (eg, C-type retroviruses)
bud through the basolateral membrane, which may enable the virus to enter the bloodstream or lymphatic system as a prelude to establishing systemic infection (Fig. 2.13).

QUANTITATIVE ASSAYS OF VIRUSES

The study of basic virus processes and virus-based diseases often requires the researcher or clinician to know how much virus exists in a given sample. The reproducibility of both in vitro and in vivo experiments depends upon using a consistent amount of virus to initiate an infection. In assessing clinical cases, it may be important to determine the quantity of virus in various tissues or fluids as a part of the determination of pathogenicity and to select the correct specimens for diagnostic testing. A
common metric used to assess the effectiveness of antiviral drugs is to compare the viral load (or “burden”) in clinical specimens before and after drug treatment. The answer to the question as to how much virus is present in an individual sample or specimen may not be simple, and is test dependent. There are two general types of viral quantification tests; specifically, biological assays and physical

assays. Quantifying virus in a single sample using different assays will often yield different answers, and it is essential to understand the reasons for these differences. Physical assays that do not depend on any biological activity of the virus particle include electron microscopic particle counts,
hemagglutination, immunological assays such as antigencapture enzyme-linked immunosorbent assay (ELISA) tests and, most recently, quantitative PCR assays.

Biological assays that depend on a virus particle initiating a successful replication cycle include plaque assays and various endpoint titration methods. The difference between the amount of virus detected
using a physical assay such as particle counting by electron microscopy and a biological assay such as a plaque assay is often referred to as the particle to pfu ratio. In virtually all instances, the number of physical particles exceeds the number determined in a biological assay. For some viruses this ratio may be as high as 10,000:1, with ratios of 100:1 being common (Table 2.2). The reasons for the higher number of physical particles as compared with infectious particles are virus dependent and include: (1) the assembly process is inefficient and error prone, and morphologically complete particles can be formed without the correct nucleic acid component; (2) not all virions that bind a receptor or initiate the entry and uncoating processes are successful in establishing a productive infection; (3) the replication process is highly error prone (RNA viruses), and virus stocks can contain particles with lethal mutations; (4) virus stocks are produced or maintained under suboptimum conditions such that infectious particles are inactivated; (5) tests for infectivity are performed in animals or cells that are not optimum for detecting infectious particles; (6) host cell
defenses prevent some infectious particles from successfully completing the replication process. The choice of host or host cell for the biological assays is a critical determinant for defining the amount of infectious virus in a sample. It is not unusual for assays in the natural host animal to provide the highest estimates of infectious units, as available cell cultures may be a poor substitute for the
target cells in the animal.

Physical Assays

Direct Particle Counts by Electron Microscopy The most direct method to determine the concentration of virus particles in a sample is to visually count the particles using an electron microscope. This process is not performed routinely because it requires expensive equipment and highly trained technicians. In this assay the virus sample is first mixed with a sample of standard particles
(eg, latex beads) of known concentration. The virus/standard particle mixture is then observed using an electron microscope, and the numbers of virus particles and standard particles are counted separately. The number of virus particles counted is easily converted into a concentration
(eg, virus particles/mL) by multiplying the ratio of the virus particle count/standard particle count by the known concentration of the standard particles. This procedure is most accurate for nonenveloped viruses that produce highly stable virus particles with unique geometric shapes such as picornaviruses, reoviruses, and adenoviruses. This process cannot assess biological activity of the preparation, but it can be used to assess whether the

particles contain nucleic acid, as visual observation can be used to differentiate empty capsids from complete particles.

Hemagglutination

As mentioned earlier in this chapter, some virus-infected cells acquire the ability to bind red blood cells on their surface (hemadsorption) due to interactions between surface-expressed viral proteins and ligands on the red blood cell. The free virus particles of some viruses are also able to bind to red blood cells, and when mixed together will cause the cells to aggregate into a lattice of cross-linked cells. This property is called hemagglutination, and can be used as the basis for quantifying viruses that possess this activity (eg, influenza A virus). The hemagglutination assay cannot accurately determine the number of virus particles present in a sample (ie, virus particles/mL); but it is useful for comparing the relative concentrations of a virus between samples, such as those obtained from multiple infected hosts, or those collected sequentially from an individual host on different days or times. To perform this assay the virus-containing sample is first processed in a serial twofold dilution series (typically in a 96-well microtiter plate). A solution containing red blood cells is then added to each sample well. After a defined period of time the wells are observed visually for the presence of hemagglutinated red blood cells which appear as a thin continuous layer of cells covering the bottom surface of the well. Nonagglutinated red blood cells settle into a small "button" of cells in the center of the well. The "HA titer" of the stock virus sample is reported as the inverse of the highest dilution that completely agglutinates the red blood cells (Fig. 2.14).

Quantitative Polymerase Chain Reaction Assays

With the development of real-time (quantitative) PCR assays (see Chapter 5: Laboratory Diagnosis of Viral Infections), it is now possible to determine the concentration of a virus-specific nucleic acid in a test sample. PCR can detect nucleic acid sequences in virtually any context, not just in a virus particle. The increased sensitivity of PCR over virus isolation in many instances is achieved by detecting nonvirion nucleic acid in tissue samples. To use PCR correctly to quantify virus, it is necessary first to treat the suspension with nucleases to degrade all nonvirion nucleic acid. Virion-associated nucleic acid will be protected by the intact virus particle. With copy number controls being included in the assay as a basis for comparison, the concentration of the target nucleic acid in the treated sample can be determined. This type of assay does not detect empty capsids (those that do not contain viral nucleic acid), and it is not influenced by the infectivity of the preparation.

Biological Assays

Plaque Assays

Perhaps no other procedure in virology has contributed as much to the development of the field as the plaque assay. The plaque assay was originally developed by d'Herelle in 19151917, in his initial studies on bacteriophage. The assay is elegantly simple and is the most accurate of the quantitative biological assays. To perform a plaque assay with bacteriophage, the sample is first processed in a serial 10-fold dilution series in a bacterial culture medium. A suspension of host bacteria in a melted culture medium (top agar) is then added to each diluted sample.

This mixture is then poured onto a nutrient agar culture plate to distribute the bacteriophage/bacteria suspension evenly across the surface of the plate where it will rapidly cool and solidify. The dishes are placed into an incubator and over time the host bacteria divide and produce a visible “lawn” of bacteria over the surface of the agar plate. Bacteria that are infected with bacteriophage die and release progeny virions which in turn infect and kill neighboring bacteria. Eventually, enough bacterial cells are killed so that a clear area of cell-free agar is observed.

These clear areas are referred to as plaques. It is important to note that although an extremely high number of bacteriophage will be present in each plaque, the plaque originated from the infection of one bacterial cell by a single bacteriophage; and therefore, each plaque represents one bacteriophage present in the sample that was plated. No plaques should appear on uninfected control plates. If the original stock sample has a high concentration of bacteria, then the plates containing the low dilution samples will be completely or nearly completely cleared due to most or all of the bacteria being infected and killed. Plates used to assay the very highest dilution samples may have only a few plaques or none at all.

Somewhere between these extremes, plates will be identified that contain a number of plaques that can be accurately counted, and these plates will be used to determine the concentration (titer) of the bacteriophage in the original sample. This will be achieved by taking into account the sample dilution, volume tested, and the plaque count. In 1953, the bacteriophage plaque assay was modified for use with the newly developed tissue culture systems and animal viruses. This assay works best with cytopathic viruses that induce the lysis of their host cell. Although variations of the assay exist, in general, it is performed as follows. The stock virus sample is processed in a serial dilution series as described above. Liquid growth medium is removed from plates containing monolayers of cultured cells, and each diluted virus sample is then overlaid onto a monolayer of cells at a standard volume that minimally covers the cells. The plates are then incubated for a short period to allow the virus to bind and enter the cells, and then the cells are overlaid with nutrient agar. The nutrient agar overlay prevents newly produced progeny virions from spreading freely to distal regions of the plate, but it does not restrict the movement of these virions to immediately adjacent cells. Eventually, virus will spread from the original host cell to infect enough neighboring cells to form a focus of infected cells that can be visualized when stained with a vital dye (Fig. 2.15). Both immunofluorescent and immunohistochemical staining procedures have been developed for conducting plaque assays with noncytopathic viruses (Fig. 2.3).

In addition to its use to quantify the amount of virus in a sample, the plaque assay established a fundamental principle applicable to the vast majority of animal viruses; namely that a single virus particle was sufficient to establish a productive infection. This was proven by determining that the number of plaques in an assay increased in a linear fashion when plotted against the dilution factor, that is, the plaque number followed a one-hit kinetic curve. This is not the case for many

plant viruses, in which segmented genomes are incorporated into separate virus particles and a productive infection requires coinfection of a cell by multiple viruses. Plaque assays were also instrumental in early studies of viral genetics, as plaque variants either occurring naturally or induced chemically could be selected by isolating virus from individual plaques (biologically cloned) and studied to determine the
impact of the mutation on viral growth properties.

Endpoint Titration Assays

Before the development of the plaque assay for animal viruses, and for noncytopathic viruses that do not produce plaques, the quantification of virus stocks was achieved by inoculating virus into test animals or embryonated eggs. As with the plaque assay, these assays begin with the serial dilution of the sample or specimen. Each diluted sample is then inoculated into one or more test animals or
eggs. A successful infection could be scored directly, being inferred from the death of the animal or egg, or indirectly by confirming an immune response to the virus in the infected host. At low dilutions, all animals would become infected whereas, at high dilutions, none of the animals would become infected. At some intermediate dilution only some of the animals or eggs would show evidence of infection. Two methods were devised (ReedMuench and SpearmanKarber) to use the results
(ie, number of infected vs number of uninfected at each dilution tested) to calculate the dilution of the virus that would infect 50% of the test animals. In this case, the titer of the stock virus would be expressed as an infectious dose 50 (ID50) (Table 2.3).
If the virus causes the death of the animal or egg, then this assay can be used to determine its lethal dose 50 (LD50) or egg infectious dose 50 (EID50), respectively. Endpoint titration assays can also
be performed in cultured cells and in this version of the assay the titer of the virus is reported as the tissue culture infectious dose 50 (TCID50). Although not as accurate as plaque assays and not as amenable to statistical analysis, the TCID50 endpoint assay is easier to set up and automate than the plaque assay.

SPECIAL CASE OF DEFECTIVE INTERFERING (DI) PARTICLES

This chapter will conclude with a brief description of a special class of replicating virus particle referred to as a defective interfering (DI) particle. DI particles have been identified in most virus families. These “DI” particles are deemed defective because they cannot replicate autonomously, but instead require the presence of a helper virus to provide the function(s) that the defective particle lacks.
The helper virus is usually the homologous virus from which the defective particle was derived. As their name implies, DI particles interfere with the replication of the helper virus and usually decrease the yield of the helper virus in mixed infections. Defective particles are assembled from the same structural proteins as their nondefective parent virus; however, the genomes of DI particles
are defective and lack variable amounts of the normal genomic sequence. Although the genomes of defective particles are incomplete, they do retain the cis-acting sequences required for their replication and the sequences required for their encapsidation. DI particles derived from viruses with segmented genomes, such as influenza viruses and

reoviruses, tend to have genomes in which one or more gene segment have significant deletions.

Similarly, DI particles derived from viruses with a nonsegmented genome contain genomes with various degrees of deleted sequence. For example, DI particles of vesicular stomatitis virus may lack up to two-thirds of the normal genome. Morphologically, DI particles usually resemble the parental virions; however, with vesicular stomatitis virus, their normally bullet-shaped virions are
shorter than wild-type virions. In the jargon used to describe these particles, normal vesicular stomatitis virus virions are called B particles and the DI particles are called truncated or T particles.
The truncated genomes of DI particles are generated through aberrant replication and/or recombination events that lead variably to the mutation of gene sequences, sequence deletions, transpositions, duplications, and even the insertion of gene sequences derived from host
DNA or RNA. Once generated, numbers of defective particles increase greatly upon serial passage, particularly when infections are performed at a high multiplicity of infection. The rapid increase in defective particle formation under these conditions is thought to result from one or more of the following mechanisms: (1) their shortened genomes require less time to be replicated, thus over time the viral polymerase would replicate more defective genomes than full-length genomes of the
helper virus; (2) the defective genomes are often transcriptionally inactive, thus, they would be less often diverted to serve as templates for transcription of mRNA; (3) they may have enhanced affinity for the viral replicase, giving them a competitive advantage over their full-length counterparts.
In essence, DI particles appear to interfere with the replication of their helper viruses by outcompeting the helper virus for critical rate-limiting virus components such as the replicase enzyme and/or structural proteins.

Our knowledge of DI particles derives mostly from studies of viral infections of cultured cells. However, DI particles are also generated by some viruses during in vivo infections (eg, dengue, measles, hepatitis C, hepatitis A, and influenza A viruses), and evidence suggests that they can interfere with replication of the helper virus in vivo and alter the pathogenesis of the infection. This phenomenon has been repeatedly demonstrated in experimental animal model systems, but demonstrating a role
for DI particles in altering the course of natural infections has been more difficult.
Defective particles may alter the pathogenesis of infection with the helper virus in vivo by directly interfering with their replication (as described above), and/or by stimulating antiviral innate immune responses, such as the induction of type I interferon and pro-inflammatory cytokines. Because of their ability to interfere with virus replication and to alter the pathogenesis of infections with their parental virus, and in some
cases that of closely related heterologous viruses, DI particles have been studied for their potential use as antiviral agents.

Cytopathic effects produced by different viruses. The cell monolayers are shown as they would normally be viewed by phase contrast microscopy, unfixed and unstained. (A) Avian reovirus in Vero cells with prominent syncytium (arrow). (B) Untyped herpesvirus

in feline lung cell. (C) Bovine
viral diarrhea virus in primary bovine kidney cells. (D) Parainfluenza virus 3 in Vero cells detected by hemadsorption of chicken red blood cells. Courtesy of E. Dubovi, Cornell University.

Single-cell replication cycle of a representative rhabdovirus (vesicular stomatitis virus, VSV). The virion binds to a cellular receptor and enters the cell via receptor-mediated endocytosis (1). The acidic environment of the endosome lumen induces conformational changes in the spike glycoproteins which in turn mediate fusion between the viral envelope and the endosome membrane. Membrane fusion releases the alpha helical viral nucleocapsid into the cytoplasm of the host cell (2). The nucleocapsid consists of the (2) strand RNA coated throughout its length with nucleocapsid proteins and a small number of L and P proteins, which catalyze viral RNA synthesis. The (2) strand RNA serves as the template for transcription of five subgenomic mRNAs by the L and P proteins (3). The mRNAs encoding the N, P, M, and L proteins are translated by free cytoplasmic ribosomes (4), while the mRNA encoding the G protein is translated by ribosomes bound to the endoplasmic reticulum (5). Newly synthesized N, P, and L proteins participate in viral RNA replication. This process begins with synthesis of a complementary full-length (1) strand, which is also in the form of a ribonucleoprotein containing the N, L, and P proteins (6). This RNA in turn serves as a template for the synthesis of progeny (2) strand RNAs in the form of nucleocapsids (7). Some of these newly synthesized (2) strand RNAs are used as templates for additional transcription of mRNAs (8). Newly synthesized G proteins enter the secretory pathway (9), where they are glycosylated, oligomerized, and transported to the plasma membrane (10). Progeny nucleocapsids and M proteins are transported to the plasma membrane (11 and 12), where association with regions containing the G proteins initiates assembly and budding of progeny virions (13). From Flint, S.J., Enquist, L.W., Racaniello, V.R., Skalka, A.M., 2008. Principles of Virology, third ed., vol. 1, p. 534. Copyright r Wiley (2008), with permission.

Single-cell replication cycle of a simple retrovirus. The virus attaches by binding of the viral envelope protein to specific receptors on the surface of the cell (1). The viral core is deposited into the cytoplasm following fusion of the viral envelope with the plasma membrane (2). Entry
of some beta- and gammaretroviruses may involve endocytic pathways. The viral RNA genome is reverse transcribed by the virion reverse transcriptase (RT) within a subviral particle (3). The product of reverse transcription is a linear, double stranded, complementary DNA (cDNA) with ends
(long terminal repeats, LTRs) that are shown juxtaposed in preparation for integration. Viral DNA and the integrase (IN) enzyme gain access to the nucleus with the help of intracellular trafficking machinery or, in some cases, by exploiting nuclear disassembly during mitosis (4). Integrative recombination catalyzed by IN results in insertion of the viral cDNA into a host cell chromosome, which establishes the provirus (5). Transcription of the proviral DNA by RNA polymerase II produces full-length RNA transcripts (6). Some full-length transcripts are exported from the nucleus and serve
as mRNAs (7), which are translated by cytoplasmic ribosomes to form the viral Gag and GagPol polyprotein precursors (8). Some full-length transcripts which are destined to become encapsidated as progeny viral genomes associate into dimers (9). Other full-length

transcripts are spliced within
the nucleus before being exported to the cytoplasm (10). These spliced mRNAs encode the Env polyprotein precursor and are translated by ribosomes bound to the endoplasmic reticulum (11). The Env glycoproteins are transported through the Golgi apparatus where they are processed and eventually cleaved by a cellular enzyme to form the mature SUTM spike complex (12). Mature envelope proteins are delivered to the surface of the infected cell (13). Internal virion components (viral RNA, Gag and GagPol precursors) assemble at budding sites containing the viral spikes (14). Type C retroviruses (eg, alpharetroviruses and lentiviruses) assemble at the inner face of the plasma membrane, as illustrated. Other types (A, B, and D) assemble on internal cellular membranes. The nascent virions bud from the surface of the cell (15). Maturation (and infectivity) requires the action of the virus-encoded protease (PR), which is itself a component of a core precursor polyprotein (GagPol in the model represented here). During or shortly after budding, PR cleaves the Gag and GagPol precursors at specific sites to yield the individual viral proteins (16). This process yields functional forms of RT and IN, and frees the NC, CA, and MA proteins to assemble into the internal structures of the virion (eg, nucleocapsids, capsid, and matrix). From Flint, S.J., Enquist, L.W., Racaniello, V.R., Skalka, A.M., 2008. Principles of Virology, third ed., vol. 1, p. 531. Copyright r Wiley (2008), with permission.

Maturation of enveloped viruses. (A) Viruses that possess a matrix (and some viruses that lack a matrix) bud through a patch of the plasma membrane in which glycoprotein spikes (peplomers) have accumulated over matrix proteins. (B) Most enveloped viruses that lack a matrix bud into cytoplasmic vesicles (rough endoplasmic reticulum or Golgi), pass through the cytoplasm in smooth vesicles, and are released from the cell by exocytosis.

Sites of budding of various enveloped viruses. Viruses that bud from apical surfaces are in position to be shed in respiratory or genital secretions or intestinal contents. Viruses that bud from basal surfaces are in position for systemic spread via the bloodstream (ie, viremia) or the lymphatics. Some viruses, such as flaviviruses, bunyaviruses, and coronaviruses, take a more circuitous route in exiting the cell (see specific chapters in Part II). Viruses that do not bud usually are released only via cell lysis.

VIRUS REPLICATION

Viruses cannot reproduce on their own. They must invade a cell, take over the cell's internal machinery and instruct the machinery to build enzymes and new viral structural proteins. Then they copy the viral genetic material enough times so that a copy can be placed in each newly constructed virus. Finally, they leave the host cell.
In order for viruses to reproduce, they must complete these 4 steps:
1) Adsorption and penetration.
2) Uncoating of the virus.
3) Synthesis and assembly of viral products (as well as inhibition of the host cell's own DNA, RNA and protein synthesis).
4) Release of virions from the host cell (either by lysis or budding).

Adsorption and Penetration

Fig. 23-15. The viral particle binds to the host cell membrane. This is usually a specific

interaction in which a viral encoded protein on the capsid or a glycoprotein embedded in the virion envelope binds to a host cell membrane receptor. Unlike the bacteriophage virion (see Chapter 3 on Bacterial Genetics), which injects its DNA, these viruses are completely internalized, capsid and nucleic acid. This internalization occurs by endocytosis or by fusion of the virion envelope with the host cell membrane.

Uncoating

The nucleic acid is released from the capsid into the nucleus or cytoplasm.

Transcription, Translation, Replication RNA Viruses

These viruses usually undergo transcription, translation, and replication in the cytoplasm. Positive stranded RNA viruses are the equivalent of preformed messenger RNA (mRNA). As soon as they invade the cell they are ready for translation. These viruses immediately use the host cell's ribosomal proteins and enzymes to translate their positive RNA into an RNA dependent RNA polymerase to make negative stranded copies of their RNA for replication.

Negative stranded RNA viruses have a bit of a problem. They cannot translate into protein because they are a negative strand (copy of mRNA) so they need to carry with them in the virion a viral RNA dependent RNA polymerase to first make a positive strand copy which can then be translated into viral proteins.

Fig. 23-16. Positive (+) stranded RNA virus replication. Positive strand RNA viruses first have to make the RNA dependent RNA polymerase by protein translation of their positive strand of RNA (which is like mRNA).

Assembly and Release

The structural proteins and genome (RNA or DNA) assemble into the intact helical or icosahedral virion. The virion is then released. Naked virions: The cell may lyse and release the virions, or the virions may be released by reverse phagocytosis (exocytosis). Enveloped virions: The newly formed naked virion acquires its new "clothing" by budding through the Golgi apparatus, nuclear membrane, or cytoplasmic membrane, tearing off a piece of host cell lipid bilayer as it exits.

HOST CELL OUCOM

Death: With the viral infection, the host cell's own function shuts down as the cell is commandeered for virion replication. This can result in cell death. Transformation: Infection can activate or introduce oncogenes. This results in uncontrolled and uninhibited cell growth. Latent infection: The virus can survive in a sleeping state, surviving but not producing clinically overt infection. Various factors can result in viral reactivation. Chronic slow infection: Some viruses will cause disease only after many years, often decades, of indolent infection.

Genomic Replication Strategies of Viruses The old terms "eclipse phase" or "latent period" describe that part of a virus life cycle when no infectious virus can be extracted from cells which had just been exposed to infectious virions: a good illustration of the concept in terms of a virus assay experiment is shown here. What happens once a virus is uncoated, or partially uncoated, depends largely upon what sort of virus it is. The Baltimore Classification of viruses by their genome types and replication strategies makes it fairly easy to predict the broad sort of strategy that a virus with a given genome will

employ in order to get replicated. This classification was originally devised by David Baltimore; it originally only had six categories, but the discovery of "DNA retroviruses" or PARARETROVIRUSES in the 1980s has necessitated a new Class VII.

The concept of a virus as an organism challenges the way we define life: viruses do not respire, nor do they display irritability; they do not move and nor do they grow, however, they do most certainly reproduce, and may adapt to new hosts. By older, more zoologically and botanically biased criteria, then, viruses are not living. However, this sort of argument results from a "top down" sort of definition, which has been modified over years to take account of smaller and smaller things (with fewer and fewer legs, or leaves), until it has met the ultimate "molechisms" or "organules" - that is to say, viruses - and has proved inadequate.

If one defines life from the bottom up - that is, from the simplest forms capable of displaying the most essential attributes of a living thing - one very quickly realises that the only real criterion for life is: The ability to replicate and that only systems that contain nucleic acids - in the natural world, at least - are capable of this phenomenon. This sort of reasoning has led to a new definition of organisms: "An organism is the unit element of a continuous lineage with an individual evolutionary history." The key words here are UNIT ELEMENT, and INDIVIDUAL: the thing that you see, now, as an organism is merely the current slice in a continuous lineage; the individual evolutionary history denotes the independence of the organism over time. Thus, mitochondria and chloroplasts and nuclei and chromosomes are not organisms, in that together they constitute a continuous lineage, but separately have no possibility of survival, despite their independence before they entered initially symbiotic, and then dependent associations. The concept of replication is contained within the concepts of individual viruses constituting continuous lineages, and having an evolutionary history. Thus, given this sort of lateral thinking, viruses become quite respectable as organisms: they most definitely replicate, their evolution can (within limits) be traced quite effectively, and they are independent in terms of not being limited to a single organism as host, or even necessarily to a single species, genus or phylum of host.

"Viruses are entities whose genomes are elements of nucleic acid that replicate inside living cells using the cellular synthetic machinery, and cause the synthesis of specialised elements [virions] that can transfer the genome to other cells". SE Luria, JE Darnell, D Baltimore and A Campbell (1978)

"Virus are submicroscopic, obligate intracellular parasites...[and] · Virus particles (virions) are formed from the assembly of pre-formed components; · Virus particles themselves do not "grow" or undergo division; · Viruses lack the genetic information which encodes apparatus necessary for the generation of metabolic energy or for protein synthesis (eg: ribosomes)". AJ Cann (1997). Principles of molecular virology, 2nd Edition. Academic Press, San Diego.

The concept of a virus as an organism challenges the way we define life: viruses do not respire, nor do they display irritability; they do not move and nor do they grow, however, they do most certainly reproduce, and may adapt to new hosts. By older, more zoologically and botanically biased criteria, then, viruses are not living. However, this sort of argument results from a "top down" sort of definition, which has been modified over years to take account of smaller and smaller things (with fewer and fewer legs, or leaves), until it has met the ultimate "molechisms" or "organules" - that is to say, viruses - and has proved inadequate.

Classical Properties of Living Organisms: · Reproduction · Nutrition · Respiration · Irritability · Movement · Growth · Excretion More modern definitions include the storage and replication of genetic information as nucleic acid, and the presence of or potential for, enzyme catalysis.

Other Autonomous or Semi-Autonomously Replicating Genomes There are a number of types of genomes which have some sort of independence from cellular genomes: these include "retrons" or retrotransposable elements, bacterial and fungal (and eukaryotic organelle) plasmids, satellite nucleic acids and satellite viruses which depend on helper viruses for replication, and viroids. A new class of agents - PRIONS - appear to be "proteinaceous infectious agents" (see also here for an ICTV description, here for some local information and more links).

Plasmids Plasmids may share a number of properties with viral genomes - including modes of replication, as in ss circular DNA plasmids and viruses - but are not pathogenic to their host organisms, and are transferred by conjugation between cells rather than by free extracellular particles. Satellite Nucleic Acids Certain viruses have associated with them nucleic acids that are dispensable in that they are not part of the genome, which have no (or very little) sequence similarity with the viral genome, yet depend on the virus for replication, and are encapsidated by the virus. These are mainly associated with plant viruses and are generally ssRNA, both linear and circular - however, a circular ssDNA satellite of a plant geminivirus has recently been found. Satellite Viruses There are also viruses which depend for their replication on HELPER VIRUSES: a good example is tobacco necrosis satellite virus (sTNV), which has a small piece of ssRNA which codes only for a capsid protein, and depends for its replication on the presence of TNV. Another good example is the hepatitis delta agent with its circular ssRNA genome. The adeno-associated viruses (AAVs) are also satellite viruses dependent on the linear dsDNA adenoviruses for replication, but which have linear ssDNA genomes and appear to be degenerate or defective parvoviruses.

Viroids Viroids are small naked circular ssRNA genomes which appear rodlike under the EM, which are capable of causing diseases in plants. They code for nothing but their own structure, and are presumed to replicate by somehow interacting with host RNA polymerase, and to cause pathogenic effects by interfering with host DNA/RNA metabolism and/or transcription. A structurally similar disease agent in humans is the hepatitis B virus-dependent hepatitis delta agent, which additionally codes for a structural protein.

Retroid Elements and Retroviruses Retroviridae [ssRNA(+) viruses replicating via a longer-than-genome-length dsDNA intermediate], Hepadnaviridae, caulimoviruses and badnaviruses [family Caulimoviridae, gapped circular dsDNA viruses replicating via longer-than-genome-length RNA intermediates] all share the unlikely attribute of the use of an enzyme complex consisting of a RNA-dependent DNA polymerase/RNAse H in order to replicate. They share this attribute with several retrotransposons, which are eukaryotic transposable cellular elements with striking similarities with retroviruses [such as the yeast Ty element, the mammalian LINE-1 elements, and the Drosophila copia element]; and with retroposons, which are eukaryotic elements which transpose via RNA intermediates, but share no obvious genomic similarity with any viruses other than the

reverse transcriptase. Bacteria such as E coli also have reverse- transcribing transposons - known as retrons - but these are very different to any of the eukaryotic types while preserving similarities in certain of the essential reverse transcriptase sequence motifs. All of these elements are collectively known as RETROELEMENTS; the fact that the reverse transcriptases of all of them have some amino acid identity suggests a common evolutionary origin. Several reviewers have pointed out that just such an enzyme as reverse transcriptase would have been necessary for the transition from what is widely believed to have been an RNA world - that is, where all the extant organsisms had RNA genomes - to the present world in which all cellular organisms have DNA genomes.

Элементы Ретроида и Ретровирусы

Viruses with RNA genomes which use RNA-dependent RNA polymerases for their replication may be the only remnants of that preDNA era; however, cellular elements and viruses which use reverse transcriptase may share a common origin as cell-derived "modules" coding for a reverse transcriptase, which evolved to become retrons and retroposons and retrotransposons. Addition of structural proteins may have allowed evolution of retroviruses. The evolution of the DNA retroviruses - Hepadnaviridae, caulimo- and badnaviruses - is more obscure; it appears as though these arose from retrotransposon-like sequences, but this probably occurred near the origin of of these types of element as they are so diverse in sequence and genome organisation.

It is believed that retrotransposons may contribute substantially to the evolution of their hosts. Evidence for this has been obtained by studying human LINE-1s (Long Interspersed Nuclear Elements) - a group of retrotransposable elements which make up approximately 15 % of the human genome. The vast majority of LINE-1s are no longer retrotransposition competent and it is believed that in humans only between 30 and 60 full length LINE-1s are currently active. There is strong evidence from sequences in the sequence databases to suggest that active LINE-1s play an important role in "exon shuffling" (belived to be the major mechanism of macro-evolution whereby entirely new genes are created by reshuffling the components of older genes). The most compelling evidence that LINE-1s do facilitate exon shuffling, however, is the experimental demonstration that they are not only able to move large amounts of non-LINE-1 exonic DNA but also insert this DNA into unrelated expressed genes to obtain chimeras which encode active hybrid gene products.

VIRIONS are virus particles: they are the INERT CARRIERS of the genome, and are ASSEMBLED inside cells, from virus-specified components: they do not GROW, and do not form by DIVISION. They may be regarded as the EXTRACELLULAR PHASE of the virus: they are exactly analogous to "spacecraft" in that they take viral genomes from cell to cell, and they protect the genome in inhospitable environments in which the virus cannot replicate.

Helical Nucleocapsids This is one of the SIMPLEST FORMS of viral capsid: the protein is "wound on" to the viral nucleic acid (generally ssRNA, though M13 and other filamentous phage virions contain circular ssDNA) in a simple HELIX, like a screw (see the diagram for tobacco mosaic virus, below).

In the case of TMV this is the entire virion: this is also the case for all RODLIKE and FILAMENTOUS virions where no membranes are involved. This includes all Tobamoviridae, Potyviridae, and Closteroviridae, but NOT Filoviridae, like Ebola virus (see here). In other cases, filamentous helical nucleocapsids may be enclosed within

matrix protein and a membrane studded with spike proteins: excellent examples of this are PARAMYXOVIRIDAE, images of which can be found here, at Linda Stannard's site.

Isometric Nucleocapsids These are built up according to simple structural principles, as amply outlined here, and in more detail here. Put simply, nearly all isometric virions are constructed around a BASIC ICOSAHEDRON, or solid with 20 equilateral trinagles for faces. It suffices to say that the "quasi-icosahedral" capsid is possibly Nature's most popular means of enclosing viral nucleic acids; they come in many sizes, from tiny T=1 structures (Nanoviruses, eg: banana bunchy top virus; 18 nm diameter) to huge structures such as those of Iridoviridae or Phycodnaviridae (over 200 nm diameter). A good example of a simple structure is illustrated below in the animated GIF: this shows cowpea chlorotic mottle (CCMV) virion surface structure (courtesy J-Y Sgro), which is composed of 180 copies of a single coat protein molecule.

The different colours in the picture represent different "positional states" of the capsid protein: subunits around 5-fold rotational axes of symmetry are BLUE, and cluster as PENTAMERS; subunits around 3-fold axes are RED and GREEN to reflect their different 2-fold symmetries; they cluster as HEXAMERS around "local 6-fold axes". Another recent example - that of turnip yellow mosaic virus (TYMV) - is given here. This has exactly the same basic structure, with a single type of coat protein subunit, only the pentamer-hexamer clustering is more pronounced A more complex capsid - that of the common-cold-causing Rhinovirus R16 (family: Picornaviridae), with 60 copies of 4 proteins in a T=3 structure - is shown below (animation modified from one by J-Y Sgro). This shows a capsid with a cutaway, to reveal internal structure. BLUE subunits around 5-fold axes are VP1; RED and GREEN are VP3 and VP2 respectively; YELLOW subunits (seen only internally) are VP4. The VP4 subunits are formed by autocatalytic cleavage of VP0 (into VP2 and VP4) upon binding of a "procapsid" with viral genomic ssRNA. See here for further details of picornaviruses, here for a scheme showing picornavirus assembly, and here for a scheme outlining polyprotein processing of picornaviruses, and here for material from the Leicester course.

Prion Diseases This document describes infectious agents which (almost certainly) do not have a nucleic acid genome. It seems that a protein alone is the infectious agent. The infectious agent has been called a prion. A prion has been defined as "small proteinaceous infectious particles which resist inactivation by procedures that modify nucleic acids". The discovery that proteins alone can transmit an infectious disease has come as a considerable surprise to the scientific community. Prion diseases are often called spongiform encephalopathies because of the post mortem appearance of the brain with large vacuoles in the cortex and cerebellum. Probably most mammalian species develop these diseases. Specific examples include: Scrapie: sheep TME (transmissible mink encephalopathy): mink CWD (chronic wasting disease): muledeer, elk BSE (bovine spongiform encephalopathy): cows Humans are also susceptible to several prion diseases: CJD: Creutzfeld-Jacob Disease GSS: Gerstmann-Straussler-Scheinker syndrome FFI: Fatal familial Insomnia Kuru Alpers Syndrome These original classifications were based on a clinical evaluation of a patients family history symptoms and are still widely used, however more recent and accurate molecular diagnosis of the disease is gradually taking the place of this classification. The incidence of sporadic CJD is about 1 per million per year. GSS occurs at about 2% of the rate of CJD. It is estimated that 1 in 10,000 people are infected with CJD at the time of death. These figures are likely to be underestimates since prion diseases may be misdiagnosed as other neurological disorders. The diseases are

characterised by loss of motor control, dementia, paralysis wasting and eventually death, typically following pneumonia. Fatal Familial Insomnia presents with an untreatable insomnia and dysautonomia. Details of pathogenesis are largely unknown. Visible end results at post-mortem are non-inflammatory lesions, vacuoles, amyloid protein deposits and astrogliosis.

GSS is distinct from CJD, it occurs typically in the 4th-5th decade, characterised by cerebellar ataxia and concomitant motor problems, dementia less common and disease course lasts several years to death. (Originally thought to be familial, but now known to occur sporadically as well). CJD typically occurs a decade later has cerebral involvement so dementia is more common and patient seldom survives a year (originally thought to be sporadic, but now known to be familial as well). FFI pathology is characterised by severe selective atrophy of the thalamus. Alpers syndrome is the name given to prion diseases in infants. Scrapie was the first example of this type of disease to be noticed and has been known about for many hundreds of years. There are two possible methods of transmission in sheep: 1. Infection of pasture with placental tissue carrying the agent followed by ingestion,or direct sheep-lamb transmission i.e. an acquired infection. 2. Parry showed considerable foresight by suggesting that it is not normally an infectious disease at all but a genetic disorder. He further suggested that selective breeding would get rid of the disease.

Humans might be infected by prions in 2 ways: 1. Acquired infection (diet and following medical procedures such as surgery, growth hormone injections, corneal transplants) i.e. infectious agent implicated. 2. Apparent hereditary mendelian transmission where it is an autosomal and dominant trait. This is not prima facie consistent with an infectious agent. This is one of the features that single out prion diseases for particular attention. They are both infectious and hereditary diseases (?see below). They are also sporadic, in the sense that there are also cases in which there is no known risk factor although it seems likely that infection was acquired in one of the two ways listed above. Kuru is the condition which first brought prion diseases to prominence in the 1950s. Found in geographically isolated tribes in the Fore highlands of New Guinea. Established that ingesting brain tissue of dead relatives for religious reasons was likely to be the route of transmission. They ground up the brain into a pale grey soup, heated it and ate it. Clinically, the disease resembles CJD. Other tribes in the vicinity with same religious habit did not develop the disease. It is speculated that at some point in the past a tribe member developed CJD, and as brain tissue is highly infectious this allowed the disease to spread. Afflicted tribes were encouraged not to ingest brain tissue and the incidence of disease rapidly declined and is now almost unknown.

Evidence suggests that a prion is a modified form of a normal cellular protein known as PrPc (for cellular), a normal host protein encoded by a single exon of a single copy gene. This protein is found predominantly on the surface of neurones attached by a glycoinositol phospholipid anchor, and is protease sensitive. Thought to be involved in synaptic function. The modified form of PrPc which may cause disease i.e. the prion is known as PrPsc (for scrapie) which is relatively resistant to proteases and accumulates in cytoplasmic vesicles of diseased individuals. It has been proposed that PrPsc when introduced into a normal cell causes the conversion of PrPc into PrPsc. Process is unknown but it could involve a chemical or conformational modification.

The Virus Life Cycle Viruses have a defined "life cycle" as do any other type of organisms; however, given that they are obligate intracellular parasites, this cycle revolves around: getting into a host cell replicating there, and getting out again. For eighteen years now I have taught this cycle under the heading "Entrance, Entertainment, and Exit*", as this is the best mnemonic I know to remind one of the process. Other courses tend to label these steps as (for example) Virus Entry Into Cells Replication of Viruses Assembly and Release of Virions

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Pathogenesis of Viral Infections and Diseases

Viral infection is not synonymous with disease, as many viral infections are subclinical (syn., asymptomatic, inapparent), whereas others result in disease of varying severity that is typically accompanied by characteristic clinical signs in the affected host (Fig. 3.1). Amongst many other
potentially contributing factors, the outcome of the virushost encounter is essentially the product of the virulence of the infecting virus on the one hand and the susceptibility of the host on the other. The term virulence is used as a quantitative or relative measure of the pathogenicity of the infecting virus—that is, a virus is said to be either pathogenic or nonpathogenic, but its virulence is stated in relative terms ("virus A is more virulent than virus B" or "virus strain A is more virulent in animal species Y than species Z"). The terms pathogenicity and virulence refer to the capacity of a virus to cause disease in its host, and are unrelated to the infectivity or transmissibility (contagiousness) of the virus.

For viruses to cause disease they must first infect their host, spread within the host, and damage target tissues.

To ensure their propagation, viruses must then be transmitted to other susceptible individuals—that is, they must be shed within secretions or excretions into the environment, be taken up by another host or a vector, or be passed congenitally from mother to offspring. Viruses have developed a remarkable variety of strategies to ensure their own survival. Similarly, individual viruses cause disease through a considerable variety of distinct pathogenic mechanisms.

INTERPLAY OF VIRAL VIRULENCE AND HOST RESISTANCE, OR SUSCEPTIBILITY
FACTORS IN MANIFESTATION OF VIRAL DISEASES

Viruses differ greatly in their virulence, but even in a population infected by a particular virus strain there are usually striking differences in the outcome of infection between individual animals. Similarly, there is much variation amongst viruses of the same species and the determinants of viral virulence are often multigenic, meaning that several viral genes contribute to the virulence of individual viruses. The determinants of host resistance/susceptibility are usually multifactorial, and include not only
a variety of host factors but environmental ones as well.

Вирусы сильно различаются по своей вирулентности, но даже в популяции, зараженной определенным штаммом вируса, обычно есть разительные различия в исходе инфекции между отдельными животными. Аналогичным образом, существует много различий между вирусами одного и того же вида, и детерминанты вирусной вирулентности часто являются мультигенными, что означает, что несколько вирусных генов способствуют вирулентности отдельных вирусов. Детерминанты устойчивости / восприимчивости хозяина обычно многофакторные и включают в себя не только множество факторов хозяина, но также и факторы окружающей среды.

The advent and application of molecular technologies has facilitated mapping of virulence determinants in the genome of many viruses (eg, by whole-genomic sequencing of virus strains, and manipulation of molecular clones), as well as resistance/susceptibility determinants in the genome of experimental animals. Virus strain differences may be quantitative, involving the rate and yield of virus replication, lethal dose, infectious dose, the number of cells infected in a given organ, or they may be qualitative, involving organ or tissue tropism, extent of host-cell damage, mode and efficacy of spread in the body, and character of the disease they induce.

Assessment of Viral Virulence

There is wide variation in the virulence of viruses, ranging from those that almost always cause inapparent infections, to those that usually cause disease, to those that usually cause death. Meaningful comparison of the virulence of viruses requires that factors such as the infecting dose of the virus and the age, sex, and condition of the host animals and their immune status be equal; however, these conditions are never met in nature, where heterogeneous, outbred animal populations are the rule and the dynamics of exposure and viral infection are incredibly varied. Hence, subjective and vague terminology may be used to describe the virulence of particular viruses in domestic and wild animals. Precise measures of virulence are usually derived only from assays in inbred animals such as mice. Of course, such assays are only feasible for those viruses that grow in mice, and care must always be exercised in extrapolating data from laboratory mice to the host species of interest.

The virulence of a particular strain of virus administered in a particular dose, by a particular route, to a particular age and strain of laboratory animal may be assessed by determining its ability to cause disease, death, specific clinical signs, or lesions. The dose of the virus required to cause death in 50% of animals (lethal dose 50, LD50) has been a commonly used measure of virulence, but is now passing out of favor in the research arena for ethical reasons. For example, in the susceptible BALB/c strain of mouse, the LD50 of a virulent strain of ectromelia virus is 5 virions, as compared with 5000 for a moderately attenuated strain and about 1 million for a highly attenuated strain. Viral virulence can also be measured in experimental animals by determining the ratio of the dose of a particular strain of virus that causes infection in 50% of individuals (infectious dose 50, ID50) to the dose that kills 50% of individuals (the ID50:LD50 ratio). Thus, the ID50 of a virulent strain of ectromelia virus in BALB/c mice is 2 virions and the LD50 about 5 virions, whereas for resistant C57BL/6 mice the ID50 is the same but the LD50 is 1 million virions. The severity of an infection, therefore, depends

on the interplay between the virulence of the virus and the resistance of the host. Viral virulence also can be estimated through assessment of the severity, location, and distribution of gross, histologic, and ultrastructural lesions in affected animals.

Determinants of Viral Virulence

The advent of molecular biology has facilitated determination of the genetic basis of virulence of many viruses, along with other important aspects of their replication. Specifically, the role of potential determinants of virulence identified by genetic sequence comparison of viruses of defined virulence can be confirmed unequivocally by manipulation of molecular clones of the virus in question. This "reverse genetics" strategy utilizing molecular (infectious) clones was first widely employed using complementary DNA (cDNA) copies of the entire genome of simple positive-strand RNA viruses such as alphaviruses and picornaviruses, where RNA transcribed from the full-length cDNA copies (clones) of the genomes of such viruses is itself capable of initiating the viral replication cycle following transfection into cells. The genomic RNA of negative-sense RNA viruses such as rhabdoviruses is not in and of itself infectious, but infectious virus can be recovered from cDNA clones if viral proteins supporting genome replication are also produced in cells transfected with genome-length RNA transcripts.

Viruses exhibit host and tissue specificity (tropism), usually more than is appreciated clinically. Mechanistically, the organ or tissue tropism of the virus is an expression of all the steps required for successful infection, from the interaction of virus attachment molecules and their cellular receptors to virus assembly and release (see Chapter 2: Virus Replication). Organ and tissue tropisms also involve all stages in the course of infection in the whole host animal, from the site of entry, to the major target organs responsible for the clinical signs, to the site involved in virus release and shedding.

Caution should be exercised in attributing characteristics of viral epidemics solely to the virulence of the causative virus, as there typically is considerable variation in the response of individual infected animals, both within and between animal species. For example, during the epizootic of West Nile virus infection that began in North America in 1999, approximately 10% of infected horses developed neurological disease (encephalomyelitis) and, of these, some 3035% died. Neuroinvasive disease was
even less common in humans infected with this same strain of West Nile virus, whereas infected corvids (crows and their relatives) almost uniformly developed disseminated, rapidly fatal infections.

Determinants of Host Resistance/ Susceptibility As just described for West Nile virus, genetic differences in host resistance/susceptibility to viral infections are most obvious when different animal species are compared. Viral infections tend to be less pathogenic in their natural host species than in exotic or introduced species.

For instance, myxoma virus produces a small benign fibroma in its natural host, which are wild rabbits of the Americas (Sylvilagus spp.), but the same virus almost invariably causes a fatal generalized infection in the European rabbit, Oryctolagus cuniculus. Likewise, zoonotic (transmitted from animal to human) infections caused by arenaviruses, filoviruses, paramyxoviruses, coronaviruses, and many arboviruses are severe in humans but mild or subclinical in their reservoir animal hosts.

The innate and adaptive immune responses to particular viral infections differ greatly from

one individual to another (see Chapter 4: Antiviral Immunity and Virus Vaccines). Studies with inbred strains of mice have confirmed that susceptibility to specific viruses may be associated with particular major histocompatibility (MHC) antigen haplotypes, presumably because of their central role in directing the nature of the adaptive immune response generated to the infecting virus. Similarly,
studies with genetically modified mice have unequivocally confirmed the critical role of innate immune
responses, especially those associated with the interferon system, in conferring antiviral resistance and protection.
Expression of critical receptors on target cells is a fundamental determinant of host resistance /susceptibility to a particular virus. The more conserved or ubiquitous the receptor, the wider the host range of the virus that exploits it; for example, rabies virus, which uses sialylated gangliosides in addition to the acetylcholine receptor, has a very wide host range, but infection is restricted
narrowly to a few host cell types, including myocytes, neurons, and salivary gland epithelium. Changes in viral attachment proteins can lead to the emergence of variant viruses with different tropism and disease potential. For example, porcine respiratory coronavirus arose from transmissible gastroenteritis virus, which is strictly an enteric pathogen, through a substantial deletion in the
gene encoding the viral spike protein that mediates virus attachment. This change affected the tropism of the virus as well as its transmissibility.

Physiologic Factors Affecting Host Физиологические факторы, влияющие на хозяина
Resistance/Susceptibility In addition to innate and adaptive immune responses, a considerable variety of physiologic factors affect host resistance/susceptibility to individual viral diseases, including age, nutritional status, levels of certain hormones, and cell differentiation. Viral infections tend to be most serious at both ends of life—in the very young and the very old. Rapid physiologic changes occur during the immediate postpartum period and resistance to the most severe manifestations of many intestinal and respiratory infections builds quickly in the neonate. Maturation of the immune system is responsible for much of this enhanced, age-related resistance, but physiologic changes also contribute. Malnutrition can also potentially impair immune responsiveness in adults, but it is often difficult to distinguish adverse nutritional effects from other factors found in animals living in very adverse environments.
Certain infections, particularly herpesvirus infections, can be reactivated during pregnancy, leading to abortion or perinatal infection of the progeny of infected dams. The fetus itself is uniquely susceptible to a number of different viral infections, reflecting immaturity of the immune system, immaturity of biological barriers (eg, the bloodbrain barrier) and increased permissiveness of rapidly dividing cell populations, the latter being abundant in developing tissues. Cellular differentiation and the stage of the cell cycle may affect susceptibility to infection with specific viruses.
For example, parvoviruses replicate only in cells that are in the late S phase of the cell cycle, so the rapidly dividing cells of bone marrow, intestinal epithelium, and the developing fetus are vulnerable. The rapidly dividing, often migratory cell populations that occur during embryogenesis in the developing fetus are exquisitely susceptible to infection and injury by a number of viruses,

notably several highly teratogenic viruses that infect the developing central nervous system (CNS).
Almost all viral infections are accompanied by fever. In classic studies of myxoma virus infection in rabbits, it was shown that increasing body temperature increased protection against disease, whereas decreasing temperature increased the severity of infection. Blocking the development of fever with drugs (eg, salicylates) increased mortality. Similar results have been obtained with ectromelia and coxsackievirus infections in mice.
In contrast, fever does not accompany viral infection in certain poikilotherms (eg, fish), in which this response is probably of no or lesser selective advantage.
The immunosuppressive effects of increased concentrations of corticosteroids, whether endogenous or exogenous in origin, can reactivate latent viral infections or exacerbate active mild or subclinical viral infections, such as those caused by herpesviruses. This mechanism probably contributes to the increased incidence of severe viral infections that occurs in settings in which animals are
stressed as a result of transport and/or introduction into crowded environments, such as animal shelters and feedlots. Products of host inflammatory and innate immune responses also probably contribute to the transient immunosuppression and other general signs that can accompany
viral infections.
Like other microorganisms, viruses must gain entry into their host's body before they can exert their
pathogenic effects; entry of virus into the host can occur through any of a variety of potential routes, depending on the properties of the individual virus (Table 3.1).

Routes of Virus Entry Пути проникновения вирусов

Viruses are obligate intracellular parasites that are transmitted as inert particles. To infect its host, a virus must first attach to and infect cells at one of the body surfaces, either the integument or a mucosal surface. The skin that covers the animal body externally has a relatively impermeable outer layer of keratin, and initiation of infection may require that this barrier be compromised or even bypassed via a wound such as a needle stick, insect or animal bite. Barriers to the initiation of infection on mucosal surfaces are much less formidable, specifically on the mucosal epithelial lining of the respiratory, gastrointestinal, and urogenital tracts and the nonkeratinized epithelial lining of the conjunctiva and cornea of the eyes. In animals without significant areas of keratinized epithelium
(eg, fish), the skin and gills serve as an extensive mucosal surface that is the initial site of infection with many viruses. Virus replication may subsequently be limited to the body surface through which the virus entered or the virus may be disseminated to replicate in multiple tissues, with subsequent shedding from body surfaces that are either the same or different from the route of entry (Fig. 3.2).

Entry via the Respiratory Tract

The mucosal surfaces of the respiratory tract are lined by epithelial cells that can potentially support the replication of viruses, so defenses are necessary to minimize the risk of infection. The respiratory tract from the nasal passages to the distal airways in the lungs is protected by the "mucociliary blanket," which consists of a layer of mucus produced by goblet cells that is kept in continuous flow
by the coordinated beating of cilia on the luminal surface of the epithelial cells that line the

nasal mucosa and airways. Inhaled virions can be trapped in the viscous mucus layer and then carried by ciliary action from the nasal cavity and airways to the pharynx, where they are then swallowed or coughed out. The distance to which inhaled particles penetrate into the respiratory tract is inversely
related to their size, so that larger particles (greater than 10 μm in diameter) are trapped on the mucociliary blanket lining the nasal cavity and airways and small particles (less than 5 μm in diameter) can be inhaled directly into the airspaces of the lungs (alveoli), where they are ingested by resident alveolar macrophages.

The respiratory system is also protected by innate and adaptive immune mechanisms that operate at all mucosal surfaces (see Chapter 4: Antiviral Immunity and Virus Vaccines), including specialized lymphoid aggregates that occur throughout the respiratory tree [eg, nasal-associated lymphoid tissue (NALT) and tonsils, and bronchusassociated lymphoid tissue (BALT)]. Despite its protective
mechanisms, however, the respiratory tract is perhaps the most common portal of virus entry into the body. Environmental factors may enhance infection by compromising defense mechanisms. For example, exposure to ammonia vapor causes ciliary stasis, and serous effusions associated with inflammation can dilute the viscosity of the mucus layer, both of which can enhance a virus' ability to attach to specific receptors on epithelial cells within the mucosa. After invasion, some viruses remain localized to the respiratory system or spread from cell to cell to invade other tissues, whereas many others become widely disseminated via lymphatics and/or the bloodstream.

Entry via the Gastrointestinal Tract

A substantial number of viruses (enteric viruses) are spread to susceptible hosts by ingestion of viruscontaminated food or drink. The mucosal lining of the oral cavity and esophagus (and forestomachs of ruminants) is relatively refractory to viral infection, with the notable exception of that overlying the tonsils, thus enteric viral infections typically begin within the mucosal epithelium of the stomach and/or intestines. The gastrointestinal tract is protected by several different defenses,
including acidity of the stomach, the layer of mucus that tenaciously covers the mucosa of the stomach and intestines, the antimicrobial activity of digestive enzymes as well as that of bile and pancreatic secretions, and innate and adaptive immune mechanisms, especially the activity of defensins and secretory antibodies such as immunoglobulin (Ig) A, the latter produced by B lymphocytes in
the gastrointestinal mucosa and mucosa-associated lymphoid tissues (MALTs). Despite these protective mechanisms, enteric infection is characteristic of certain viruses that first infect the epithelial cells lining the gastrointestinal mucosa or the specialized M cells that overlie intestinal lymphoid aggregates (Peyer's patches).

In general, viruses that cause purely enteric infection, such as rotaviruses and enteroviruses, are acid and bile resistant. However, there are acid- and bile-labile viruses that cause important enteric infections; for example, transmissible gastroenteritis virus (a coronavirus) is protected during passage through the stomach of young pigs by the buffering action of suckled milk. Not only do some
enteric viruses resist inactivation by proteolytic enzymes in the stomach and intestine, their infectivity is actually increased by such exposure. Thus cleavage of an outer capsid

protein by intestinal proteases enhances the infectivity of rotaviruses. Whereas rotaviruses and coronaviruses are major causes of viral diarrhea in animals, the great majority of enteric infections caused by enteroviruses, adenoviruses and many other viruses are typically subclinical. Some parvoviruses, morbilliviruses, amongst others, can also cause gastrointestinal infection and diarrhea, but only after reaching cells of the gastrointestinal tract in the course of generalized (systemic) infection after viremic spread.

Entry via the Skin

The skin is the largest organ of the body, and its dense outer layer of keratin provides a mechanical barrier to the entry of viruses. The low pH and presence of fatty acids in skin provide further protection, as do various other components of innate and adaptive immunity, including the presence of migratory dendritic cells (Langerhans cells) within the epidermis itself. Breaches in skin integrity such as
insect or animal bites, cuts, punctures, or abrasions predispose to viral infection, which can either remain confined to the skin, such as the papillomaviruses, or disseminate widely. Deeper trauma can introduce viruses into the dermis and subcutis, where there is a rich supply of blood vessels, lymphatics, and nerves that can individually serve as routes of virus dissemination. Generalized infection of the skin, such as occurs in lumpy skin disease, sheeppox, and others, is the result not of localized cutaneous infection but of systemic viral spread via viremia.

One of the most efficient ways by which viruses are introduced through the skin is via the bite of arthropods, such as mosquitoes, ticks, Culicoides spp. (hematophagous midges or "gnats"), or sandflies. Insects, especially flies, may act as simple mechanical vectors ("flying needles"); for example, equine infectious anemia virus is spread among horses, rabbit hemorrhagic disease virus
and myxoma virus are spread among rabbits, and fowlpox virus is spread among chickens in this way. However, most viruses that are spread by arthropods replicate in their vector, the defining feature of a "biological" vector.
Viruses that are both transmitted by and replicate in arthropod vectors are called arboviruses.
Infection can also be acquired through the bite of an animal, as in rabies, and introduction of a virus by skin penetration may be iatrogenic—that is, the result of veterinary or husbandry procedures. For example, equine infectious anemia virus has been transmitted via contaminated needles, twitches, ropes, and harnesses, and orf virus and papillomaviruses can be transmitted via ear tagging, tattooing, or virus-contaminated inanimate objects (fomites).

Entry via Other Routes

Several important pathogens (eg, several herpesviruses and papillomaviruses) are spread through the genital tract, and this is known as venereal transmission. Small tears or abrasions in the penile mucosa and the epithelial lining of the vagina may occur during sexual activity and facilitate
transmission. The conjunctiva, although much less resistant to viral invasion than the skin, is constantly cleansed by the flow of secretion (tears) and mechanical wiping by the eyelids; some adenoviruses and enteroviruses, however, gain entry at this site, and a

substantial number of
viruses can be experimentally transmitted by this route.

Host Specificity and Tissue Tropism

The capacity of a virus to infect cells selectively in particular organs is referred to as tropism (either cell or organ tropism), which is dependent on both viral and host factors. At the cellular level, there must be an interaction between viral attachment proteins and matching cellular receptors. Although such interactions are usually studied in cultured cells, the situation is considerably more complex in vivo. Not only do some viruses require several cellular receptors/coreceptors (see Chapter 2: Virus
Replication), some viruses utilize different receptors on different cells; for example, canine distemper virus uses CD150 (signaling lymphocyte activation molecule, SLAM) to infect cells of the lymphoid system, an important step in multisystemic viral spread, whereas it attaches to nectin 4 to target the epithelial cells that mediate viral shedding. Expression of receptors can be dynamic; for example, it has been shown experimentally that animals treated with neuraminidase have substantial protection
against intranasal infection with influenza virus that lasts until the neuraminidase-sensitive receptors have regenerated. Receptors for a particular virus are usually restricted to certain cell types in certain organs, and only these cells can be infected. In large part, this accounts for both the tissue and organ tropism of a given virus and the pathogenesis of the disease caused by the virus.

The presence of critical receptors is not the only factor that determines whether the cell may become infected. Cells must support viral entry following receptor binding and the viral genome must be presented with factors required for transcription and genome replication. These requirements are not met by all cell types and thus represent a determinant of viral tropism. For example, paramyxoviruses may require extracellular proteases to activate their fusion protein, the fusion protein mediating
viral entry following attachment. This is the case for Sendai virus, where specialized cells in the bronchioles of rats (Clara cells) secrete a protease required for productive viral infection of the lung. Similarly, papillomaviruses, retroviruses and several herpesviruses rely on the interaction between host proteins and viral genomic elements known as enhancers to support viral gene expression. Viral enhancers are gene activators that increase the efficiency of transcription of viral or cellular genes; specifically, they are short, often tandem-repeated sequences of nucleotides that may contain motifs representing DNAbinding sites for various cellular or viral site-specific DNA-binding proteins (transcription factors). Viral enhancers augment binding of DNA-dependent RNA polymerase to promoters, thereby accelerating transcription. Because many of the transcription factors affecting
individual enhancer sequences in viruses are restricted to particular cells, tissues, or host species, they can determine the tropism of viruses and can act as specific virulence factors. For example, the genomic DNA of papillomavirus contains enhancers that are active only in keratinocytes and, indeed, only in the subset of these cells in which papillomavirus replication occurs.

Relationship between initiation of infection, spread, total viral load (burden), and clinical signs in a multisystemic infection, illustrated here by canine distemper virus infection of a young immunologically

naı ̈ve dog. Peak virus shedding occurs at the point of epithelial infection and peak viral burden in the host. Clinical signs, reflecting cumulative effects of virus replication in multiple organ systems, are not manifest until after significant virus shedding has begun. The onset of immune-mediated viral clearance correlates with the appearance of clinical signs of infection. Courtesy of M. Oglesbee and S. Niewiesk, The Ohio State University.

Mechanisms of Viral Spread and Infection of Target Organs

The ability to restrict viral infection to the body surface that is the point of entry, as contrasted to multisystemic dissemination of virus infection, has profound implications on virus shedding and thus transmission of infection within a population of susceptible animals (Fig. 3.2). From the virus' standpoint, the challenge to local spread is the ability to infect a sufficient number of epithelial
cells to support a level of shedding that assures transmission. The benefits of local spread are more limited opportunities for the immune system to disrupt the course of infection. In contrast, multisystemic spread may introduce virus to many body surfaces that can participate in shedding, and the surface area supporting replication may be much greater than can be achieved via local spread. The challenges to the virus during multisystemic spread include the numerous opportunities for the immune system to disrupt the infection cycle, the potential need to infect multiple cell types, and the need to balance cytopathic effects with the requirement for viable cells to support step-wise spread throughout the body.

In pioneering experiments in 1949, Frank Fenner used ectromelia virus (the agent of mousepox) as a model system that first revealed the sequence of events leading to systemic infection and disease. Groups of mice were inoculated in the footpad of a hind limb, and at daily intervals their organs were titrated to determine the amount of virus present. Fenner showed that, during the incubation period,
infection spread through the mouse body in a step-wise fashion. The virus first replicated locally in tissues of the footpad and then in the draining lymph nodes. Virus produced in these sites then gained entry into the bloodstream, causing a primary viremia, which brought the virus to its initial target organs (organ tropism), especially the spleen, lymph nodes, and the liver. Virus produced in
the target organs—ie, the spleen and liver—caused a secondary viremia that disseminated virus to the skin and mucosal surfaces. Infection in the skin caused a macular and papular rash from which large amounts of virus were shed, leading to contact exposure of other mice. Infection ultimately resulted in tissue necrosis, this being the cause of death, but not until spread within the host and shedding
from the host was achieved. This pattern has subsequently been demonstrated for many viruses of veterinary medical relevance, and can be illustrated by canine distemper virus infection of young immunologically naıve dogs (Fig. 3.3).

Following aerosol exposure, canine distemper virus replicates in lymphoid tissues associated with the respiratory tract, resulting in primary viremia and infection of lymphoid tissues throughout the body, including the thymus and spleen. This amplifies viral burden in the host and leads to secondary viremia with infection of multiple epithelial compartments, some of which are highly efficient at viral shedding (eg, respiratory, urothelial, and conjunctival mucosa) and some of which play either a subordinate or no role in shedding and transmission (eg, integument, odontogenic epithelium, gastric

mucosa).
Clinical signs coincide with the peak of viral shedding, with fever signaling the onset of adaptive immune responses that drive viral clearance. Death, if it occurs, reflects the combination of immune suppression and compromised mucosal barriers that facilitate secondary microbial infections (eg, bacterial bronchopneumonia). Death may also reflect viral infection of brain, a by-product of
the secondary viremia. However, these events occur only after the infection cycle is complete and shedding has occurred.

Local Spread on Epithelial Surfaces

Viruses first replicate in epithelial cells at the site of entry and produce a localized infection, often with associated virus shedding directly into the environment from these sites. The spread of infection along epithelial surfaces occurs by the sequential infection of neighboring cells, which, depending on the individual virus, may or may not precede spread into the adjacent subepithelial tissues and beyond.
In the skin, papillomaviruses and poxviruses such as orf virus remain confined to the epidermis, where they induce localized proliferative lesions, whereas other poxviruses such as lumpy skin disease virus spread widely after cutaneous infection to involve other organ systems.
Viruses that enter the body via the respiratory or intestinaltracts can quickly cause extensive infection of the mucosal epithelium, thus diseases associated with these infections progress rapidly after a short incubation period. In mammals, there is little or no productive invasion of subepithelial tissues of the respiratory tract after most influenza and parainfluenza virus infections, or in the intestinal tract following most rotavirus and coronavirus infections. Although these viruses apparently enter lymphatics and thus have the potential to spread, they usually do not do so, because appropriate viral receptors or other permissive cellular factors such as cleavage-activating proteases or transcription enhancers are restricted to epithelial cells, or because of other physiological constraints.
Restriction of viral infection to an epithelial surface should never be equated with lack of virulence or disease severity. Although localized, injury to the intestinal mucosa caused by rotaviruses and coronaviruses can result in severe and, especially in neonates, even fatal diarrhea. Similarly, influenza virus infection can cause extensive injury in the lungs, leading to acute respiratory distress syndrome and possibly death.

Subepithelial Invasion and Lymphatic Spread

A variety of factors probably contribute to the ability of some viruses to breach the epithelial barrier and to invade the subepithelial tissues, including (1) targeted migration of virus within phagocytic leukocytes, specifically dendritic cells and macrophages, and (2) directional shedding of viruses from the infected epithelium (see Chapter 2: Virus Replication). Dendritic cells are abundant in the skin and at all mucosal surfaces, where they constitute a critical first line of immune defense, both innate and adaptive (see Chapter 4: Antiviral Immunity and Virus Vaccines). Migratory dendritic cells (such as Langerhans cells in the skin) “traffic” from epithelial surfaces to mucosa-associated lymphoid tissue (MALT), which would include lymphoid organs such as tonsils and Peyer’s patches, and the adjacent (draining) regional lymph node. Infection of these migratory dendritic cells may be responsible for the initial spread of alphaviruses, bluetongue, African horse sickness and other orbiviruses, and feline and simian human immunodeficiency viruses, amongst many others. Directional release of virus into the

lumen of the respiratory or intestinal tracts facilitates local spread to the surface of contiguous epithelial cells and immediate shedding into the environment, whereas shedding from the basolateral cell surface of epithelial cells potentially facilitates invasion of subepithelial tissues and subsequent virus dissemination via lymphatics, blood vessels, or nerves.

Many viruses that are widely disseminated in the body following infection at epithelial surfaces are first carried to the adjacent (regional) lymph nodes through the afferent lymphatic drainage (Fig. 3.4). Within the draining lymph node, virions may be inactivated and processed by macrophages and dendritic cells so that their component antigens are presented to lymphocytes to stimulate
adaptive immune responses (see Chapter 4: Antiviral Immunity and Virus Vaccines). Some viruses, however, replicate efficiently in macrophages (eg, many retroviruses, orbiviruses, filoviruses, canine distemper virus and other morbilliviruses, arteriviruses such as porcine reproductive and respiratory syndrome virus, and some herpesviruses), and/or in dendritic cells and lymphocytes.
From the regional lymph node, virus can spread to the bloodstream in efferent lymph, and then quickly be disseminated throughout the body, either within cells or as cell-free virions. Blood-filtering organs, including the lung, liver, and spleen, are often target organs of viruses that cause disseminated infections.

Normally, there is a local inflammatory response at the site of viral invasion, the severity of which reflects the extent of tissue damage. Inflammation leads to characteristic alterations in the flow and permeability of local blood vessels, as well as leukocyte trafficking and activity. Some viruses take advantage of these events to infect cells that participate in this inflammatory response, which
in turn can facilitate spread of these viruses either locally or systemically. Local inflammation may be especially important to the pathogenesis of arthropod-transmitted viruses because of the marked reaction at the site of virus inoculation induced by the bite of the arthropod vector.

Spread via the Bloodstream: Viremia

The blood is the most effective vehicle for rapid spread of virus through the body. Initial entry of virus into the blood after infection is designated primary viremia, which, although usually inapparent clinically (subclinical), leads to the seeding of distant organs. Virus replication in major target organs leads to the sustained production of much higher concentrations of virus, producing a secondary viremia (Fig. 3.5) and infection in yet other parts of the body that ultimately results in the clinical manifestations of the associated disease.

In the blood, virions may circulate free in the plasma or may be contained in, or adsorbed to, leukocytes, platelets, or erythrocytes (red blood cells). Parvoviruses, enteroviruses, togaviruses, and flaviviruses typically circulate free in the plasma. Viruses carried in leukocytes, generally lymphocytes
or monocytes, are often not cleared as readily or in the same way as viruses that circulate in the plasma. Specifically, cellassociated viruses may be protected from antibodies and

other plasma components, and they can be carried as “passengers” when leukocytes that harbor the virus emigrate
into tissues. Individual viruses exhibit tropism to different leukocyte populations; thus monocyte-associated viremia is characteristic of canine distemper, whereas lymphocyteassociated viremia is a feature of Marek’s disease and bovine leukosis. Erythrocyte-associated viremia is characteristic of
infections caused by African swine fever virus and bluetongue virus. The association of bluetongue virus with erythrocytes facilitates both prolonged viremia by delaying immune clearance, and infection of the hematophagous (blood-feeding) Culicoides midges that serve as biological vectors of the virus. A substantial number of viruses, including equine infectious anemia virus, bovine viral diarrhea virus, and bluetongue virus, associate with platelets during viremia—an interaction that might facilitate infection of endothelial cells. Neutrophils, like platelets, have a very short lifespan; neutrophils also possess powerful antimicrobial mechanisms and they are rarely infected, although they may contain phagocytosed virions.

Virions circulating in the blood are removed continuously by macrophages, thus viremia can typically be maintained only if there is a continuing introduction of virus into the blood from infected tissues or if clearance by tissue macrophages is impaired. Although circulating leukocytes can themselves constitute a site for virus replication, viremia is usually maintained by infection of the parenchymal cells of target organs such as the liver, spleen, lymph nodes, and bone marrow. In some infections, such as African horse sickness virus and equine arteritis virus infections of horses, viremia is largely
maintained by the infection of endothelial cells and/or macrophages and dendritic cells. Striated and smooth muscle are an uncommon site for viral replication, not representing a target organ essential to completion of the viral infection cycle within the host, but nonetheless significant from a clinical standpoint due to the clinical signs associated with inflammation of the muscle (eg, the myositis that may accompany influenza virus infections).

There is a general correlation between the magnitude of viremia generated by blood-borne viruses and their capacity to invade target tissues. Certain neurotropic viruses are virulent after intracerebral inoculation, but avirulent when given peripherally, because they do not attain viremia titers
sufficient to facilitate invasion of the nervous system. The capacity to produce viremia and the capacity to invade tissues from the bloodstream are, however, two different properties of a virus. For example, some strains of Semliki Forest virus (and certain other alphaviruses) have lost the capacity to invade the CNS while retaining the capacity to generate a viremia equivalent in duration and magnitude
to that produced by neuroinvasive strains.
Viruses that circulate in blood, especially those that circulate free in plasma, encounter, amongst many others, two cell types that exert especially important roles in etermining the subsequent pathogenesis of infection: macrophages and vascular endothelial cells.

The role of viremia in the spread of viruses through the body, indicating sites of replication and important routes of shedding of various viruses. Subepithelial invasion and

spread of infection is associated with a primary round of replication that leads to primary viremia. That viremia infects target organs that further amplify viral burden, resulting in a high-level secondary viremia. Secondary viremia may result in the infection of target organs that are conducive to viral shedding, transmission of infection via arthropod vectors, or infection of organs that are a dead end for transmission (eg, brain). Courtesy of M. Oglesbee and S. Niewiesk, The Ohio State University.

Virus Interactions with Monocytes and Macrophages

Macrophages are bone marrow-derived mononuclear phagocytic cells that are present in all compartments of the body. Their precursors are monocytes in the blood, the largest of the leukocytes. Monocytes migrate into tissues to become part of the normal resident macrophage population found in submucosal connective tissue, spleen and bone marrow, alveoli of the lung, sinusoids of lymph nodes and liver, and parenchyma of the brain (ie, brain microglia). Monocytes also migrate into areas of inflammation to supplement the macrophage population.

Macrophages are generally considered to play host protective roles in microbial infection (Fig. 3.6). They may phagocytize and thus inactivate viruses and, together with dendritic cells, have a critical role in antigen processing and presentation to other immune cells that is central to the initiation of adaptive immune responses (see Chapter 4: Antiviral Immunity and Virus Vaccines). They also initiate innate immune responses because of their ability to detect the presence of pathogen-associated molecular patterns ("microbial signatures") through specific receptors—eg, Toll-like receptors. Toll-like receptor signaling is an important basis for the production of type I interferons that restrict viral virulence.

In contrast to these protective roles, macrophages may contribute to the spread of virus infection and/or tissue damage. Some viruses exhibit a specific tropism for macrophages, where they replicate to high levels. Venezuelan equine encephalitis virus is one such virus, where replication of the virus in macrophages determines the level of viremia which in turn facilitates invasion of the CNS. Viral replication in macrophages may also be envisioned to reduce the contributions of these cells to innate and adaptive antiviral immune responses, thus indirectly contributing to viral burden and spread. Productive infection of macrophages may facilitate local viral spread to neighboring parenchymal cells, as has been suggested for infectious canine hepatitis virus infection of dogs where viral antigen is detected in both hepatocytes and sinusoidal macrophages (Kupffer cells) of the liver. Virus infection of macrophages may enhance inflammatory responses that contribute to tissue injury. For example, hemorrhagic viral fevers caused by Ebola and bluetongue viruses are characterized by induction of inflammatory and vasoactive mediators such as tissue necrosis factor (TNF) by macrophages and dendritic cells, and these cytokines contribute to the pathogenesis of disease.

It should be emphasized that virus infection of macrophages may reflect interaction of viral attachment proteins with specific host cell receptors, or simply an indirect consequence of the phagocytic mechanisms employed by these cells. Although macrophages are inherently efficient phagocytes, this capacity is even further enhanced after their activation by certain microbial products and cytokines such as interferon-γ.

Macrophages also have Fc receptors and C3 receptors that further augment their ability to ingest opsonized virions, specifically those virions that are coated with antibody or complement molecules. For viruses that are capable of replicating in macrophages, opsonization of virions by antibody can actually facilitate antibody-mediated enhancement of infection, which may
be a major pathogenetic factor in human dengue and several retrovirus infections. Virus infection of monocytes should be considered in this discussion, having potential to profoundly influence viral spread by exploiting the tendency of these cells to migrate into tissues as part of an inflammatory response or simply to replenish the normal resident macrophage population. Monocyte infection is a
form of cell-associated viremia that is important to the pathogenesis of lentivirus infections and a proposed mechanism for neuroinvasion by paramyxoviruses.

In many instance, the contribution of virus interaction with macrophages is more difficult to define in terms of its protective versus detrimental role to the host. Macrophages are heterogeneous in their functional activity, which can vary markedly depending on their location and state of activation; even in a given tissue or site there are subpopulations of macrophages that differ in phagocytic activity and
in susceptibility to viral infection. Differences in virusmacrophage interactions may account for differences in the virulence of closely related viruses, individual strains of the same virus, and differences in host resistance.

Types of interactions between viruses and monocytes and macrophages. Virus may exploit these cells to facilitate spread or to generate viral progeny following infection. Alternatively, macrophages may
restrict virus replication and take on a host defense role which includes initiation of innate immune and pro-inflammatory responses. Innate immune responses include production of type 1 interferon (IFN) and presentation of viral antigen, both of which facilitate subsequent adaptive immunity to the virus. Pro-inflammatory responses include production of cytokines such as tumor necrosis factor (TNF). While these pro-inflammatory responses can mediate host protective responses, excesses can paradoxically contribute to manifestations of disease. Courtesy of M. Oglesbee and S. Niewiesk, The Ohio State University.

Virus Interactions with Vascular Endothelial Cells

The vascular endothelium with its basement membrane constitutes the bloodtissue interface and may represent a barrier for particles such as virions in locations where endothelial cells are nonfenestrated and joined together by tight junctions. The degree of barrier function varies between tissue compartments, being greatest in the brain and eye. Parenchymal invasion by circulating virions
depends on crossing such barriers, often in capillaries and venules, where blood flow is slowest and the vascular wall is thinnest. Virions may move passively between or through endothelial cells and the basement membrane of small vessels, be carried within infected leukocytes (socalled “Trojan horse” mechanism), or infect endothelial cells and “grow” their way through this barrier, with infection of the luminal aspect of the cell and release from the basal aspect. This subject has been studied most

intensively in relation to viral invasion of the CNS, but it also applies to invasion of many tissues during generalized infections.

Endothelial infection may be clinically inapparent, reflecting a noncytopathic infection that facilitates viral spread. Alternatively, infection of endothelial cells may be characterized by vascular injury that results in widespread hemorrhage and/or edema, contributing to the pathogenesis of the so-called hemorrhagic viral fevers. Virus-induced endothelial injury leads to vascular thrombosis and, if widespread, disseminated intravascular coagulation (consumptive coagulopathy). However, it is likely that inflammatory and vasoactive mediators produced by virus-infected macrophages and dendritic cells, such as tissue necrosis factor, also contribute to the pathogenesis of vascular injury in hemorrhagic viral fever (Fig. 3.6).

Spread via Nerves

Although infection of the CNS can occur after hematogenous spread, invasion via the peripheral nerves is also an important route of infection—eg, in rabies, Borna disease, and several alphaherpesvirus infections (eg, B virus encephalitis, pseudorabies, and bovine herpesvirus 5 encephalitis). Herpesviruses can travel to the CNS in axon cytoplasm and, while doing so, also sequentially infect Schwann cells of the nerve sheath. Rabies virus and Borna disease virus also travel to the CNS in axon cytoplasm, but usually do not infect the nerve sheath. Sensory, motor, and autonomic nerves may be involved in the neural spread of these viruses. As these viruses move centripetally, they must cross cellcell junctions. Rabies virus and pseudorabies virus can efficiently traverse synaptic junctions (Fig. 3.7).

In addition to passing centripetally from the body surface to the sensory ganglia and from there to the brain, herpesviruses can move through axons centrifugally from ganglia to the skin or mucous membranes. This is the same phenomenon that occurs after reactivation of latent herpesvirus infections and the subsequent production of recrudescent epithelial lesions. Centrifugal spread through axons is also the mechanism by which rabies virus reaches salivary glands from the brainstem, with salivary gland infection being important to viral shedding. Viruses can also use olfactory nerve endings in the nares as sites of entry, including rhabdoviruses (eg, rabies virus and vesicular stomatitis virus), herpesviruses, and paramyxoviruses. They gain entry in the special sensory endings of the olfactory neuroepithelial cells where they cause local infection and progeny virus (or subviral entities containing the viral genome) then travel in axoplasm of olfactory nerves directly to the olfactory bulb of the brain.

Mechanisms of Virus Shedding

Shedding of infectious virions is crucial to the maintenance of infection in populations (see Chapter 6: Epidemiology and Control of Viral Diseases). For viruses that replicate only at epithelial surfaces, exit of infectious virions usually occurs from the same organ system involved in virus entry (eg, the respiratory or gastrointestinal system; Fig. 3.2). In generalized viral infections, shedding can occur from a variety of sites (Fig. 3.5), and some viruses are shed from several sites. The amount of virus shed in an excretion or secretion is important in relation to transmission. Very low

concentrations may be irrelevant unless very large volumes of infected material are involved; however, some viruses occur in such high concentrations that a minute quantity of virus-laden secretion or excretion can readily lead to transmission to the next animal host. Enteric viruses are in general more resistant to inactivation by environmental conditions than respiratory viruses; especially when suspended in water and protected from light, such viruses can persist in the environment for some time.

Viruses such as influenza and the pneumoviruses that typically cause localized infection and injury of the respiratory tract are shed in mucus and are expelled from the respiratory tract during coughing or sneezing. Viruses are also shed from the respiratory tract in several systemic infections. Enteric viruses such as rotaviruses are shed in the feces, and the more voluminous the fluid output the greater is the environmental contamination they cause. A few viruses are shed into the oral cavity from infected salivary glands (eg, rabies virus and cytomegaloviruses) or from the lungs or nasal mucosa during infection of the respiratory system. Salivary spread depends on activities such as licking, nuzzling, grooming, or biting. Virus shedding in saliva may continue during convalescence or recurrently thereafter, especially with herpesviruses.

The skin is an important source of virus in diseases in which transmission is by direct contact or via small abrasions: papillomaviruses and some poxviruses and herpesviruses employ this mode of transmission. Although skin lesions are produced in several generalized diseases, the skin is not generally a source of significant viral shedding. Exceptions include vesicular diseases such as foot-and-mouth disease, vesicular stomatitis, and swine vesicular disease, where the causative viruses are produced in great quantities in vesicles within the mucosa and skin of affected animals; virus is shed from these lesions after the vesicles rupture. Localization of virus in the feather follicles is important in the shedding of Marek's disease virus by infected chickens.

Urine, like feces, tends to contaminate food sources and the environment. A number of viruses (eg, infectious canine hepatitis virus, foot-and-mouth disease viruses, and the arenaviruses) replicate in tubular epithelial cells in the kidney and are shed in urine. Canine distemper virus replicates in transitional epithelium of the renal pelvis, ureters and urinary bladder, also contributing to urinary viral shedding or "viruria." Viruria is prolonged and common in equine rhinitis A virus infection and lifelong in arenavirus infections of reservoir rodent species; it constitutes the principal mode of contamination of the environment by these viruses.

Several viruses that cause important diseases of animals are shed in the semen and are transmitted during coitus; for example, equine arteritis virus can be shed for months or years in the semen of apparently healthy carrier stallions, long after virus has been cleared from other tissues. Similarly, viruses that replicate in the mammary gland are excreted in milk, which may serve as a route of transmission—eg, caprine arthritisencephalitis virus, mouse mammary tumor virus, and some of the tick-borne flaviviruses. In salmonid fish, the fluid surrounding eggs oviposited during spawning may contain high concentrations of viruses such as infectious

hemopoietic necrosis virus, which is an important mode of virus transmission in both hatchery and wild fish populations.

Although not "shedding" in the usual sense of the word, blood and tissues from slaughtered animals must be considered important sources of viral contagion. Virus-laden blood is also the basis for transmission when it contaminates needles and other equipment used by veterinarians and others treating or handling sick animals. Similarly, the use of virus-contaminated fetal bovine serum can result in similar contamination of biological products.

Virus Infection Without Shedding (p. 74)

Many sites of virus replication might be considered "dead ends" from the perspective of natural spread. Infection of the brain may not result in shedding in the case of paramyxoviruses, although it is significant from the standpoint of clinical disease. Transmission may occur in instances where infected nervous tissues and muscle are ingested by carnivores and omnivores. Similarly, classical swine fever (hog cholera) and African swine fever have been translocated to different regions and countries through feeding garbage containing contaminated pork scraps. The prion diseases are an analogous example, where the unprecedented epizootic of bovine spongiform encephalopathy (mad cow disease) in the United Kingdom was spread widely amongst cattle by the feeding of contaminated meat and bone meal containing bovine offal that included nervous tissue.

Some viruses, notably retroviruses and bovine virus diarrhea virus are also transmitted directly in the germplasm or by infection of the avian egg or developing mammalian embryo. Despite the lack of horizontal transmission, these vertically transmitted viruses accomplish the same ends as those shed into the environment—that is, transmission to new hosts and perpetuation in nature Many sites of virus replication might be considered "dead ends" from the perspective of natural spread. Infection of the brain may not result in shedding in the case of paramyxoviruses, although it is significant from the standpoint of clinical disease. Transmission may occur in instances where infected nervous tissues and muscle are ingested by carnivores and omnivores. Similarly, classical swine fever (hog cholera) and African swine fever have been translocated to different regions and countries through feeding garbage containing contaminated pork scraps. The prion diseases are an analogous example, where the unprecedented epizootic of bovine spongiform encephalopathy (mad cow disease) in the United Kingdom was spread widely amongst cattle by the feeding of contaminated meat and bone meal containing bovine offal that included nervous tissue.

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MECHANISMS OF VIRAL INJURY AND DISEASE

The most common adaptation of a virus to a host involves infection, spread, and shedding with minimal if any adverse effects on the host. Medically relevant virus infections are distinct in that infection causes tissue injury and thus disease (Fig. 3.8). Tissue injury may

facilitate virus propagation within or transmission between hosts, and at minimum should not interfere with these processes
if the virus is to be maintained within a specific population of animals. Virus-induced cytopathic effects may induce inflammatory and physiological responses such as coughing and sneezing that facilitate shedding and transmission. Induction of diarrhea is another means of facilitating transmission by enhancing environmental contamination with progeny virus. Virus-induced immune suppression may confound host attempts at clearance and thus benefit viral spread, while also predisposing the
infected host to secondary microbial infections. Tissue injury may reflect host defense mechanisms that include apoptosis or immune responses that target virus-infected cells. In other instances, damage to the host may be a consequence of virus replication in which there is no known advantage to either the virus or host, or reflects a byproduct of infection with no significant impact on transmission. The latter includes many instances where viruses infect the CNS, resulting in congenital malformations in fetuses or neonates, or clinically significant inflammatory disease in older animals. Host species is a significant variable when considering the potential of a virus to cause
disease, where a given virus may cause clinically inapparent infection in a reservoir species and clinical disease in a species to which the virus is less adapted. Mechanisms of virus-induced tissue injury may be considered "direct" when they are a direct consequence of virus replication within a cell or tissue, and "indirect" when the injury is mediated by a host immune or inflammatory response.

Types of VirusCell Interactions

Virus-induced tissue injury reflects viral cell and tissue tropism, and the mode of replication within the infected cells. As described in the preceding section, cellular tropism of viruses is determined by the presence of appropriate cellular receptors and an environment that is conducive to virus gene expression and replication. The latter may include the expression of cell-type-specific proteases, transcription factors, and other factors required for viral replication. Cells are said to be permissive to infection if they provide such an environment. Viruses typically encode genes that modulate host-cell functions for their own benefit and, of course, the host has elaborate innate defenses to restrict viral functions (see Chapter 4: Antiviral Immunity and Virus Vaccines). Permissiveness may thus also reflect the ability of a virus to inhibit innate antiviral defense mechanisms. Viral and cellular factors
that influence the outcome of infection are often in delicate balance and easily shifted one way or the other. The dynamic nature of the viruscell relationship is defined in terms that describe the degree of damage to the infected cell and the production of viral progeny. Cytopathic infections are characterized by loss of cell functions that are essential to survival. Cell degeneration and necrosis or virus-induced apoptosis are final outcomes of cytopathic infections. These infections are alternatively described as cytocidal (meaning "cell death") or cytolytic (meaning "cell lysis" or "rupture"). Cell lysis is required for release of nonenveloped viral progeny, whereas progeny of enveloped viruses can be released by budding from viable cells. Cell maintenance functions are preserved in noncytopathic infections. Noncytopathic infections can be clinically significant when they disrupt cell specialized functions. For example, noncytopathic infections of neurons may cause loss of impulse conduction, and noncytopathic infection of oligodendrocytes
may result in loss of myelin formation, both of which contribute to clinical neurological

disease despite survival of the infected cells. A noncytopathic viruscell relationship may give rise to a persistent infection due to survival of the cell, the inability of immune mechanisms to eliminate the virus, and a low level of virus replication that assures persistence of the virus' genetic information.
Persistence may be associated with production of viral progeny (productive infections) or the absence of viral progeny (nonproductive infections), whereas cytopathic infections are generally productive. A persistent productive infection may result in viral carriers capable of lifelong shedding, and may continually seed infections within the host and stimulate immune and inflammatory responses that contribute to chronic disease.

Latent infection may be viewed as a type of persistent infection in which the viral genome is not transcribed and so there is no production of viral proteins or progeny. The viral genome is maintained indefinitely in the cell, either by the integration of the viral nucleic acid into the hostcell DNA or by carriage of the viral nucleic acid in the form of an episome, and the infected cell survives and
may divide repeatedly. As such, latent infections are restricted to infection by DNA viruses or RNA viruses capable of generating DNA copies of their genome.
Clinical significance of these infections is that virus gene expression can be periodically reactivated, giving rise to the production of viral protein and infectious viral progeny. This is the case of neurons latently infected with herpesviruses, where reactivation results in progeny production that in turn is amplified by productive cytopathic infections of other tissues. Persistent or latent infections with oncogenic viruses may also lead to cell transformation, as described later in this chapter. The various types of interaction that can occur between virus and cell are summarized in Table 3.2 and in Fig. 3.8.

Cytopathic Changes in Virus-Infected Cells

Cytopathic viral infections ultimately kill the cells in which they replicate, by preventing synthesis of host macromolecules (as described below), by producing degradative enzymes or toxic products, or by inducing apoptosis. In a productive infection of tissue culture cells, the first round of virus replication yields progeny virions that spread through the medium to infect both adjacent and distant cells; all cells in the culture may eventually become infected. Cells exhibit biochemical and structural changes that are collectively referred to as a cytopathic effect. Some cytopathic effects have a light microscopic appearance that is characteristic of the particular virus involved, and is therefore an important preliminary clue in the identification of clinical isolates in the diagnostic laboratory (see Chapters 2 and 5: Virus Replication and Laboratory Diagnosis of Viral Infections).

Other changes reflect disruption of cellular processes that are less specific to the infecting virus. Apoptotic cells have a characteristic light microscopic appearance, although this host defense mechanism can be elicited by members of numerous virus families. Similarly, virusinduced metabolic and toxic insults to the cell may result in morphological changes indicative of cell degeneration and
necrosis, the cumulative effect of numerous insults that may be triggered by a number of different viruses. Cytopathic effects should be viewed in context of their relationship to viral replication; to what degree is the change unique to a particular group of viruses, and does the change influence the

virus' ability to produce progeny? Inclusion bodies are sites of viral transcription and genome replication in the cell that are readily apparent in cells by light microscopy. DNA viruses that replicate
in the nucleus utilize cell machinery to varying degrees in support of transcription and genome replication. Host cell DNA may be displaced from the nuclear matrix by the viral genome, resulting in chromatin margination along the nuclear membrane as aggregates of viral nucleic acid and protein accumulate. The result is an intranuclear inclusion—an aggregate of uniform staining that is distinct from nuclear structures observed in uninfected cells.

Stains used routinely in diagnostic settings yield red signal for protein, and blue signal for nucleic acid.
Intranuclear inclusions typically stain red, indicative of the high viral protein content, whereas the marginalized chromatin is blue. Intranuclear inclusions are characteristic of cells infected with herpesviruses and adenoviruses. Occasionally an RNA virus will induce structures known as nuclear bodies, a type of intranuclear inclusion that is host in origin but rich in viral protein. These structures
are thought to regulate RNA processing within the cell, and are the basis for the intranuclear inclusions of canine distemper virus infection. Cytoplasmic inclusions are typical of viruses replicating to high levels in the cytoplasm, again reflecting aggregates of viral genomes engaged in
transcription and replication. Cytoplasmic inclusions are typical of infections caused by poxviruses, paramyxoviruses, rhabdoviruses, and reoviruses (Fig. 2.2). The diagnostic importance of these structures is illustrated by the fact that some of these inclusions are known by specific names, such as the "Negri bodies" of rabies virus infected neurons.

Inhibition of Host Cell Protein Production while viral protein synthesis continues is a characteristic of many viral infections. This shutdown is particularly rapid and profound in picornavirus infections, but it is also pronounced in togavirus, influenza virus, rhabdovirus, poxvirus, and herpesvirus infections. With some other viruses, the shutdown occurs late in the course of infection and is more gradual, whereas with noncytocidal viruses, such as pestiviruses, arenaviruses, and retroviruses, there is no
dramatic inhibition of host-cell protein synthesis, and no cell death. Viruses have evolved numerous mechanisms to interfere with host-cell mRNA transcription, processing, and translation. Inhibition of both host-cell DNA replication and mRNA transcription is a consequence of DNA virus infection when cellular machinery is redirected to viral templates. Inhibition may reflect a broader strategy by the virus to preserve nucleotide pools in support of virus replication, and to diminish cellular mRNA levels that would otherwise compete with viral mRNA for translational machinery. This phenomenon is observed
during replication of viruses in several different families, including poxviruses, rhabdoviruses, reoviruses, paramyxoviruses, and picornaviruses. In some instances, this inhibition may be the indirect consequence of viral effects on host-cell protein synthesis that decrease the availability of transcription factors required for DNA-dependent RNA polymerase activity.

Inhibition of processing and translation of host-cell messenger RNA occurs during

replication of vesicular stomatitis viruses, influenza viruses, and herpesviruses, through interference with the splicing of cellular primary mRNA transcripts that are needed to form mature mRNAs. In some instances, spliceosomes are formed, but subsequent catalytic steps are inhibited. For example, a
protein synthesized in herpesvirus-infected cells suppresses RNA splicing and leads to reduced amounts of cellular mRNAs and the accumulation of primary mRNA transcripts. In addition to interference with host-cell mRNA transcription and processing, viruses may produce factors that bind to ribosomes and inhibit cellular mRNA translation. Viral proteins may inhibit the processing and
transport of cellular proteins from the endoplasmic reticulum, and this inhibition may lead to their degradation.

This effect is seen in lentivirus and adenovirus infections. Influenza viruses remove the 5’ cap structure of cellular mRNAs to initiate synthesis of viral mRNAs, the cap being required for translation. Other viruses simply produce viral mRNAs in large quantities in order to assure translation, outcompeting cellular mRNAs for cellular translation machinery by mass action. The cumulative effect of inhibition of host-cell protein synthesis and depletion of nucleotide pools can be the loss of cellular homeostasis, resulting in a sequence of degeneration and necrosis. This progression is relatively nonspecific as to cause, with similar changes being induced by physical or chemical insults. The most common early and potentially reversible change is cloudy swelling, a change associated with increasing permeability of the cellular membranes leading to swelling of the nucleus, distention of the endoplasmic reticulum and mitochondria, and rarefaction of the cytoplasm. Later in the course of many viral infections the nucleus becomes condensed and shrunken, and cytoplasmic density increases. Cell destruction can be the consequence of further loss of osmotic integrity and leakage of lysosomal enzymes into the cytoplasm. This progression is consistent with the so-called common terminal pathway to cell death. In contrast to these nonspecific changes are toxicities induced by viral proteins that interfere with cellular membrane or cytoskeletal structure and function.
Interference with Cellular Membrane Function can affect the participation of cellular membranes in many phases of virus replication, from virus attachment and entry, to the formation of replication complexes, to virion assembly. Viruses may alter plasma membrane permeability, affect ion exchange and membrane potential, or induce the synthesis of new intracellular membranes or the rearrangement of previously existing ones. For example, a generalized increase in membrane permeability occurs early during picornavirus, alphavirus, reovirus, rhabdovirus, and adenovirus infections. Early changes in cell structure often are dominated by proliferation of various cell membranes: for example, herpesviruses cause increased synthesis, even reduplication, of nuclear membranes; flaviviruses cause proliferation of the endoplasmic reticulum; picornaviruses and caliciviruses cause a distinctive proliferation of vesicles in the cytoplasm; and many retroviruses cause peculiar fusions of cytoplasmic membranes.

Enveloped viruses specifically direct the insertion of their surface glycoproteins, including fusion proteins, into host-cell membranes as part of their budding process, and this may lead to membrane fusion between infected and uninfected cells, resulting in the formation of a multinucleated syncytium. This activity is restricted to enveloped viruses whose

fusion proteins are activated when viral
membrane glycoproteins come in contact with a cellular receptor. Normally this process allows fusion of the virion envelope with the cytoplasmic membrane of a target cell during the initiation of an infection, allowing entry the viral genome into the cytoplasm. In the course of virus replication however, these same fusion proteins are inserted into the cytoplasmic membrane of the infected
cell in preparation for viral budding

If viral membrane glycoproteins engage receptors on neighboring cells, the fusion proteins may be activated to cause fusion of one cell membrane with another, giving rise to the syncytial cell. Syncytia are a conspicuous feature of infection of cell monolayers in culture by lentiviruses, coronaviruses, paramyxoviruses, and some herpesviruses.
Syncytia may also be observed in tissue of infected animals, particularly for paramyxoviruses; for example, in horses infected with Hendra virus and cattle infected with respiratory syncytial virus. Syncytium formation has been suggested as a means by which viruses spread in tissues: fusion bridges may allow subviral entities, such as viral nucleocapsids and nucleic acids, to spread while avoiding host defenses. Relevance of this mechanism is likely limited to specific cell types, being implicated as a means for neuron-to-neuron spread of rhabdoviruses and paramyxoviruses where fusion events are likely restricted to very small cell contact points within the synapse.
Viral proteins (antigens) inserted into the host-cell plasma membrane may constitute targets for specific humoral and cellular immune responses that cause the lysis of the infected cell. This may happen before significant progeny virus is produced, thus slowing or arresting the progress of infection and hastening recovery (see Chapter 4: Antiviral Immunity and Virus Vaccines). It is for this reason that accumulation of viral membrane glycoproteins occurs late in the infection cycle, in preparation for viral budding. Although these viral proteins are attractive targets for immune clearance, the host response may also contribute to immune-mediated tissue injury and disease. Viral antigens may also be incorporated in the membrane of cells transformed by viruses, and play an important role in immune-mediated resolution or regression of viral papillomas, for example.
It should be noted that cell surface expression of membrane glycoproteins was exploited early in the development of diagnostic tests (see Chapter 5: Laboratory Diagnosis of Viral Infections). Many of these viral proteins have the potential to bind glycoproteins expressed on the surface of red blood cells in a species specific manner. Cells in monolayer cultures infected with influenza viruses, paramyxoviruses, and togaviruses, all of which bud from the plasma membrane, acquire the ability
to adsorb erythrocytes in a phenomenon termed hemadsorption (

The viral membrane glycoprotein serves as a receptor for ligands on the surface of erythrocytes. The same glycoprotein spikes are responsible for hemagglutination in vitro—that is, the agglutination of erythrocytes by free viral particles. Hemadsorption and hemagglutination are not known to play a part in the pathogenesis of viral diseases.
Disruption of the Cell Cytoskeleton causes changes in cell shape (eg, rounding) that are characteristic of many viral infections. The cytoskeleton is made up of several filament systems, including microfilaments (eg, actin), intermediate filaments (eg, vimentin), and microtubules (eg, tubulin). The cytoskeleton is responsible for the structural integrity of the cell, for the transport of organelles through the cell, and for certain cell motility

activities. Particular viruses may damage specific filament systems: for example, canine distemper virus, vesicular stomatitis viruses, vaccinia virus, and herpesviruses cause a depolymerization of actin-containing microfilaments, and enteroviruses induce extensive damage to microtubules. Such damage contributes to the drastic cytopathic changes that precede cell lysis in many infections. The elements of the cytoskeleton are also employed by many viruses in the course of their replication: in virus entry, in the formation of replication complexes and assembly sites, and in virion release.

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. It was long thought that viruses killed cells exclusively 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. There are two distinct cellular pathways that trigger apoptosis, 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 antiapoptotic (eg, Bcl-2) and proapoptotic (eg, 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-membrane receptors, which are members of the tissue necrosis factor (TNF) receptor family (TNF, Fas, and others). Thus binding of tissue necrosis factor 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.

Noncytopathic Changes in Virus-Infected Cells

Noncytopathic viral infections usually do not kill the cells in which replication occurs. On the contrary, they often cause persistent infection during which infected cells produce and release virions but cellular metabolism that is essential to maintaining homeostasis is either not affected or is minimally affected. In many instances, infected cells even continue to grow and divide. This type of interaction can occur in cells infected with several kinds of RNA viruses, notably pestiviruses, arenaviruses, retroviruses, and some paramyxoviruses. Nevertheless, with few exceptions (eg, some retroviruses), there are slowly progressive changes that ultimately lead to cell death. In the host animal, cell replacement occurs so rapidly in most organs and tissues that the slow fallout of cells as a result of persistent infection may have no effect on overall function, whereas terminally

differentiated cells such as neurons, once destroyed, are not replaced, and persistently infected differentiated cells may lose their capacity to carry out specialized functions.

Viruses such as the pestiviruses, arenaviruses, Bornavirus, and retroviruses that do not shut down hostcell protein, RNA, or DNA synthesis and that do not rapidly kill their host cells, can produce important pathophysiologic changes in their hosts by affecting crucial functions that are associated neither with the integrity of cells nor their basic housekeeping functions. Damage to the specialized functions of differentiated cells may still affect complex regulatory, homeostatic, and metabolic functions, including those of the central nervous system, endocrine glands, and immune system.
Вирусы, такие как пестивирусы, аренавирусы, борнавирусы и ретровирусы, которые не блокируют синтез белка, РНК или

Virus-Mediated Tissue and Organ Injury

The severity of a viral disease is not necessarily correlated with the degree of cytopathology produced by the causative virus in cells in culture. Many viruses that are cytocidal in cultured cells do not produce clinical signs in vivo (eg, many enteroviruses), whereas some that are noncytocidal in vitro cause lethal disease in animals (eg, rabies virus). Further, depending on the organ affected, cell and
tissue damage can occur without producing clinical signs of disease—for example, a large number of hepatocytes (liver cells) may be destroyed in Rift Valley fever in sheep without obvious clinical signs. When damage to cells does impair the function of an organ or tissue, this may be relatively insignificant in a tissue such as skeletal muscle, but potentially devastating in organs such as the
heart or the brain. Likewise, virus-induced inflammation and edema are especially serious consequences in organs such as the lungs and CNS.
Mechanisms of Viral Infection and Injury of Target Tissues and Organs The mechanisms by which individual viruses cause injury to their specific target organs are described in detail under individual virus families in Part II of this book, thus the objective of this section is to provide a brief overview of
potential pathogenic mechanisms that viruses can use to cause injury in their target tissues.

Viral Infection of the Respiratory Tract

Viral infections of the respiratory tract are extremely common, especially in animals housed in crowded settings. Individual viruses exhibit tropism for different levels of the respiratory tract, from the nasal passages to the pulmonary airspaces (terminal airways and alveoli), but there is considerable overlap. Tropism of respiratory viruses is probably a reflection of the distribution of appropriate receptors and intracellular transcriptional enhancers, as well as physical barriers, physiological factors, and immune parameters. For example, bovine rhinitis viruses (Family Picornaviridae) replicate in the nasal
passages because their replication is optimized at lower temperatures, whereas bovine respiratory syncytial virus (Family Paramyxoviridae) preferentially infects epithelial cells lining the terminal airways; thus rhinitis viruses may cause mild rhinitis, whereas respiratory syncytial virus is
the cause of bronchiolitis and bronchointerstitial pneumonia. Some viruses cause injury to the type I or type II pneumocytes lining the alveoli, either directly or indirectly; if

extensive, injury to type I pneumocytes leads to acute respiratory distress syndrome, whereas injury to type II pneumocytes delays repair and healing in the affected lung.

Influenza viruses replicate in both the nasal passages and airways of infected mammals, but influenza virus infection is typically confined to the lung because of the requirement for hemagglutinin cleavage by tissue-specific proteases. However, highly virulent influenza viruses such as the EurasianAfrican H5N1 virus can spread beyond the lungs to cause severe generalized (systemic) infection and disease. The ability of this virus to escape the lung may be related to its tropism for type I neumocytes that line alveoli, and its ability to cause systemic disease may reflect the fact that its hemagglutinin can be cleaved by proteases that are present in many tissues.
Similarly in birds, high-pathogenicity avian influenza viruses have several basic amino acids at the hemagglutinin cleavage site, which can be cleaved intracellularly by ubiquitous endopeptidase furins located in the trans-Golgi network in a wide variety of cell types in various tissues. In contrast, the hemagglutinin protein of low pathogenicity avian influenza viruses is cleaved extracellularly by
tissue-restricted proteases that are confined to the respiratory and gastrointestinal tracts (see Chapter 21: Orthomyxoviridae).

Regardless of the level of the respiratory tree that is initially infected, viral infection typically leads to local cessation of cilial activity, focal loss of integrity of the lining mucus layer, and multifocal destruction of small numbers of epithelial cells). Initial injury is followed by progressive infection of epithelial cells within the mucosa, and inflammation of increasing severity, with
exudation of fluid and influx of inflammatory cells. Fibrin-rich inflammatory exudate and necrotic cellular debris (degenerate neutrophils and sloughed epithelium) then accumulate in the lumen of the affected airways or passages, with subsequent obstruction and, in severe cases, increasing hypoxia and respiratory distress. The mucosa is quickly regenerated in animals that survive, and adaptive immune responses clear the infecting virus and prevent reinfection for variable periods of time
(depending on the particular virus).

In addition to their direct adverse consequences, viral infections of the respiratory tract often predispose animals to secondary infections with bacteria, even those bacteria that constitute the normal flora in the nose and throat. This predisposition can result from interference with normal mucociliary clearance as a consequence of viral injury to the mucosa, or suppression of innate immune responses. For example, cellular expression of Toll-like receptors is depressed in the lung after influenza virus infection, and thus convalescent animals may be less able to quickly recognize and neutralize invading bacteria.

This potential synergy between respiratory viruses and bacteria is compounded by overcrowding of animals as occurs during shipping and in feedlots and shelters. Environmental factors may combine to facilitate concurrent airway infection by multiple viruses and bacteria. These polymicrobial infections are facilitated by the immunosuppressive effects of stress, the induction of ciliary stasis by exposure to ammonia vapor from animal waste, and crowded humid environments that facilitate

aerosol transmission of enveloped viruses. Viral Infection of the Gastrointestinal Tract Infection of the gastrointestinal tract can be acquired either by ingestion of an enteric virus (eg, rotaviruses,
coronaviruses, astroviruses, etc.) where infection is confined to the gastrointestinal tract or as a consequence of generalized hematogenous spread during a systemic viral infection such as with certain parvoviruses (eg, feline panleukopenia, canine parvovirus), pestiviruses (eg, bovine viral diarrhea virus), and morbilliviruses (eg, canine distemper and rinderpest viruses). Enteric viral
infections usually result in rapid onset of gastrointestinal disease after a short incubation period, whereas systemic infections have a longer incubation period and are typically accompanied by clinical signs that are not confined to dysfunction of the gastrointestinal tract.

Virus-induced diarrhea is a result of infection of the epithelial cells (enterocytes) lining the gastrointestinal mucosa. Rotaviruses, astroviruses, and enteric coronaviruses characteristically infect the more mature enterocytes that line the intestinal villi, whereas parvoviruses and pestiviruses infect and destroy the immature and dividing enterocytes present in the intestinal crypts. Regardless of their site of predilection, these infections all destroy enterocytes in the gastrointestinal mucosa and so
reduce its absorptive surface, leading to malabsorption diarrhea with attendant loss of both fluid and electrolytes. The pathogenesis of enteric virus infections can be even more complex than simple virus-mediated destruction of enterocytes; for example, rotaviruses produce a protein (nsp4) that itself causes secretion of fluid into the bowel (intestinal hypersecretion), even in the absence of substantial virus-mediated damage. In suckling neonates, undigested lactose from ingested milk passes through the small bowel to the large bowel, where it exerts an osmotic effect that further exacerbates fluid loss. Neonates are also disadvantaged by the fact that the replacement rate of enterocytes is not as high as in older animals. Animals with severe diarrhea can rapidly develop pronounced dehydration, hemoconcentration, acidosis that inhibits critical enzymes and metabolic pathways, hypoglycemia,
and systemic electrolyte disturbances (typically, decreased sodium and increased potassium), and diarrhea can be quickly fatal in very young or otherwise compromised animals.

Enteric virus infections that occur via the oral route generally begin in the stomach or proximal small intestine, and they then spread caudally as a “wave” that sequentially affects the jejunum, ileum, and large bowel. As the infection progresses through the bowel, absorptive cells destroyed by the infecting virus are quickly replaced by immature enterocytes from the intestinal crypts. The presence of increased numbers of these immature enterocytes contributes to malabsorption and intestinal hypersecretion (fluid and electrolyte loss). Adaptive immune responses lead to mucosal IgA and systemic IgG production in animals that survive, conferring resistance to reinfection. Enteric virus infections in neonates are frequently associated with infections by other enteric pathogens, including bacteria (eg, enterotoxigenic or enteropathogenic Escherichia coli) and protozoa such as
Cryptosporidium spp., probably because of the common factors (crowding, poor sanitation) that predispose to these infections.

Viral Infection of the Skin

In addition to being a site of initial infection, the skin may be invaded secondarily via the bloodstream. Thus skin lesions that accompany viral infections can be either localized, such as papillomas, or disseminated. In animals, erythema (reddening) of the skin as a consequence of systemic viral infections is most obvious on exposed, hairless, nonpigmented areas such as the snout, ears, paws,
scrotum, and udder. In addition to papillomas (warts, see Chapter 11: Papillomaviridae and Polyomaviridae), virus-induced lesions that commonly affect the skin of virus-infected animals include macules (flat discolored areas of skin), papules (raised areas of skin), vesicles (fluid-filled raised areas of skin), and pustules (raised areas of skin containing leukocytes).

Viruses of particular families tend to produce characteristic cutaneous lesions, frequently in association with similar lesions in the oral and nasal mucosa, the teats and genitalia, and at the junction of the hooves and skin of ungulates. Vesicles are especially important cutaneous lesions that are characteristic of important, potentially reportable diseases of livestock; in particular, vesicle formation is characteristic of foot-and-mouth disease and other viral diseases that can mimic it, although vesicles clearly can occur in other diseases not caused by viruses. Vesicles are essentially discrete “blisters” that result from accumulation of edema fluid within the affected epidermis, or separation of the epidermis from the underlying dermis (or mucosal epithelium from the submucosa). Vesicles rupture quickly to leave focal ulcers. Papules are either localized (eg, orf) or disseminated (eg, lumpy skin disease) epithelial proliferations that are characteristic of poxvirus infections. These proliferative and raised lesions frequently become extensively encrusted with inflammatory exudate. Virus infections that result in widespread endothelial injury in blood vessels throughout the body, including those of the subcutaneous tissues, can produce subcutaneous edema and erythema or hemorrhages in the skin and elsewhere (including the oral cavity and internal organs).

Viral Infection of the Central Nervous System

The CNS (brain and spinal cord) is exquisitely susceptible to serious, often fatal injury by certain viral infections provided the virus can gain access to these tissues. Viruses can spread from distal sites to the brain via nerves (Fig. 3.7), or via the blood (Fig. 3.13). Spread via nerves may involve peripheral nerve endings or infection of olfactory neurons in the nasal cavity with subsequent viral transport by axons of the olfactory nerve directly into the brain. To spread from the blood, viruses must cross either
the bloodbrain or bloodcerebrospinal fluid barriers.

The bloodbrain barrier consists of endothelial cells that are nonfenestrated and connected by tight junctions, which in turn are surrounded by a basement membrane and astrocytes. Viruses may cross this barrier by either direct infection of endothelial cells and spread of infection to the adjacent astrocytes, or the virus may be carried across the barrier by infected leukocytes that are engaged
in immune surveillance of the CNS or inflammatory responses. The bloodcerebrospinal fluid barrier is
formed by tight junctions between epithelial cells of the choroid plexus, which are highly vascular structures producing the cerebrospinal fluids that circulate within the entricles of

the brain, the central canal of the spinal cord, and the leptomeninges that cover the surface of both brain and spinal cord. There is no barrier between the bloodstream and the epithelial cells of the plexus, and if virus can infect the choroid plexus epithelial cells, it may be shed through the cerebrospinal fluid pathways to be widely disseminated in the CNS. Collectively, a virus' ability to overcome these barriers and initiate CNS infection are known as neuroinvasiveness.

Once present within the CNS, a number of viruses can quickly spread to cause progressive infection of neurons and/or glial cells (astrocytes, microglia, and oligodendrocytes). This capability is known as neurovirulence. A virus can be poorly neuroinvasive, but if infection is initiated, exhibit a high degree of neurovirulence. Cytopathic infections of neurons, whether caused by togaviruses, flaviviruses, herpesviruses, or other viruses, leads to encephalitis or encephalomyelitis characterized by neuronal necrosis, phagocytosis of neurons (neuronophagia), and perivascular infiltrations of inflammatory cells (perivascular cuffing). The small vessels of the meninges are frequently involved in virus-induced inflammation of the CNS, either alone (meningitis) or in combination with inflammation of the brain and spinal cord (meningoencephalitis and meningomyelitis). In contrast, virulent rabies virus infection of neurons is noncytocidal and evokes little inflammatory reaction, but it is uniformly lethal for most mammalian species.

Other characteristic pathologic changes are produced by various viruses, and by prions that cause slowly progressive diseases of the CNS. In bovine spongiform encephalopathy in cattle and scrapie in sheep, for example, there is slowly progressive neuronal degeneration and vacuolization. In contrast, infection of glial cells in dogs with canine distemper leads to progressive demyelination.
In most cases, infection of the CNS seems to be a dead end in the natural history of viruses—shedding and transmission does not occur, particularly when the infection is highly cytopathic. Viruses that successfully use neurons for transmission are in the minority and they typically exhibit noncytopathic or poorly cytopathic infections. Noncytopathic infection of neurons is needed for rabies virus to complete the cycle of infection within a host. Rabies is reliant upon axon transporters of viable cells to travel from the point of inoculation to the CNS, to spread within the CNS, and to spread from the CNS to peripheral organs such as salivary glands that amplify and shed progeny virus. Cell death and the attending inflammatory response can prevent the virus from completing this cycle in more highly cytopathic infections, where the virus is less adapted to its host. The alphaherpesviruses undergo latent infection of peripheral nerves, specifically the dorsal root ganglion neurons. When reactivated, the infection is productive yet noncytopathic for the neuron. Progeny infect epithelial cells of mucosal surfaces where the infection is both productive and cytopathic. All in all, it seems anomalous that neurotropism should be the outstanding characteristic of so many of the most notorious pathogens of animals and zoonotic pathogens of humans, and yet be the characteristic least related to virus propagation in nature.

Viral Infection of the Hemopoietic System (p. 83)

The hemopoietic system includes: (1) the myeloid tissues, specifically the bone marrow and cells derived from it—erythrocytes, platelets, monocytes, and granulocytes; and (2)

the lymphoid tissues, which include the thymus, lymph nodes, spleen, mucosa-associated lymphoid tissues and, in birds, the cloacal bursa. Cells that populate the myeloid and lymphoid systems, including lymphocytes, dendritic cells, and cells of the mononuclear phagocytic system (monocytes and macrophages) are all derived from bone marrow (or equivalent hemopoietic tissue) precursors. It is therefore convenient to group these cells and tissues under the heading of the hemopoietic system and to dispense with historical terminology such as "lymphoreticular" or "reticuloendothelial" systems. Importantly, lymphocytes and mononuclear phagocytes (blood monocytes, tissue macrophages, dendritic cells) are responsible for adaptive immunity (see Chapter 4: Antiviral Immunity and Virus Vaccines), thus viral infections of these cells can have profound effects on immunity.

Infection and damage to mononuclear phagocytes can inhibit both the innate and adaptive immune response to the virus, in addition to serving as a source of progeny virions. Some of the most destructive and lethal viruses known exhibit this tropism: filoviruses, arenaviruses, hantaviruses, orbiviruses such as African horse sickness and bluetongue viruses, certain bunyaviruses such as Rift Valley fever virus, alphaviruses such as Venezuelan equine encephalitis virus, and flaviviruses such as yellow fever virus. After initial invasion, infection with these viruses begins with their uptake by dendritic cells and/or macrophages in lymphoid tissues (lymph nodes, thymus, bone marrow, Peyer's patches, and the white pulp of the spleen). Viral infection can then spread in these tissues, frequently leading to cytolysis of adjacent lymphocytes and immune dysfunction.

Viral infections can result in either specific acquired immunodeficiency or generalized immunosuppression. A relevant example of this phenomenon is provided by infection of the cloacal bursa (bursa of Fabricius) in chickens (the site of B cell differentiation in birds) with infectious bursal disease virus, which leads to atrophy of the bursa and a severe deficiency of B lymphocytes, equivalent to bursectomy. The result is an inability of severely affected birds to develop antibody-mediated immune responses to other infectious agents, which in turn leads to an increase in susceptibility to bacterial infections such as those caused by Salmonella spp. and E. coli, and other viruses. Acquired immunodeficiency syndrome (AIDS) in humans is caused by the human immunodeficiency virus (HIV), and similar viruses infect monkeys (simian immunodeficiency viruses), cattle (bovine immunodeficiency virus), and cats (feline immunodeficiency virus). In susceptible animals, these viruses individually can infect and destroy specific but different cells of the immune system, thereby causing immunosuppression of different types and severity.

Many other viruses (eg, classical swine fever virus, bovine viral diarrhea virus, canine distemper virus, feline and canine parvoviruses) that cause systemic infections, especially those that infect mononuclear phagocytes and/ or lymphocytes, may temporarily but globally suppress adaptive immune responses, both humoral and cellmediated. Affected animals are predisposed to diseases caused by other infectious agents during the period of virus-induced immunosuppression, a phenomenon that can also occur following vaccination with certain liveattenuated vaccines. The immune response to unrelated antigens may be reduced or abrogated in

animals undergoing such infections. Virus-induced immunosuppression may in turn lead to enhanced virus replication, such as the reactivation of latent herpesvirus, adenovirus, or polyomavirus infections. Similarly, immunosuppression associated with administration of cytotoxic drugs or irradiation for chemotherapy or organ transplantation can predispose to recrudescence of herpesviruses and, potentially, others.

Viral Infection of the Fetus

Most viral infections of the dam do not lead to infection of the fetus due to barrier functions provided by the placenta, although severe infections of the dam can sometimes lead to fetal death and expulsion (abortion) in the absence of fetal infection. However, some viruses can cross the placenta to infect the fetus (Table 3.3). Such infections occur most commonly in young dams (such as first-calf heifers) that are exposed during pregnancy to pathogenic viruses to which they have no immunity, as a consequence of lack of either appropriate vaccination or previous natural infection. The outcome of fetal viral infection is dependent upon the properties (virulence and tropism) of the infecting virus, as well as the gestational age of the fetus at the time of infection. Severe cytolytic infections of the fetus, especially in early gestation, are likely to cause fetal death and resorption or abortion, which also is dependent on the species of animal affected—abortion is especially common in those species in which pregnancy is sustained by fetal production of progesterone (such as sheep), whereas pregnancy is less likely to be terminated prematurely in multiparous species in which pregnancy is maintained by maternally derived progesterone (such as swine).

Teratogenic viruses are those that can cause developmental defects after in utero infection. The outcome of infections of pregnant animals with teratogenic viruses is influenced to a great extent by gestational age, which influences stages of organogenesis, degree to which biological barriers have formed in tissues such as the CNS, and degree of immune competence. Viral infections that occur during critical stages of organogenesis in the developing fetus can have devastating consequences from virus-mediated infection and destruction of progenitor cells before they can populate organs such as the brain. For example, Akabane, Cache Valley and Schmallenberg viruses, bovine viral diarrhea virus, and bluetongue virus can all cause teratogenic brain defects in congenitally infected ruminants, as can parvovirus infections in cats.

Although immune competence generally is developed by mid-gestation, viral infections before this time can lead to a weak and ineffectual immune response that leads to persistent postnatal infection, such as persistent bovine viral diarrhea virus infection in cattle and congenital lymphocytic choriomeningitis virus infection in mice.

Viral Infection of Other Organs

Almost any organ may be infected with one or another kind of virus via the bloodstream, but most viruses have well-defined organ and tissue tropisms that reflect the factors described earlier (presence of receptors, intracellular and other physiological or physical determinants of infection). The clinical importance of infection of various organs and tissues depends, in part, on their role in the physiologic well-being of the animal. In addition to the organs and tissues already described (respiratory tract, gastrointestinal tract, skin, brain and spinal cord, hemopoietic

tissues), viral infections of the heart and liver can also have especially devastating consequences. The liver
is the target of relatively few viral infections of animals, in marked contrast to the numerous hepatitis viruses (hepatitis A, B, and C viruses in particular) and other viruses (eg, yellow fever virus) that are important causes of severe liver disease in humans. In animals, Rift Valley fever virus, mouse hepatitis virus, and infectious canine hepatitis virus characteristically affect the liver, as do several abortigenic herpesviruses after fetal infections (eg, infectious bovine rhinotracheitis virus, equine herpesvirus
1, pseudorabies virus). Virus-mediated cardiac injury is relatively uncommon in animals, but is characteristic of bluetongue and some other endotheliotrophic viral infections, and alphavirus infections of Atlantic salmon and rainbow trout.

Viruses that cause widespread vascular injury can result in disseminated hemorrhages and/or edema as a result of increased vascular permeability. Vascular injury in these so-called hemorrhagic viral fevers can result either from viral infection of endothelial cells or the systemic release of vasoactive and inflammatory mediators such as tissue necrosis factor from other infected cells— particularly mononuclear phagocytes and dendritic cells. Viruses causing vascular injury include dengue virus, yellow fever virus, ebola virus, and different hantavirus infections in humans, and bluetongue and African horse sickness viruses in livestock. Widespread endothelial injury leads to thrombosis that may precipitate disseminated intravascular coagulation, which is the common pathway that leads to death of animals and humans infected with a variety of viruses that directly or indirectly cause vascular injury. Paradoxically, these infected individuals bleed profusely due to the consumption of clotting factors.

Nonspecific Pathophysiological Changes in Viral Diseases

Some of the adverse consequences of viral infections cannot be attributed to direct cell destruction by the virus, to immunopathology, or other physiological responses that may include release of endogenous adrenal glucocorticoids in response to the stress of the infection. Viral diseases are accompanied frequently by a number of vague general clinical signs, such as fever, malaise, anorexia,
and lassitude. Cytokines (interleukin-1 in particular) produced in the course of innate immune responses to infection may be responsible for some of these signs, which collectively can significantly reduce the animal's performance. Less characterized are the potential neuropsychiatric effects of persistent viral infection of particular neuronal tracts, such as that caused by Borna disease virus. Borna disease virus infection is not lytic in neurons, but induces bizarre changes in the behavior of rats, cats, and horses.

Virus-Induced Immunopathology The adaptive immune response (eg, antibodies and cytotoxic T cells) to viruses could theoretically be harmful if the elimination of virusinfected cells leads to dangerous physiological consequences (eg, damage to liver or heart). The concept of virus-induced immunopathology is based on experimental results obtained in mouse models. Antibody-mediated
immunopathology (also called type III hypersensitivity reactions) is caused by deposition of complexes of antigen and antibody (immune complexes) that initiate inflammation and tissue damage. Immune complexes circulate in blood in the course of most viral infections. The fate of the immune complexes depends on the ratio of antibody to antigen. The virus

is typically cleared by tissue macrophages in infections where there is a large excess of antibody as compared with circulating virus, or even if there
are equivalent amounts of antibody and virus. However, in some persistent infections, viral proteins (antigens) and/or virions are released continuously into the blood but the antibody response is weak and antibodies are of low avidity. In these instances, immune complexes are deposited in small blood vessels that function as filters, especially those of the renal glomeruli. Immune complexes continue to be deposited in glomeruli over periods of weeks, months, or even years, leading to their accumulation and subsequent immune-complex mediated glomerulonephritis. This phenomenon is observed in Aleutian mink disease (parvovirus infection), feline leukemia, and equine infectious anemia.
A similar pathogenesis may underlie the progression of feline infectious peritonitis, a multisystemic disease associated with coronavirus infection in cats. T cell-mediated immunopathology (also called type IV reactions or delayed hypersensitivity reactions) has only been unequivocally demonstrated in mouse models of lymphocytic choriomeningitis virus infection.

Viruses and Autoimmune Disease

It has been proposed, with little definitive evidence, that viral infections may be responsible for autoimmune diseases in animals and humans. Proposed mechanisms for this largely hypothetical phenomenon focus on either unregulated or misdirected immune responses precipitated by a viral infection, or the presence of shared or equivalent antigens on infectious agents and host cells (molecular mimicry). Molecular mimicry clearly is responsible for immune-mediated diseases initiated by microbial infection, as classically illustrated by rheumatic heart disease in humans that is initiated by group A Streptococcus infection. In viruses, individual epitopes have been identified in several viruses that are also present in animal tissue, such as muscle or nervous tissue (eg, myelin basic protein). The antibodies to these epitopes might contribute to immune-mediated tissue damage during the course of viral infection, but their pathogenic role, if any, in initiating and potentiating autoimmune disease remains uncertain.

Persistent Infection and Chronic Damage to Tissues and Organs

Persistent infections of one type or another are produced by a wide range of viruses, and are common in veterinary medicine. Apart from enteric and respiratory viruses that cause transient infections that remain localized to their respective target organs, most other categories of viral infections include examples of persistent infection. Foot-and-mouth disease, for example, usually is an acute, self-limiting infection, but a carrier state of uncertain epidemiological relevance occurs in which virus persists in the oropharynx of a very few convalescent animals. In other instances, such as those associated with immunodeficiency viral infections, persistent viral infections lead to chronic diseases, even when the acute manifestations of infection have been trivial or subclinical. Finally, persistent infections can lead to continuing tissue injury, often with an immune-mediated basis.
Persistent viral infections are important for several reasons. For example, they may be reactivated and cause recrudescent episodes of disease in the individual host, or they may lead to immune-mediated disease or to neoplasia. Persistent infection may allow survival of a particular virus in individual animals and herds, even after vaccination. Similarly, persistent infections may be of epidemiologic importance—the source of contagion in

long-distance virus transport and in reintroduction after elimination of virus from a given herd, flock, region, or country.

Persistent infections are manifest in several ways. There are persistent infections in which virus is demonstrable continuously, whether or not there is ongoing disease. Disease may develop late, often with an immunological or neoplastic basis. In other instances, disease is not manifest in persistently infected animals; for example, in the deer mouse (Peromyscus maniculatus), the reservoir rodent host of Sin Nombre virus, and the etiologic agent of hantavirus pulmonary syndrome in humans, virus is shed in urine, saliva, and feces probably for the life of the animal, even in the face of neutralizing antibody.

A striking proportion of persistent infections involve the CNS. Restrictions in antigen presentation by neurons and glia and the activity of regulatory T cells combine to tightly regulate immune responses in the CNS. This regulation is important in order to assure that immune and inflammatory responses do not disrupt the highly specialized functions of terminally differentiated neurons and myelin producing cells. Myelin also contains unique antigens capable of eliciting autoimmune reactions, further emphasizing the importance of regulating immune responses in the CNS. This environment poses an excellent opportunity for a virus to avoid immune surveillance, and neurons and glia often exhibit limited permissiveness to virus gene expression that favors a noncytopathic persistent infection.

Latent infections are a form of persistence in which infectious virus is not demonstrable except when reactivation occurs. For example, in infectious pustular vulvovaginitis, the sexually transmitted disease caused in cattle by bovine herpesvirus 1, virus usually cannot be isolated from the latently infected carrier cow except when there are recrudescent lesions. Viral latency may be maintained by restricted expression of genes that have the capacity to kill the cell. During latency, herpesviruses express only a few genes that are necessary in the maintenance of latency, notably so-called latency-associated transcripts.

During reactivation, which is often stimulated by immunosuppression and/or by the action of a cytokine or hormone, the whole viral genome is transcribed again. This strategy protects the virus during its latent state from all host immune actions that would normally result in virus clearance.

The dynamic nature of viruscell or virustissue interactions gives rise to a spectrum of clinical manifestations of disease associated with viral persistence (Fig. 3.14).

Slow infections is a clinical term used to describe a slowly progressive disease, where the initiation of infection is subclinical, and evidence of disease builds slowly as the virus persists. Persistence is associated with a progressive increase in viral burden and antigen expression and the associated inflammatory and immune responses that are the basis for disease. An example of a slow virus infection is ovine progressive pneumonia caused by retrovirus infection (see Chapter 14: Retroviridae). In chronic diseases, there may be evidence of the initiation of infection (ie, the acute clinical episode) followed by the clinical manifestations of persistence in which disease progresses more rapidly following an incubation period. Canine distemper virus

infection in the CNS can be manifest as a chronic disease following acute multisystemic infection, although the initial infection may go unrecognized and the incubation period for the appearance of clinical neurological disease may be prolonged as in a slow virus infection. Viruses that undergo latency with episodes of periodic reactivation may similarly be manifest as chronic diseases. These examples highlight the limitations of using clinical terminology to describe virus infection of a host, where the presence or absence of the virus or the different types of virustissue interactions are considered separately. To further illustrate this latter point are examples where acute infections have late clinical manifestations in which continuing replication of the causative virus is not involved in the progression of the disease. For example, in the cerebellar hypoplasia syndrome that occurs in young cats as a result of fetal infection with feline panleukopenia virus, virus cannot be isolated at the time neurologic damage is diagnosed. In fact, because of this, the cerebellar syndrome was for many years considered to be an inherited malformation.

Further, some persistent infections possess features of more than one of these categories. For example, all retrovirus infections are persistent and most exhibit features of latency, but the diseases they cause may be delayed following infection or only manifest as slowly progressive diseases.

Individual viruses employ a remarkable variety of strategies for successful evasion of host immune and inflammatory responses in vivo. These mechanisms include noncytocidal infections without expression of immunogenic proteins, replication in cells of the immune system or subversion of host innate and adaptive immunity (see Chapter 4: Antiviral Immunity and Virus Vaccines), and infection of nonpermissive, resting, or undifferentiated cells. Some viruses have evolved strategies for evading neutralization by the antibody they elicit.

Ebola virus, for example, uses an "immune decoy" to evade neutralizing antibody—specifically, a secreted viral protein that binds circulating antibody. The surface glycoproteins of filoviruses, arenaviruses, bunyaviruses (eg, Rift Valley fever virus), and some arteriviruses (eg, porcine reproductive and respiratory syndrome virus and lactate dehydrogenase-elevating virus) are heavily glycosylated, which may serve to mask the neutralizing epitopes contained in these proteins. Antigenic drift is especially characteristic of persistent RNA viral infection, particularly for lentiviruses (eg, equine infectious anemia virus). During persistent infection, sequential antigenic variants are produced, with each successive variant sufficiently different to evade the immune response raised against the preceding variant. In equine infectious anemia, clinical signs occur in periodic cycles, with each cycle being initiated by the emergence of a new viral variant. In addition to providing a mechanism for escape from immune elimination, each new variant may be more virulent than its predecessor, and this may directly affect the severity and progression of the disease. The integration of retroviral proviral DNA into the genome of the host germ-line cells assures indefinite maintenance from one generation to the next; such proviral DNA can also lead to induction of tumors (oncogenesis).

VIRUS-INDUCED NEOPLASIA

Neoplasms arise as a consequence of the dysregulated growth of cells derived from a few

or a single, genetically altered progenitor cell(s). Thus, although neoplasms are often composed of several cell types, they are considered to originate from an oligoclonal or monoclonal outgrowth of a single cell. The genetic changes that are ultimately responsible for neoplasia may be caused by naturally occurring mutations, chemical or physical agents or infectious agents including viruses, but all involve certain common cellular pathways. The discoveries of the viral etiology of avian leukemia by Ellerman and Bang and of avian sarcoma by Rous, in 1908 and 1911, respectively, were long regarded as curiosities unlikely to be of any fundamental significance.

However, study of these avian viruses and related retroviruses of mice has increased our overall understanding of neoplasia greatly, and since the 1950s there has been a steady stream of discoveries clearly incriminating other viruses in a variety of benign and malignant neoplasms of numerous species of mammals, birds, amphibians, reptiles, and fish. Many avian retroviruses are major pathogens of poultry, and several DNA viruses have been determined to be responsible for cancers in humans and animals. Any discussion of virus-induced neoplasia requires that a few commonly used terms are defined: a neoplasm is a new growth (syn. tumor) which can be benign or malignant; neoplasia is the process that leads to the formation of neoplasms (syn. carcinogenesis); oncology is the study of neoplasia and neoplasms; a benign neoplasm is a growth produced by abnormal cell proliferation that remains localized and does not invade adjacent tissue; in contrast, a malignant neoplasm (syn. cancer) is locally invasive and may also be spread to other parts of the body (metastasis). Carcinomas are cancers of epithelial cell origin, whereas sarcomas are cancers that arise from cells of mesenchymal origin. Solid neoplasms of lymphocytes are designated lymphosarcoma, malignant lymphoma, or lymphoma, whereas leukemias are cancers of hemopoietic origin characterized by circulation of cancerous cells.

The Cellular Basis of Neoplasia

Neoplasia is the result of nonlethal genetic injury, as may be acquired by chemical or physical damage, or from viral infections. Some cancers, however, arise randomly through the accumulation of spontaneous genetic mutations. A neoplasm results from the clonal expansion of cells that have suffered genetic damage, typically in one of four types of normal regulatory genes: (1) protooncogenes, which are cellular genes that regulate growth and differentiation; (2) tumor suppressor genes that inhibit growth, typically by regulating the cell cycle; (3) genes that regulate apoptosis (programmed cell death); (4) genes that mediate DNA repair. Carcinogenesis involves a multistep progression resulting from the cumulative effects of multiple mutations.

Once developed, neoplasms are: (1) self-sufficient, in that they have the capacity to proliferate without external stimuli; for example, as the result of unregulated oncogene activation; (2) insensitive to normal regulatory signals that would limit their growth, such as transforming growth factor and the cyclin-dependent kinases that normally regulate orderly progression of cells through the various phases of the cell cycle; (3) resistant to apoptosis because of either the activation of antiapoptotic molecules or the inhibition of mediators of apoptosis such as p53; (4) limitless potential for replication. Cancers also

may have the ability to invade and spread to distant tissues (metastasis), and neoplasms typically promote the proliferation of new blood vessels that support their growth.

Neoplasia, regardless of cause, is the result of unregulated cellular proliferation. In the normal sequence of events during cellular proliferation, a growth factor binds to its specific cellular receptor, leading to signal transduction that ultimately results in nuclear transcription, which in turn leads to the cell entering and progressing through the cell cycle until it divides. Proto-oncogenes are normal
cellular genes that encode proteins that function in normal cellular growth and differentiation; they include (1) growth factors; (2) growth factor receptors; (3) intracellular signal transducers; (4) nuclear transcription factors; (5) cell cycle control proteins. Oncogenes are derived by mutation of their normal cellular proto-oncogene counterparts, and the expression of oncogenes results in production of oncoproteins that mediate autonomous (unregulated) growth of neoplastic cells.
The development of cancer (malignant neoplasia) is a protracted, multistep process that reflects the accumulation of multiple mutations. Potentially neoplastic cells must bypass apoptosis (programmed death), circumvent the need for growth signals from other cells, escape from immunologic surveillance, organize their own blood supply, and possibly metastasize. Thus, tumors other than
those induced by rapidly transforming retroviruses like Rous sarcoma virus generally do not arise as the result of a single event, but by a series of steps leading to progressively greater loss of regulation of cell division.

Viruses are classified as tumor viruses if part of the viral genome is present in tumors, with expression within the tumor of some viral genes. In vitro, infection of cells with tumor viruses leads to transformation caused by specific viral genes. Infection of experimental animals leads to tumor formation that is preventable by vaccination, although this experiment cannot be performed with most
human viruses because they do not infect rodents. Oncogenic DNA viruses (eg, papillomaviruses, polyomaviruses, herpesviruses) and RNA viruses (retroviruses) have been identified in both animals and humans. DNA viruses can cause neoplasia by inhibiting tumor suppressor genes whereas RNA viruses typically activate protooncogenes. Cells transformed by nondefective retroviruses
also express the full range of viral proteins, and new virions bud from their membranes. In contrast, transformation by DNA viruses usually occurs in cells undergoing nonproductive infection in which viral DNA is integrated into the cellular DNA of the transformed cells or, in the case of papillomaviruses, polyomaviruses and herpesviruses, in which the viral DNA remains episomal. Certain virus-specific antigens are demonstrable in transformed cells.

Oncogenic RNA Viruses

Retrovirus-Induced Neoplasia Retroviruses are a significant cause of neoplasia in many species of animals, including cattle, cats, nonhuman primates, mice, and birds, among others. Their pathogenesis is linked to their propensity to integrate within the genome of host cells, thereby being infectious mutagens. The consequences of such integration are largely innocuous and clinically silent, and only seldom result in oncogenesis. As described in Chapter 14, Retroviridae, retroviruses can be biologically divided into

exogenous (horizontally transmissible) agents, or endogenous. Retroviruses can be either replication-competent or replication-defective. Oncogenic retroviruses are classified as acute transforming or chronic transforming retroviruses. These two major types of transforming retroviruses induce neoplasia in significantly different ways.

Acute Transforming Retroviruses

Acute transforming retroviruses infect mice and birds and are directly oncogenic by carrying an additional viral oncogene, v-onc, and are classified as "transducing" retroviruses. The retroviral v-onc originates from a host c-onc gene, where the transforming activity of the v-onc is accentuated by mutation. These mutations reflect the high error rate of the viral reverse transcriptase. Other viral oncogenes may induce cellular transformation simply by overexpression (from the viral promoter), independent of any mutations. These acquired genes are components of the cell signaling networks and the strongly promoted production of the viral oncoprotein will readily exceed that of the normal cellular oncoprotein. The result can be uncontrolled cell growth. Because c-onc genes are the precursors of v-onc genes, c-onc genes are also called "proto-oncogenes." Wherever acute transforming retroviruses integrate in the host genome, it is the v-onc that is directly responsible for the rapid malignant change that occurs in cells infected with these viruses. Over 60 different v-onc genes have been identified, and retroviruses have been instrumental in identifying their cellular homologues.

The v-onc is usually incorporated into the viral genomic RNA, replacing a portion of one or more normal viral genes. Because such viruses have lost some of their viral genetic sequences, they are usually incapable of replication, and are therefore termed "defective" retroviruses. An exception is Rous sarcoma virus, in that its genome contains a viral oncogene (v-src) in addition to its full complement of functioning viral genes (gag, pol, and env); thus Rous sarcoma virus is both replication-competent and an acute transforming virus. Rous sarcoma virus is one of the most rapidly acting carcinogens known, transforming cultured cells in a day or so and causing neoplasia and death in chickens in as little as 2 weeks after infection. Defective retroviruses circumvent their defective replicative ability by utilizing nondefective "helper" retroviruses for formation of infectious virions.

Replication of the defective virus is thus said to be "rescued" by helper viruses that provide the missing function (eg, an environmentally stable envelope).

Although v-onc genes often compromise retrovirus replication, v-onc genes may be acquired over time by integrated proviruses, most likely because of the effects on cell proliferation that would amplify v-onc containing cells. Cell proliferation also favors replication of helper virus that can rescue the v-onc containing defective virus, thereby facilitating direct viral dissemination of v-onc within a host.

The various v-onc genes and the proteins they encode can be assigned to major classes: growth factors (such as v-sis); growth factor receptors and hormone receptors (such as v-erbB); intracellular signal transducers (such as v-ras); and nuclear transcription factors (such as v-jun).

The oncoprotein products of the various retroviral v-onc genes act in many different ways to affect cell growth, division, differentiation, and homeostasis:

1. v-onc genes usually contain only that part of their corresponding c-onc gene that is transcribed into messenger RNA—in most instances they lack the introns that are so characteristic of eukaryotic genes.
2. v-onc genes are separated from the cellular context that normally controls gene expression, including the normal promoters and other sequences that regulate conc gene expression.
3. v-onc genes are under the control of the viral long terminal repeats (LTRs), which not only are strong promoters but also are influenced by cellular regulatory factors. For some retrovirus v-onc genes, such as myc and mos, the presence of viral LTRs is all that is needed for tumor induction.
4. v-onc genes may undergo mutations (deletions and rearrangements) that alter the structure of their protein products; such changes can interfere with normal proteinprotein interactions, leading to escape from normal regulation.
5. v-onc genes may be joined to other viral genes in such a way that their functions are modified. For example, in Abelson murine leukemia virus the v-abl gene is expressed as a fusion protein with a gag protein; this arrangement directs the fusion protein to the plasma membrane where the Abl protein functions. In feline leukemia virus, the v-onc gene fms is also expressed as a fusion protein with a gag protein, thus allowing the insertion of the Fms oncoprotein in the plasma membrane. Infection with acute transforming retroviruses may lead to transformation of every infected cell and therefore
to very rapid tumor development (sometimes within days).

Chronic Transforming Retroviruses

Chronic transforming retroviruses induce neoplasia through integration into the genome of somatic cells. Recent research suggests that the selectivity of integration sites is specific for individual retrovirus species, and thereby contribute to pathogenicity. Chronic transforming retroviruses are classified as cis- or trans-acting. “Cis-acting” retroviruses (eg, avian leukosis viruses) transform
cells by becoming integrated in the host-cell DNA close to a cell growth regulating gene, and thus usurping normal cellular regulation of this gene. These cell growth regulating host genes are termed “proto-oncogenes,” or cellular oncogenes (c-onc). Despite the terminology implying that they are oncogenic, c-onc genes are host genes that encode important cell signaling products that regulate normal cell proliferation and quiescence. The presence of an integrated provirus, with its strong promoter and enhancer elements, upstream from a c-onc gene may amplify the expression of the c-onc gene greatly. This is the likely mechanism whereby the weakly oncogenic endogenous avian leukosis viruses produce neoplasia. When avian leukosis viruses cause malignant neoplasia, the viral genome has generally been integrated at a particular location, immediately upstream from a host
c-onc gene. Integrated avian leukosis provirus increases the synthesis of the normal c-myc oncogene product 30- to 100-fold. Experimentally, only the viral LTRs need be integrated to cause this effect; furthermore, by this mechanism c-myc may also be expressed in cells in which it is not normally expressed or is normally expressed at much lower levels. Infection with cis-acting retroviruses results
in transformation of single cells (monoclonal tumor) and slow tumor formation over months.

"Trans-activating" retroviruses express viral proteins that act as oncogenes. The retroviruses that cause nasal carcinomas and pulmonary adenocarcinomas (Jaagsiekte) in sheep infect epithelial cells, and transformation is related to expression of the viral env gene. Bovine leukemia virus is an exogenous retrovirus that causes chronic leukosis and B cell lymphoma. Its Tax protein functions as a transactivator of host genes. Both the ovine retrovirus Env and the Tax proteins of bovine leukemia virus stimulate continuous cell division of infected cells, which is thought to result in an increased number of mutations and subsequently cellular transformation. Infection with transacting retroviruses leads to oligoclonal tumors which develop over months to years.

Oncogenic DNA Viruses

Apart from retroviruses, the most important oncogenic viruses in animals are DNA viruses (papillomaviruses, polyomaviruses, herpesviruses; see also Table 3.4). DNA tumor viruses interact with cells in one of two ways: (1) productive infection, in which the virus completes its replication cycle, resulting in cell lysis or (2) nonproductive infection, in which the virus transforms the cell without completing its replication cycle. During such nonproductive infection, the viral genome or a truncated version of it is integrated into the cellular DNA or the complete genome persists as an autonomously replicating plasmid (episome). The genome continues to express early genes.

The molecular basis of oncogenesis by DNA viruses is best understood for polyomaviruses, papillomaviruses, and adenoviruses, all of which contain genes that behave as oncogenes, including tumor suppressor genes. These oncogenes appear to act by mechanisms similar to those described for retrovirus oncogenes: they act primarily in the nucleus, where they alter patterns of gene expression and regulation of cell growth. The relevant proteins have a dual role in both virus replication and cell transformation. With a few possible exceptions, the oncogenes of DNA viruses have no homologue or direct ancestors (conc genes) among cellular genes of the host.

Oncogenic Papillomaviruses

Papillomaviruses produce papillomas (warts) on the skin and mucous membranes of most animal species (see Chapter 11: Papillomaviridae and Polyomaviridae). Papillomas are hyperplastic epithelial outgrowths that generally regress spontaneously. Occasionally, however, infections by some papillomavirus types may cause malignant cellular transformation, resulting in the development of cancer. Papillomaviruses are known to cause oropharyngeal and cervical squamous cell carcinomas in people. In animals, papillomaviruses are also thought to cause sarcoids in horses, and have been associated with some squamous cell carcinomas in horses, cats and dogs.

In warts, the papillomavirus DNA remains episomal, meaning it is not integrated into the host-cell DNA and persists as an autonomously replicating episome. In contrast, in human papillomavirus-induced neoplasms the viral DNA is integrated into that of the host. As the pattern of integration is clonal within cancers, each cancer cell carries at least one, and often many incomplete copies of the viral genome. The site of virus integration is random,

and there is no consistent association with cellular proto-oncogenes. For some papillomaviruses, integration disrupts one of the early genes, E2, which is a viral repressor. Other viral genes may also be deleted, but the viral oncogenes (eg, E6 and E7) remain intact. These oncogenes alter normal cell growth and division and the overexpression of E6 and E7 is considered a critical step in malignant transformation by a human papillomavirus.

It is to be stressed that the development of warts is a normal part of viral replication cycle of some papillomavirus types. However, the integration of DNA into a cell is accidental and prevents replication of the papillomavirus and only a very small proportion of papillomavirus infections result in cancer development. However, bovine papillomavirus type 1 is thought to cause equine sarcoids
predominantly through changes in cell proliferation that are mediated by the E5 oncoprotein. In contrast to human papillomavirus-induced cancers, viral integration appears to be uncommon within papillomavirus-associated cancers in animals.

Oncogenic Hepadnaviruses

Mammalian, but not avian, hepadnaviruses are associated strongly with naturally occurring hepatocellular carcinomas in their natural hosts. Woodchucks that are chronically infected with woodchuck hepatitis virus almost inevitably develop hepatocellular carcinoma, even in the
absence of other carcinogenic factors. Oncogenesis induced by mammalian hepadnaviruses is a multifactorial process, and there are differences in the cellular mechanisms responsible for carcinogenesis associated with different viruses. Whereas ground squirrel and woodchuck
hepatitis viruses activate cellular oncogenes, the mode of action of human hepatitis B virus is uncertain, as it apparently has no consistent site of integration or oncogene association. The hepatocellular regeneration accompanying cirrhosis of the liver also promotes the development
of neoplasia in hepatitis virus-infected humans, but there is no cirrhosis in the animal models. The likelihood of hepadnavirus-associated carcinoma is greatest in animals (and humans) infected at birth.

Oncogenic Herpesviruses

Marek's disease virus of chickens (gallid herpesvirus 2) transforms T lymphocytes, causing them to proliferate to produce a generalized polyclonal T lymphocyte neoplasm. The disease is preventable by vaccination with liveattenuated virus vaccines that lack the retrovirus v-onc genes that are present in Marek's disease virus. The best characterized oncogene is the Meq protein, which inhibits tumor suppressor genes and stimulates expression of proteins important for cell growth (IL-2, Bcl-2, CD30). It also binds to the promoter of and stimulates the expression of micro RNA21, which subsequently causes expression of metalloproteinases required for tissue invasion by tumor cells.

Oncogenic Poxviruses

Although some poxviruses are regularly associated with the development of benign tumor-like lesions (see Chapter 7: Poxviridae), there is no evidence that these ever become malignant, nor is there evidence that poxvirus DNA is ever integrated into cellular DNA.

A very early viral protein produced in poxvirus-infected cells displays homology with epidermal growth factor and is probably responsible for the epithelial hyperplasia characteristic of many poxvirus infections. For some poxviruses (eg, fowlpox, orf, and rabbit fibroma viruses), epithelial hyperplasia is a dominant clinical manifestation and may be a consequence of a more potent form of the poxvirus epidermal growth factor homologue.

Oncogenic in Experimental Systems: Polyomaviruses and Adenoviruses

During the 1960s and 1970s, two members of the family Polyomaviridae, murine polyomavirus and simian virus 40 (SV40), as well as certain human adenoviruses (types 12, 18, and 31) were shown to induce malignant neoplasms following their inoculation into baby hamsters and other rodents. With the exception of murine polyomavirus, none of these viruses induces cancer under natural conditions in its natural host, rather they transform cultured cells of certain other species and provide experimental models for analysis of the molecular events in cell transformation. More recently, polyomaviruses have been incriminated as the cause of cancers in both humans and animals (see Chapter 11: Papillomaviridae and Polyomaviridae).

Polyomavirus- or adenovirus-transformed cells do not produce virus. Viral DNA is integrated at several sites in the chromosomes of the cell. Most of the integrated viral genomes are complete in the case of the polyomaviruses, but defective in the case of the adenoviruses. Only certain early viral genes are transcribed, albeit at an unusually high rate. By analogy with retrovirus genes, they are now
called oncogenes. Their products, demonstrable by immunofluorescence, used to be known as tumor (T) antigens. A great deal is now known about the role of these proteins in transformation. Virus can be rescued from polyomavirus-transformed cells—that is, virus can be induced to replicate by irradiation, treatment with certain mutagenic chemicals, or cocultivation with certain types
of permissive cells. This cannot be done with adenovirustransformed cells, as the integrated adenovirus DNA contains substantial deletions.

Veterinary virology – 2019

Lecture 4. Antiviral Immunity and Virus Vaccines

As obligate intracellular organisms, viruses have coevolved with their respective host species, which in turn have evolved diverse and sophisticated capabilities to protect themselves against viral infections and their associated diseases. Viruses have also evolved a remarkable variety of strategies to avoid or subvert these host defences. 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. Innate immunity provides constant and relatively rapid protection 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 pathogen and often its close relatives. Adaptive immunity involves cell and antibody (humoral)-mediated effector mechanisms, by T and B lymphocytes, respectively. Adaptive immune responses also exhibit memory, such that the response may be quickly reactivated after reexposure to the same virus. With many systemic viral infections, immunological memory after natural infection confers long-term, often lifelong, protection against the associated disease.

The development of efficacious vaccines has substantially reduced the deleterious impact of viral infections in humans and animals. The goal of vaccination is to stimulate the adaptive immune responses that protect animals from infection with specific viruses. An increasing variety of vaccine types are now commercially available for use in both companion and production animal species. These include conventional inactivated and live-attenuated virus vaccines, recombinant viruses that express protective proteins of heterologous viruses, virus-like particles (VLPs), and DNA vaccines. Vaccines are used extensively in regulatory programs for the control of individual viral diseases of livestock, often in combination with specific management procedures. Vaccines are also a critical component of the medical care of companion pets.

IMMUNITY TO VIRUSES

Innate Immunity to Viral Infections The cells mediating innate immunity do not respond to specific viral antigens as do their counterparts in the adaptive immune response. Rather, these cells are activated by the presence of the virus, using an array of different sensors. In addition, cells of the innate system react to viral infections through production and recognition of cytokines, which are small proteins that affect the behavior of other cells. Cytokines made by lymphocytes are often termed interleukins. A key family of cytokines in the innate response to virus infection are the interferons.

Interferon Responses

In 1957, Isaacs and Lindenmann reported that influenza virus-infected cells produce a nonviral protein they termed "interferon" that can protect uninfected cells against the same (influenza virus) as well as unrelated viruses. It has since been determined that there are several
types and subtypes of interferon and that these proteins are key elements of antiviral resistance at the cellular level. They also play a central role in both innate and adaptive immune responses to viral infections. A critical class of these proteins was collectively designated as type I interferon (IFN). These include IFN-α, which is encoded by several different genes in most species (eg, 14 in cattle
and 27 in swine). How many of the IFN-α genes are used by any species in response to any infection event is not clearly defined. There are also 7 IFN-β genes in cattle and one in swine. In addition, IFN-τ, IFN-δ, IFN-ε, IFN-κ, and IFN-ω are also type I interferons. All of these protein hormones bind a common receptor, the IFN-α receptor (IFNAR). This cell surface protein is a heterodimer of
IFNAR 1 and 2, and functions to transduce a signaling cascade of enzymes including the tyrosine and Janus kinases that induce signal transducers and activators of transcription, and interferon regulatory factors. Activation of this signaling cascade ultimately results in induction of the interferon response genes in cells (Fig. 4.1). Humans and animals with deficits in signaling pathways triggered by interferon often die of common viral diseases that are not usually fatal.

Type II interferon, or IFN-γ, was originally reported as "immune interferon." This cytokine is central to many aspects of both innate and adaptive immunity and defines multiple subtypes of T lymphocytes. Type III interferon is designated as IFN-λ. In humans, these protein hormones were originally
described as members of the interleukin 10 (IL-10) cytokine family because they are bound by IFN-λ receptor 1 and IL-10 receptor 2. IFN-λ1, 2, and 3 were first described as IL-29, IL-28A, and IL-28B, respectively. As with type I and type II interferons, the IFN-λs have cytokine activities in addition to
their inherent antiviral action. In cattle and swine, there are 2 IFNλ genes reported to date, IFN-λ1 and IFN-λ3.

Induction of type I interferon in virus-infected cells involves activation via an array of cellular receptors called pattern recognition receptors, which detect pathogen-associated molecules that are broadly specific to different classes of viruses. The binding of pathogenassociated molecules to these cellular receptors stimulates the transcription of

numerous genes encoding proteins that are involved in innate and adaptive immune responses, including the activation of interferon production and secretion. Importantly, these responses may be triggered by several redundant pathways, both cytoplasmic and extracytoplasmic. One class of pattern recognition receptors are the Toll-like receptors (TLRs), so
named because of their homology to the Toll genes of Drosophila. Different Toll-like receptors detect different pathogen-associated molecular patterns (PAMPs). For instance, TLR7 and TLR8 bind single-stranded RNA (ssRNA), thus detecting RNA virus infections, which then induces production of type I interferon. This is an important response to influenza and human immunodeficiency virus infections for example. In contrast, TLR3 detects double-stranded RNA (dsRNA), a critical intermediate of
RNA virus genome replication that is not present in normal cells. These Toll-like 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. Cytosolic pathways for pathogen sensing and type I interferon 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). Other intracellular pathways include mitochondrial antiviral signaling protein (MAVS; also referred to as IPS-1), which mediates activation of transcription factors that induce interferon production (Fig. 4.1).

mediated by several distinct signaling pathways. On the binding of type I IFNs to interferon α receptor (IFNAR) on a neighbouring uninfected cell (right) multiple downstream signaling pathways can be induced leading to a diverse range of biologic effects mediated by interferon stimulated genes (ISGs).
From McNab, F., Mayer-Barber, K., Sher, A., Wack, A., O'Garra, A., 2015. Type I interferons in infectious disease. Nat. Rev. Immunol. 15, 87103, with permission.

Type I interferon released from virus-infected cells or activated innate response cells (see below) stimulates adjacent cells via interferon α receptor (IFNAR) binding (Fig. 4.1). This activates a signaling pathway leading to induction of the interferon response element. In mice, this results in the transcriptional activation of more than 300 interferon-stimulated genes (ISGs). In large mammals and
humans it is clear that a similar group of interferonstimulated genes is activated following binding of type I interferons to their specific receptors. Most of these genes encode proteins that regulate either signaling pathways or transcription factors that amplify interferon production, whereas others promote an antiviral state via cytoskeletal remodeling, apoptosis, posttranscriptional events (mRNA editing, splicing, degradation), or posttranslational modifications.
Proteins proven to be critical to the induction of the interferon-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 interferon-stimulated cells. Activities of ISG15 can regulate all aspects of the interferon 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 R (PKR) pathway is constitutively expressed at only a very low level, but is quickly upregulated by IFNAR signaling. In the presence of dsRNA, the protein kinase phosphorylates elongation (translation) initiation factor eIF2α and prevents recycling of cyclic nucleotides (GDP), which in turn halts protein synthesis. This interferon-induced pathway is especially important for inhibiting replication of reoviruses, adenoviruses, vaccinia and influenza viruses, amongst many others.

- The 2050 oligoadenylate synthetase (OAS) pathway, like the PKR pathway, is constitutively expressed only at a low level. After IFNAR stimulation and in the presence of dsRNA, this enzyme produces oligoadenylates with a distinctive 2050 linkage, as contrasted with the normal 3050 lineage. These 2050 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. In summary, type I interferon is produced after virus infection of many different types of cells, and the interferon released from these cells then induces an antiviral state in adjacent cells. In addition, cells of the innate immune system can be activated to secrete interferon by virus infection, including nonproductive infections or by their "sensing" of viral infection, which augments the level of antiviral signaling and the local antiviral state in tissue. In many instances, this response may control, or even eliminate, a viral infection before the development of systemic infection or the occurrence of overt disease. If the virus overwhelms the early innate immune response then systemic spread occurs and disease may be detected clinically.

Natural Killer Cells

Natural killer (NK) cells are specialized lymphocytes that lack an antigen-specific receptor, which can kill virusinfected cells, tumor cells and other cells they detect to be "in a state of stress". This is accomplished via engagement of a series of receptors for ligands expressed on the surface of potential target cells. As such, natural killer cells provide early and nonspecific resistance against viral infections. Natural killer cells express an extensive complex of receptors that recognize particular patterns of expression of their respective ligands on host cells. The receptors on natural killer cells are both activating and inhibitory, and the function of natural killer cells is stringently regulated by the balance of activating and inhibitory signals from these receptors. For example, one of the primary receptors on natural killer cells binds to class I major histocompatibility complex (MHC) proteins and this binding provides a negative (inhibitory) signal for natural killer cell activation. This allows natural killer cells to "scan" tissue without harming healthy cells, which are recognized as "self." A common effect of virus infection is reduced expression of class I MHC protein on the surface of the infected cell. The lack of sufficient MHC ligand to bind the natural killer cell inhibitory receptor results in activation signals that reach the necessary threshold for cell activation. Virus-infected cells also express stress receptors that bind activating

receptors on natural killer cells, and thus the balance of signal favors “antigen-independent” activation and the resulting killing response (Fig. 4.2).

The receptors mediating activation of natural killer cells to target cell killing, or the inhibition of that activation, are encoded in two large families of genes. The killer immunoglobulin-like receptors (KIR) are encoded by a cluster of genes in the leukocyte receptor complex.
In humans and cattle, these receptors are highly polymorphic and individuals tend to have unique allelic patterns of expression. Mice lack KIR genes altogether, and KIR gene transcription products in horses have yet to be described. Only a single KIR gene transcript has been demonstrated in swine. The second receptor complex expressed by natural killer cells is the NK receptor complex.
In this locus are the NK2G genes and the Ly49 genes. Mice express many Ly49 genes as do horses, whereas pigs, cattle, cats and dogs express a single gene product and humans lack a functional Ly49 gene. There are other receptor genes encoded in the MHC locus of these species including
NKp30 and NKp44 or 46, depending on the species.

NKp46, also designated CD335, is the classic natural killer cell marker. However, cells of other lineages that are not natural killer cells can express this protein and kill other cells in a natural killer cell-like manner (ie, antigen nonspecific). Most notable of these are γδT cells (see below).
Natural killer cells kill virus-infected cells by the same pathway utilized by antigen specific, cytotoxic
T lymphocytes (CTL), which is by inducing apoptosis (ie, programmed cell death, or “cell suicide”—see
Chapter 3: Pathogenesis of Viral Infections and Diseases).
This cytocidal activity is central to the control of viral infections because it can eliminate infected cells (virus factories) before they can produce and release progeny virions. Like cytotoxic T lymphocytes, natural killer cells have cytosolic granules that contain the proteins perforin, granzyme A and
granzyme B (Fig. 4.2). When activated by the stimulatory receptor-binding process, these granules are orientated toward the target cell and then released. Perforin creates pores in the target cell membrane through which the granzyme proteins enter and once inside, these proteins induce apoptosis of the target cell.

Natural killer (NK) cell destruction of a virus-infected cell. Virusinfected cells express multiple stress indicators and virus infection inhibits expression of cell proteins. The NK cell’s multiple activating and inhibiting receptors are bound, and when activational stimuli overcome inhibition, cell killing is initiated. The cytotoxic granules orient to the cell junction and are released. The perforin creates access to
the target cell cytosol delivering the granzymes, which are serine proteases that mediate target cell death by multiple pathways. Courtesy of J.R. Patch, W.T. Golde, Plum Island Animal Disease Center, USDA.

Natural killer cells also express CD16, a surface receptor for the Fc portion of immunoglobulin G molecules (FcRγIII). This receptor allows natural killer cells to bind and lyse antibody-coated target cells through the process of antibody-dependent cellular cytotoxicity. This results in a killing activity identical to the cell-killing mechanism just described, but bypassing all of the natural killer cell receptors. Finally, natural killer cells also can mediate functions in addition to direct killing. Notably,
natural killer cells are very efficient at type II interferon (IFNγ) production and secretion following activation. IFN-γ secretion by natural killer cells creates a strong inflammatory environment, activates other cells of the innate and the adaptive immune system, and induces an antiviral state in cells at the site of inflammation.

T Cells in Innate Immunity

The antigen-specific receptor on T cells is expressed as a heterodimer in a complex with subunits of the nonpolymorphic protein CD3. The receptor heterodimer is comprised of α and β chains requiring the CD3 complex for cell surface expression; these so-called αβ T cells are required for adaptive immune responses. There is also a unique subset of T cells that plays a prominent role in innate
immunity, but with a different receptor comprised of a γ and δ chain expressed as a heterodimer in association with CD3; thus, these are termed γδ T cells. These γδ T cells can express a series of scavenger receptors including those in the WC1 family. In mice, there are no circulating γδ cells but
they constitute up to 5% of peripheral blood mononuclear cells in humans, especially in newborns. In pigs and calves, up to 50% of circulating lymphocytes can be γδ T cells, and 2030% in adult swine and cattle. These cells function in adaptive immune responses via antigen-specific interactions
with the T cell receptor, but they also can be activated in a nonspecific manner in response to cellular stress such as that associated with virus infection. Specifically, γδ T cells can respond to infection by expressing NKp46 and killing virusinfected cells in a natural killer cell-like manner. These cells
while concurrently making strong cytokine responses, particularly production of IFNγ.

Innate Responses of Dendritic Cells

Dendritic cells (DCs) are critical to the initiation of the adaptive immune response but are also central to innate immunity. There are a number of dendritic cell subtypes, with overlapping (common) functions as well as capacities unique to each subtype. The classical dendritic cell is of bone marrow myeloid lineage, expresses a high density of class II MHC proteins, and is highly phagocytic in the naïve state. These dendritic cells can also respond to stimulation by pathogen associated signatures (PAMPs) by secreting large amounts of type I interferon (notably IFNα and IFNβ).
Other dendritic cells of the myeloid lineage populate the skin, both the dermis and epidermis. A substantial portion of dendritic cells in the skin are a specialized subset of cells called Langerhans cells, which have been described in many species of mammal. A unique dendritic cell population, first described in pigs and subsequently in mice, humans, cattle, and nonhuman primates, is termed the plasmacytoid dendritic cell. These differentiate from a lymphoid lineage and have a distinct morphology from myeloid dendritic cells. Plasmacytoid dendritic cells were first described as natural interferon producing cells as they are remarkably efficient in the production and secretion of type I

interferon in response to virus infection.

Adaptive Immunity to Viral Infections

The adaptive immune response to viral infection requires recognition and binding of antigen by specific receptors on T and B lymphocytes. Induction of an adaptive immune response occurs in lymph nodes and is initiated by pathogen stimulated dendritic cells that migrate through afferent
lymphatics from the site of infection to the draining lymph node. A primary adaptive immune response takes several days to develop and involves clonal expansion of lymphocytes bearing identical antigen-specific receptors and the differentiation of these lymphocytes into effector cells. The adaptive immune response consists of two main arms: humoral immunity, mediated by antibodies secreted by terminally differentiated B lymphocytes called plasma cells, and cell-mediated immunity, driven by αβ T cell receptor expressing lymphocytes (Fig. 4.3). Antibodies bind antigen directly in its native conformation on the pathogen surface and protect the host by clearing extracellular viruses, whereas T lymphocytes recognize processed antigen in the form of peptides bound to MHC molecules at the cell surface and so target virus-infected cells. Once a virus infection is cleared from the host, a proportion of the antigen-specific lymphocytes can develop into long-lived memory cells that can rapidly respond to the pathogen should it be encountered again; this establishment of immunologic memory is a hallmark of adaptive immunity and the basis of vaccination.

FIGURE 4.3 The principal classes of lymphocytes and their functions in adaptive immunity. From Kumar, V., Abbas, A.K., Fausto, N., Aster, J., 2010. Robbins & Cotran Pathologic Basis of Disease, eighth ed. Elsevier-Saunders, Philadelphia, PA, p. 185. Copyright r Saunders/Elsevier (2010), with permission.

Dendritic Cells Link Innate and Adaptive Immune Responses

Classical dendritic cells are “professional” antigenpresenting cells (APCs), as they have a unique capacity to stimulate T cell responses to infectious agents, including viruses. Langerhans cells and other dendritic cells at epithelial surfaces exist as immature cells that are equipped to capture antigens and pathogens by phagocytosis. Many viruses directly infect dendritic cells.

Pathogen infection or exposure results in engagement of Toll-like receptors or other pathogen recognition receptors leading to interferon production, secretion, and signaling that induces a process known as maturation in which the dendritic cell transitions from innate immune responses to antigen-presenting cell function. A critical feature of dendritic cell maturation is the switch in chemokine (chemokines are cytokines that attract other cells via the process of chemotaxis) receptor expression from CCR5 to CCR7, thereby guiding dendritic cell migration. In addition, there is an accompanying change in the functional capacity of the migrating dendritic cell such that phagocytic capacity is reduced, interferon production is lost, and production of cytokines that activate naive T cells and B cells increases. Mature dendritic cells also have upregulated expression of MHC and costimulatory molecules that are particularly important in stimulating antigen-specific naı¨ve T cells resident in the lymph node paracortex. Dendritic cell and T cell engagement is facilitated by expression of adhesion molecules LFA-1 and CD2 on the T cell, and ICAM-1, ICAM-2, and CD58 on the dendritic cell. The mature dendritic cell

provides three different kinds of signal to the naı¨ve T cell. Binding of the MHC/peptide complex to the T cell receptor/CD3 complex provides the first signal, and the second is mediated by binding of costimulatory molecules on the dendritic cell with CD28 on the T cell. These two signals promote activation and survival of the T cell. The third signal mediated by cytokines produced by the dendritic cell leads to T cell differentiation, as discussed below.

Recognition and Killing of Virus-Infected Cells by Cytotoxic T lymphocytes (CTLs)
Destruction of infected cells by cytotoxic T lymphocytes expressing αβ T cell receptors is the principal mechanism utilized by the adaptive immune system to control intracellular virus infections (Fig. 4.3).
Cytosolic viral proteins within the infected cell are digested by a multicatalytic protease complex called the proteasome, which delivers short peptides to the endoplasmic reticulum through a pair of energy-dependent transporters known as TAP (transporters associated with antigen processing; TAP1 and TAP2). Within the endoplasmic reticulum, peptides are further trimmed to lengths of 811 amino acids and engage a series of chaperone molecules that allow peptides with the compatible sequence to bind nascent MHC class I molecules forming in the endoplasmic reticulum. The stable MHC class I/peptide complex is shuttled through the Golgi apparatus for presentation at the surface of the infected
cell. T lymphocytes that bear antigen receptors that recognize the specific MHC class I/peptide complex presented by the infected cell bind the complex and become activated. T cells targeting infected cells in this manner also express the CD8 coreceptor which binds an invariant region of MHC class I protein and provides the signals that are essential for an effective T cell response (Fig. 4.4).

Antigen processing and display by major histocompatibility complex (MHC) molecules. A. In the class I MHC pathway, peptides are produced from proteins in the cytosol and transported to the endoplasmic reticulum (ER), where they bind to class I MHC molecules. The peptide MHC complexes are transported to the cell surface and displayed for recognition by CD81 T cells. B. In the class II MHC pathway, proteins are ingested into vesicles and degraded into peptides, which bind to class II MHC molecules being transported in the same vesicles. The class II-peptide complexes are expressed on the cell surface and recognized by CD41 T cells. From Kumar, V., Abbas, A.K., Fausto, N., Aster, J., 2010. Robbins & Cotran Pathologic Basis of Disease, eighth ed. Elsevier-Saunders, Philadelphia, PA, p. 192. Copyright r Saunders/Elsevier (2010), with permission.

As previously described for natural killer cells, killing of virus-infected cells by cytotoxic T lymphocytes is achieved by release of cytotoxic granules at the interface between the receptor-bound T cell and a virus-infected cell, a region known as the immunologic synapse. Cytotoxic granules contain perforin that facilitates entry of cytotoxic proteins into target cell membranes; granzymes, a family of serine
proteases; and granulysin, a cytotoxic protein, combine to mediate target cell apoptosis and death (Fig. 4.5). Cytotoxic CD8 T cells can also release cytokines that act either locally or at a distance to impact virus infection. The principal cytokine produced by effector CD8 T cells is IFN-γ, which

can block virus replication and even eliminate virus from infected cells without inducing cell death.
Antigens generated within a virus-infected cell are designated as endogenous antigens and their presentation by MHC class I molecules can occur in essentially any cell in the body, providing an effective means for CD8 T cell recognition and subsequent elimination of infected cells. Endogenous antigens are not the exclusive source of antigenic peptide for MHC class I loading, however, as
peptides from extracellular sources, including phagocytized dead and dying cells that are infected with pathogens, can enter the MHC class I pathway through a process known as cross-presentation. This pathway is important in allowing dendritic cells not directly infected with a pathogen to engage and stimulate naı ̈ve virusspecific CD8 T cells in the lymph node paracortex during the initial establishment of a primary adaptive response.

Cytotoxic T lymphocyte (CTL) killing of a virus-infected cell. Virus proteins are degraded into peptides and subsequently bound by recently synthesized class I MHC proteins which are transported to the cell surface. The CTL with a T cell receptor specific for that peptide/MHC combinatorial determinant can “see” the cell is infected by this binding interaction. Once multiple receptors are bound, coreceptors including CD8 also bind and a tight cellular junction is established. The cytotoxic granules orient to the cell junction and release. Like natural killer (NK) cells, the perforin creates access to the target cell cytosol delivering the granzymes, which are serine proteases that mediate target cell death by multiple pathways. Courtesy of J.R. Patch, W.T. Golde, Plum Island Animal Disease Center, USDA.

CD4 Helper T Cells in Immunity to Virus Infection

A second population of αβ T cells bears the CD4 coreceptor and, once activated, these cells can differentiate into several subsets of functionally distinct effector cells based on the type of cytokines they produce (Fig. 4.3). Although CD4 T cells can participate directly in the killing of virusinfected cells (ie, as cytotoxic T lymphocytes), that function is more characteristic of CD8 T cells and is mediated by only a minor population of virus-reactive CD4 T cells. Rather, CD4 T cells play an especially important
role in antiviral immunity by facilitating both cellmediated and humoral immune responses, hence the term T helper cell. There are at least five different subsets of CD4 T cells that are specialized in providing help to immune responses to infections with different classes of pathogens. T-helper 1 (TH1) cells produce IFN-γ and activate macrophages, which facilitates killing of intracellular pathogens phagocytized by macrophages, creating the antiviral state in IFN-γ receptor expressing cells, and inducing differentiation of discrete aspects of B lymphocyte function. T-helper 2 (TH2) cells produce interleukins (IL-4, IL-5, and IL-6) that recruit eosinophils, mast cells and basophils, providing protection at mucosal surfaces. Both TH2 cells and T follicular helper cells (TFH) cells that reside in B cell follicles of lymph nodes, engage B cells and promote antibody production via secretion of IL-4 and
IL-13. TH17 cells produce IL-17 and IL-21 that induce fibroblasts and epithelial cells to recruit neutrophils to sites of microbial infection during the early stages of an adaptive immune response. A final class of CD4 T cell is the regulatory T cell, a heterogeneous population of cells that suppress T cell activity and limit autoimmunity. These cells are

characterized by production of antiinflammatory
cytokines such as IL-10 and transforming growth factor.

CD4 T cells recognize antigenic peptides presented by MHC class II proteins, which are limited in expression to antigen presenting cells (Dendritic cells, macrophages and B cells). In many species of large mammal, including primates, cattle, and swine, T cells can be activated to MHC class II gene expression and contribute to antigen presentation thereby expanding secondary adaptive immune
responses. Peptides presented by MHC class II molecules derive largely from exogenous antigens, those antigens that are made outside of the cell, such as endocytosed virus particles and particulate antigens derived from dead and dying cells. Antigen taken up by cells from the extracellular space is internalized into endosomes which become acidified, activating proteases that degrade antigen into peptide fragments and individual amino acids that are then available for new protein synthesis. Critical
for adaptive immunity, these peptides are also available for binding to MHC class II molecules (Fig. 4.4).

Newly formed MHC class II molecules in the endoplasmic reticulum are protected from binding peptides within that compartment by a chaperone protein called invariant chain, which blocks the peptide-binding groove of the molecule and targets delivery of MHC class II to a low-pH
compartment. Proteases process the invariant chain leaving a truncated form of protein termed class II-associated invariant chain peptide (or CLIP) that continues to protect the peptidebinding groove. Vesicles containing endocytosed exogenous proteins (eg, viral proteins) fuse with vesicles containing
MHC class II molecules and antigenic peptides displace the CLIP chaperone protein, facilitated by the MHC class IIlike molecule HLA-DM. The fully formed MHC class II/peptide complex is transported to the cell surface where a T cell receptor with specificity for the particular peptide/MHC combination can bind to form a trimolecular complex of T cell receptor, MHC class II and peptide. This interaction is
further facilitated by the CD4 coreceptor expressed by these T cells, which binds to an invariant region of MHC class II and promotes an effective T cell response (Fig. 4.4).

A key function of effector CD4 T cells is to provide help to CD8 T cells, an essential step in the activation of cytotoxic T lymphocytes in the majority of viral infections.
Within lymph nodes, CD4 T cells engage virus-derived peptides bound to MHC class II presented by antigen presenting cells such as dendritic cells, which also engage naı ¨ve CD8 T cells through presentation of different viral peptides in the context of MHC class I. The effector CD4 T cell expresses
CD40 ligand that binds CD40 on the dendritic cell, thus activating the dendritic cell and inducing the upregulation of essential costimulatory molecules such as CD80 and CD86 that are required for activation of the CD8 T cell. Effector CD4 T cells also secrete abundant IL-2 that drives CD8 T cell proliferation. Further, CD4 T cells are essential in the effective activation and differentiation of B cells in most humoral immune responses, as detailed below.

T Cell Memory

Memory is a critical aspect of adaptive immune responses, in contrast to innate immunity where there is no recall on reexposure to specific antigens. During the period of antigenic stimulation, a portion of the reactive helper (TH) and cytotoxic (CTL) T cells differentiate into memory T cells. These cells return to quiescence and reside primarily in the local (ie, "draining") lymph nodes and to a lesser extent, other lymphoid organs like the spleen. When there is a subsequent exposure to the virus,
these cells mediate the recall response. The induction and maintenance of T cell memory is a critical aspect of vaccination. In swine and cattle, memory T cells express both CD4 and CD8. Humans and nonhuman primates also have a small percentage of peripheral T cells that express both CD4 and CD8 whereas this phenomenon is very rare in mature T cells of mice.

Humoral Immunity to Virus Infection

Humoral immunity is mediated by antibodies (syn. immunoglobulins (Ig)), which are the effector molecules of B lymphocytes (Fig. 4.3). Immunoglobulins consist of a combination of proteins called heavy and light chains, which each have variable (V) and constant (C) regions.
The antigen-binding region is unique to each antibody and is formed by the combined V regions of both heavy and light chains at one end of the molecule. The C region of the heavy chain is at the other end of the molecule, called the Fc region, and determines both the class of antibody and its functional specialization. There are four different classes of secreted antibody. IgM antibodies are found primarily in blood and are the first antibodies produced during a developing immune response. IgG is the principal class of antibody in blood and extracellular fluid, and exists as several different subtypes. IgA is the main antibody in secretions of the respiratory, genital and gastrointestinal tracts. IgE is present at very low concentrations in blood and extracellular fluid and mediates allergic reactions. A fifth class of antibody, IgD, is expressed almost exclusively as a cell surface molecule by naı ¨ve B cells.
To generate antibody a B cell must first encounter and bind epitopes on accessible proteins of the virus through engagement of its B cell receptor, the cell surface version of immunoglobulin. IgM and IgD are expressed by naı ¨ve B cells of many species of mammal, whereas only IgM is expressed in other species. The binding of antigen to its specific receptor initiates internalization of the virus particle and its subsequent degradation in acidified vesicles.

Within these vesicles, viral peptides, including those derived from internal proteins that are not accessible to the B cell receptor, are loaded onto MHC class II molecules for presentation at the cell surface. Virus-specific CD4 T cells engage the MHC class II/peptide complex and deliver activating and survival signals to the B cell in the form of CD40 ligand (which engages CD40 on the B cell) and cytokines, inducing proliferation and differentiation of B cells into antibody-secreting cells. Engagement of CD4 T cells also promotes the formation of a germinal center within the lymph node cortex, the site of intense B cell proliferation and death.

During an ongoing humoral immune response, B cell expansion/proliferation is characterized by somatic hypermutation and isotype class switching within the immunoglobulin genes. Both processes are critical to generation of effective antiviral immunity. Somatic hypermutation in the V-region of the immunoglobulin gene locus of B

cells occurs spontaneously during B cell activation and leads to affinity maturation. This process ensures antibodies are generated with increasing affinity for antigen as the immune response evolves. Thus, while the first antibodies made following a virus infection are low-affinity IgM antibodies, as the immune response matures there is a switch to high-affinity IgG and IgA antibodies produced by the concurrent events of somatic hypermutation and affinity maturation. Germinal center B cells that produce immunoglobulins with increasing affinity for antigen as a result of somatic hypermutation will preferentially survive, as the process of antigen binding, degradation, and presentation on MHC class II molecules to CD4 T cells is sustained even as antigen diminishes.

Isotype class switching involves genetic rearrangement of the C region of the immunoglobulin heavy chain gene and results in replacement of the original Cμ heavy chain, encoding IgM, with an alternative C region. Switching to a Cγ heavy chain results in production of IgG molecules, whereas expression of Cε or Cα results in production of IgE or IgA molecules, respectively. Further, in mammalian species, these B cells maintain expression of the membrane form of the antibody as the antigen receptor, or B cell receptor, while also secreting antibody. The two forms of the antibody, membrane and secreted, are coexpressed by alternate mRNA splicing of the membrane or secretory sequences to the end of the rearranged/spliced antibody mRNA. B cells that terminally differentiate into plasma cells are solely dedicated to synthesis and secretion of antibody and no longer express surface antibody nor undergo somatic mutation or further class switching. These cells have a finite life span. As antigen diminishes, for instance when a virus infection is controlled, some B cells, especially those in the lymphoid tissues, return to a resting state and can remain surface immunoglobulinexpressing until a new encounter with the same antigen.

Under the influence of cytokines produced by T cells, these cells can become memory B cells with a very long life span, just as with T cells. Together, these are the cells that mediate the recall response.

Which antibody class is selected during isotype class switching is a function of the cytokines the B cell is exposed to, with IL-4 inducing IgG1 and IgE expression and IFN-γ inducing IgG3 and IgG2a production in mice.

However, individual animal species exhibit a variety of different immunoglobulin isotypes as the duplications leading to large immunoglobulin supergene families occurred after speciation. For instance, Bos taurus cattle have three IgG isotypes; IgG1, IgG2, and IgG3, although the IgG3 constant region gene is not used. Because the IgG1 isotype is secreted in mucosal fluids, older reports assume there is no IgA in cattle. Bovine IgA was discovered only later and has unique expression profiles relative to bovine IgG1, but is a minor antibody in mucosal secretions of cattle. Likewise, IgG2 can be expressed in lower concentrations than IgG1 in serum, but can dominate the antibody response in some instances. In swine there are six IgG isotypes. IgG 2, 4, and 6 differ by only a few amino acids and their functions are identical and redundant, but they are distinct genes and are all expressed in individual animals. Porcine IgA is the predominant antibody isotype in mucosal secretions of swine, as is the case for humans. For species other than mouse and human, data are limited relating certain cytokines with induction of class switch to

particular immunoglobulin
isotypes in activated B cells.

Species-specific differences also occur in the expression of the light chain of antibody molecules. For example, humans express both κ and λ light chains, but mice express only κ and horses only λ. Cattle, swine, canines, and felines express a mixture, like humans. The combinatorial interaction between heavy and light chains determines the properties of the antigen binding cleft, and mutations that are under selection pressure by antigen driven somatic mutation, are focused in this region.
Specifically, mutations that yield higher affinity for antigen are selected and propagated.

Antiviral Functions of Antibodies

Neutralizing antibodies can be important both in mediating virus clearance during primary viral infections and in preventing reinfection with viruses to which the animal previously has been exposed. Virus neutralization occurs in vivo when antibody binds to its complementary epitope on the virus surface, preventing virus from binding to and/or productively infecting target cells. All other functions of antibodies are dependent upon the class of immunoglobulin and are mediated by the Fc region at the end of the antibody molecule distant to the binding portion.

One of these functions is activation of complement, a system of plasma proteins that are activated through sequential proteolytic cleavage reactions resulting in production of a number of immunologically active proteases. IgM is the most effective antibody class at activating complement as it exists in a pentameric form, providing multiple Fc regions for the binding of C1q, the first protein in the classical pathway of complement activation. In viral infections, complement activation leads to more efficient activation of B cells through binding to complement receptor 2 (CD21), a component of the B cell coreceptor complex. Another major function of antibody in viral infections is opsonization, which facilitates binding of the Fc portion of antibody to various Fc receptors on effector cells. Different cell types express different sets of Fc receptors, and the antibody class thus determines which type of
cell will be engaged in an immune response. Many Fc receptors are expressed by phagocytes and facilitate phagocytosis of antibody-coated particles. In addition, natural killer cells express FcγRIII (CD16) that can bind to the Fc portion of IgG after it has attached to viral proteins expressed at the surface of infected cells. This binding results in activation of the natural killer cell and killing of
the virus-infected cell through the process of antibodydependent cellular cytotoxicity that was described earlier.

Passive Immunity

A critical aspect of adaptive immunity in veterinary species involves maternal immunity that is “passively” transferred to neonatal animals. For most mammalian species, neonates are born with a naı ¨ve immune system. The final stages of immunological development occur after birth,
following separation from the maternal blood and population by the microbiome. During pregnancy, placental structure influences immunoglobulin transfer and only in a few species, notably humans and to a lesser extent carnivores, does antibody, usually of the

IgG isotype, cross the placenta to circulate in the fetus. In most mammals, including all farm animal species, passive transfer of antibodies occurs through the neonate's ingestion of colostrum immediately after birth. Colostrum contains immunoglobulin at 10100-fold its concentration in milk.

Colostrum is also a source of maternally-derived leukocytes (1 million/mL in cattle), which are absorbed and enter the neonate's circulation. In addition, colostrum contains bioactive compounds that may influence gut mucosal development and provides a source of bacteria that colonize the neonatal gastrointestinal tract, which is increasingly recognized to play a central role in normal development of the immune system.

Vaccination of pregnant animals can influence the specificity of antibodies present in colostrum and can be used to provide pathogen-specific passive protection of the neonate. When the newborn ingests colostrum from its mother, the transfer of immunoglobulins and leukocytes provide passive protection until it is able to generate its own adaptive immune responses. As such, vaccination schedules are arranged with knowledge of when the species being treated develops the autonomous capacity for mounting the immune response. Vaccination before the newborn's immune system is fully functional may result in a weak or ineffectual response, potentially compromising vaccine effectiveness. Furthermore, the presence of maternal antibodies can clear viral antigens in the vaccine and prevent induction of an effective immune response. Therefore, vaccination against common viral diseases of livestock and companion pets often starts when the animal is a few weeks or months of age, when maternal antibodies have waned and the individual is capable of developing a strong immune response.

Viral Mechanisms of Avoidance and Escape Избегание и побег

Viruses have developed remarkably sophisticated mechanisms to avoid the various host protective immune 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, and antigenic drift, individual viruses have also developed diverse and complex mechanisms of avoiding protective host innate and adaptive immune responses. Examples of these mechanisms will be discussed in this section but the reader also should consult the chapters on individual virus families for specific examples.

Shutdown of Host Macromolecule Synthesis Отключение синтеза макромолекул хозяина

Many viruses initiate infection within the cell by inhibiting 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 and antiviral cytokines such as Type I interferon. 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 rapid replication cycles.

Avoidance of Cytotoxic T lymphocyte (CTL)-Mediated Killing уклонение

Cytotoxic T lymphocyte-mediated killing of virusinfected cells requires the presentation of viral antigens on the surface of the infected cell in the context of the appropriate class I MHC molecule (Fig. 4.4). Thus, viruses have developed different strategies to suppress the normal expression of class I MHC proteins, which prevents cytotoxic T lymphocyte-mediated lysis of virusinfected cells by removing the ligand for the T cell receptor. 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; and (4) production of virus-encoded homologs of class I MHC molecules that can bind β2 microglobulin and viral peptides, but are otherwise dysfunctional as ligands for the CTL response.

Prevention of Natural Killer (NK)-Cell-Mediated Lysis of Virus-Infected Cells

In contrast to cytotoxic T lymphocyte-mediated lysis, which requires the presence of appropriate concentrations of class I MHC antigen on the surface of virus-infected cells, natural killer-cell-mediated lysis of virus-infected cells is promoted by reduced levels of class I MHC antigen on the cell surface (Fig. 4.2). Also important to natural killer cell activity is the balance of inhibitory molecules (such as class I MHC) and stimulatory molecules (such as heat-shock proteins) on the cell surface. Some viruses evade the natural killer cell response by selectively inhibiting cellular production and expression of molecules that provide stimulatory signals for natural killer cell activity.

Interference With Apoptosis

In addition to apoptosis induced by natural killer cell or cytotoxic T lymphocyte-mediated cell lysis, virus infection alone can initiate apoptosis via either the extrinsic (death receptor) or intrinsic (mitochondrial) pathways (see Chapter 3: Pathogenesis of Viral Infections and Diseases).

Apoptosis is the process of programmed cell death, essentially a mechanism of cell suicide that can be activated to eliminate viral factories before virus replication is complete. Apoptosis is especially deleterious to the relatively slow-growing DNA viruses (eg, poxviruses, herpesviruses, and adenoviruses), 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 cell death— 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 tissue necrosis factor (TNF) so that it cannot initiate the extrinsic (death receptor) pathway, or molecules that specifically block the signaling cascade initiated by death receptor activation; (3) production of virus-encoded homologs of antiapoptotic 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 that are apparently used by a myriad of viral proteins.

Counter Defences Against Cytokines Встречная защита против цитокинов

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 nonfunctional 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-1, see earlier discussion of the antiviral state) that trigger production of type I interferon and other antiviral cytokines, or with the signaling pathways activated by the binding of interferon to its receptor. Collectively, these virus-encoded proteins can modulate the activities of a wide variety of critical cytokines such as interleukins (IL-1, IL-6, IL-8), types I and II interferon, and tissue necrosis factor to the replicative benefit of the virus, by either inhibiting or promoting specific cytokine-mediated functions.

Evasion of the Antiviral State Уклонение от антивирусного состояния

Viruses have evolved elaborate strategies to circumvent the activity of important interferon-induced antiviral effector mechanisms such as the protein kinase (PKR) and 20-50 oligoadenylate synthetase (OAS) pathways (see earlier discussion of the antiviral state). 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. In addition, viruses may produce nonfunctional enzyme homologs and/or stimulate pathways that downregulate activity and function of these protective antiviral pathways. Other virus-encoded proteins sequester dsRNA, which is a critical cofactor 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 (see individual virus chapters).

Virus-Specific Gene Silencing Pathways Вирус-специфические генные пути глушения

Cells utilize small, interfering, RNA molecules (RNAi) to “silence” specific genes to regulate normal cellular processes. They can also utilize this same process to interfere with virus replication by producing RNAi that are complementary to specific viral genes. In turn, viruses have developed counter defenses to cellular antiviral RNA interference pathways, either by the production of virusencoded proteins or small interfering mRNA molecules that inhibit key steps of the relevant cellular pathway that leads to production of RNAi. Further, some 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 (Table 4.1).
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 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 (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 live-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 liveattenuated virus vaccine mimics a subclinical infection. The individual virus strain incorporated in a liveattenuated virus vaccine may be derived from any one of several sources.

Avirulent Viruses in Heterologous Species

The original vaccine (vacca meaning cow) introduced by Edward Jenner in 1798 for the control of human smallpox, utilized cowpox virus, a zoonotic pathogen (see Chapter 7: Poxviridae). 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 poxvirus disease, myxomatosis, with the naturally avirulent Shope rabbit
fibroma virus.

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" virus (syn. "wild-type" virus) 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 leads to attenuation. With the recent advent of next generation genome sequencing procedures, the genetic basis of virulence and attenuation has been established with some viruses, human poliovirus for example, 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 that result from infections with some wild-type or "field" viruses, liveattenuated 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 live-attenuated virus vaccines, there is a
strong perceived need to replace what some 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. However, the regulatory approval process for commercial use of genetically engineered vaccines in animals can be more complicated than it is for traditional live-attenuated virus vaccines.

Attenuation of Viruses by Serial Passage in Heterologous Hosts

Serial passage in a heterologous host was an 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 vaccine viruses propagated in embryonated eggs can cross the placenta of ruminants vaccinated during pregnancy, with resultant fetal infection and associated
developmental defects or loss. Similarly, embryonatedegg-propagated African horse sickness virus, which is not naturally zoonotic, caused devastating consequences in humans infected after aerosol
exposure to this vaccine virus.

Attenuation of Viruses by Selection of Mutants and Reassortants

The observation that temperature-sensitive mutants (viruses that are unable to replicate satisfactorily at certain temperatures, usually including normal body temperature) generally display reduced virulence suggested that they might make satisfactory live-attenuated vaccines, although some viruses with temperaturesensitive 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 33C 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.

Nonreplicating Virus Vaccines

Inactivated (Killed) 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 some rabies virus vaccines, and ethylenimine, which is used in the manufacture of some 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.

Furthermore, production of inactivated virus vaccines requires the initial production of large quantities of virulent virus prior to its inactivation, which itself can pose a considerable threat if this virus escapes from the production facility into the environment.

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 for influenza. Examples include vaccines against herpesviruses, influenza viruses, and coronaviruses.

Vaccines Produced Using Recombinant DNA and Related Technologies

Molecular biology and its many associated technologies have 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.

Attenuation of Viruses by Gene Deletion or Site-Directed Mutagenesis

The problem of the reversion to virulence of live attenuated virus vaccines (ie, 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 nonessential 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 ELISA 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 for infectious bovine rhinotracheitis virus (bovine herpesvirus-1) has also been developed.

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 DIVA.

Subunit Vaccines Produced by Expression of Viral Proteins

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 posttranslational 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.

Baculovirus-expressed E2 protein is a highly effective subunit vaccine against classical swine fever virus, as is the capsid protein of porcine circovirus 2. Expression of protective viral antigens in plant cells can theoretically provide a 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. Similarly, bacterial expression systems based on

Escherichia coli are very effective and efficient at generating large quantities of vaccine antigen, and such a system is used for the production of VP2 protein of infectious pancreatic necrosis virus used as a vaccine for salmon.

Viral Proteins that Self-Assemble Into Virus-like Particles (VLPs)

The expression of genes encoding the capsid proteins of viruses within certain families of nonenveloped icosahedral viruses leads to the self-assembly of the individual capsid proteins into VLPs that can be used as a vaccine. This strategy has been developed for various picornaviruses, caliciviruses, rotaviruses, and orbiviruses, and an effective VLP-based vaccine has been developed recently against human genital papillomaviruses. Baculovirusexpressed capsid protein of porcine circovirus 2 selfassembles into VLPs and this vaccine confers protective immunity against porcine-circovirus-associated diseases such as multisystemic wasting disease. The advantage of recombinant VLPs 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 with some constructs, stability of the VLP after production, and less effective immunity as compared with some existing vaccines.

Viruses as Vectors for Expression of 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 nonreplicating ("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 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, adenoviruses, herpesviruses, and adeno-associated viruses (which are parvoviruses). Vaccination of animals with a significant number of different recombinant poxvirusvectored 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 vacciniarabies 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 HIVrecombinant 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 liveattenuated 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 the livers of virus-infected rabbits. Similarly, recombinant adenoviruses successfully have been developed for immunization of animals against diseases such as rabies (in wildlife) and foot-and-mouth disease (in livestock).

A number of DNA virus vectored vaccines have also been developed for use in poultry, including recombinant turkey herpesvirus-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 (which is caused by another herpesvirus) and the other diseases represented in the construct (Newcastle disease, infectious laryngotracheitis, and 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 (nonpathogenic) 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.

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 briefly 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. 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 (eg, vesicular stomatitis virus), as have other positive-sense RNA viruses such as the 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 coexpress the GP5 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 nonstructural proteins of Venezuelan equine encephalitis virus and the structural protein genes of equine arteritis virus. A similar strategy has been used to make a porcine epidemic diarrhea virus (a coronavirus) vaccine for pigs using Venezuelan equine encephalitis virus replicons expressing the porcine epidemic diarrhea virus spike gene.
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 cocultivation 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.

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 "naked" DNA vaccine was developed to protect salmon against infectious hematopoietic necrosis virus, and a DNA-based vaccine to prevent West Nile disease in horses was approved for use in 2005 but has since been discontinued. Indeed, commercial utilization of this strategy in veterinary vaccines has been slow, and a DNA vaccine is yet to be approved for use in humans.

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 13 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

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. A commercial subunit vaccine based on infectious pancreatic necrosis virus VP2 gene expressed by E. coli is effective in controlling this disease in salmonids.

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 structure, 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 different strains of a particular virus and, therefore, may elicit a cross-reactive T cell response in some hosts. However, the MHC proteins that bind these peptides are highly polymorphic within any species and even more so between species. That makes the identification of common peptide epitopes across strains of the virus and all of the genotypes of animals responding to the virus very challenging. Today's sophisticated bioinformatics capabilities make this approach more viable.

Vaccine Adjuvants

The immunogenicity of inactivated vaccines, especially that of purified protein vaccines and synthetic peptides, usually needs to be enhanced to optimize their utility. 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.

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) to 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 polyphospazene 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 nonpyrogenic 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. As discussed earlier in this chapter, viruses contain characteristic signatures designated as PAMPs that efficiently stimulate pathogen recognition receptors in dendritic cells and other innate cells that are critical in induction of adaptive immune responses. These PAMPs include ssRNA, dsRNA, and certain viral proteins.
Whole-virus vaccines, both live-attenuated and killed, often retain these microbial signatures because the vaccines include intact virions, promoting vaccineinduced responses. TLR9 recognizes DNA molecules with methylation patterns not usually found in eukaryotic cells, and cytosine guanine oligonucleotides (CpG ODNs) have been developed in an effort to activate the TLR9 pathway in conjunction with various antigens and DNA vaccines. Other vaccine adjuvants in development or under evaluation include polyinosinic:polycytidylic acid, which resembles viral dsRNA and stimulates TLR3, and saponin, an amphipathic glyocide derived from tree bark. Enhanced production of cytokines induced by the innate immune response can be achieved by expressing relevant 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 encoding 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, 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 liveattenuated 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.
In many species, 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 lifelong, 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. 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. It is also proposed that interference can occur between different attenuated viruses contained in certain vaccine formulations. Special difficulties also complicate vaccination against viruses known to establish persistent infections, such as herpesviruses and retroviruses. These vaccines must be remarkably effective if it is to prevent, not only the primary disease, but also the
establishment of lifelong latency. Live-attenuated virus vaccines are generally more effective in eliciting cellmediated immunity than inactivated ones, however, they also carry some risk of themselves establishing persistent infections in the immunized host.

Potential Adverse Effects of Vaccines Under-Attenuation

Some live-attenuated virus vaccines cause clinical signs in some vaccinated animals—in effect, a mild, or even severe 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 appropriately evaluated 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. An additional unintended consequence of live-attenuated virus vaccines is the potential for transmission of viable vaccine virus from one animal to another, as has been reported among unvaccinated livestock adjacent to animals that were vaccinated with live-attenuated bluetongue virus vaccine. The unintended, natural transmission of live-attenuated vaccine viruses provides an opportunity for them to revert to
virulence through genetic instability or recombination with “field” viruses.

Genetic Instability and Recombination

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, liveattenuated vaccine viruses are incapable of such spread, but in those that do there may be an accumulation of mutations (reversions) 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, nonreplicating vaccine.

Temperature-sensitive mutants of bovine viral diarrhea virus have also proven to be genetically unstable. A more recent and ominous concern regarding genetic alteration of vaccine viruses comes from Australia, where the concurrent use of different infectious laryngotracheitis virus vaccines in poultry led to the emergence and spread of a novel recombinant virulent virus derived from distinct
live-attenuated vaccine strains. Similarly, it is abundantly clear that live-attenuated vaccine strains of segmented RNA viruses such as bluetongue virus and African horse sickness virus (both orbiviruses) can reassort their genes with either field viruses or other vaccine viruses, in both the insect vector and animal host, to create novel progeny with potentially undesirable properties.

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 also 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-andmouth 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 (eg, the contamination of Marek's disease vaccine with reticuloendotheliosis virus). Another important
source of virus contaminants is fetal bovine serum, used universally in cell cultures; all batches of fetal bovine serum 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 wholevirus vaccines, as some viruses are more resistant to inactivation than others; the prion agents are notoriously resistant to traditional methods of sterilization, for example. In some instances serious adverse effects relating to use of attenuated virus vaccines have an unknown
origin; for example the chimeric West Nile vaccine based on yellow fever virus was highly effective at preventing West Nile disease in horses but was recalled after multiple reports of acute anaphylaxis, colic, respiratory distress and death following vaccination of horses.

Adverse Effects in Pregnant Animals

Live-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 nonimmune 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 Nonreplicating 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 nonreplicating vaccines, and others wherein contaminating viruses survived the inactivation process.

Vaccination Policy and Schedules

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 typically modest 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.

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 inoculations an individual animal must receive. 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 many 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 vulnerable 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 liveattenuated 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 antibodagainst 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 (eg, 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 (eg, 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 live-attenuated virus vaccines are abortigenic or teratogenic, inactivated vaccines are generally recommended for vaccination of pregnant animals.

Availability and Recommendation of 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 viral diseases such as foot-and-mouth disease. There are also different requirements appropriate to various types of livestock husbandry (eg, for intensively raised dairy cattle as compared to freeranging beef cattle and their calves, or cattle in feedlots; also 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: Таким образом, читатель обращается к специализированным организациям, которые публикуют рекомендации по вакцинации пример
horses (American Association of Equine Practitioners
http://www.aaep.org/vaccination_guidelines.htm)),
cats (American Association of Feline Practitioners
(http://www.catvets.com/professionals/guidelines/publications/?Id5176)), and
dogs (American Animal Hospital Association
(http://secure.aahanet.org/eweb/dynamicpage.aspx?
site5resource&webcode5CanineVaccineGuidelines)).
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, 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

Poultry production is economically important worldwide, an estimated .$20 billion per year industry in the United States for example. To help protect this industry, 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.
In commercial aquaculture, vaccination is used to prevent infectious hematopoietic necrosis and infectious pancreatic necrosis in salmonids. Vaccines to these diseases include DNA and subunit protein vaccines that are administered either by injection or orally. A live-attenuated virus vaccine against cyprinid herpes virus 3 infection of koi carp (Cyprinus carpio haematopterus) was recently approved for use in Israel; this vaccine has a genetic deletion that allows differentiation between vaccinated and infected fish. 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 to 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 receptor such as the TLRs that lead to proinflammatory 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. Similarly, it appears that the innate

immune response induces an antiviral state in addition to priming adaptive immunity in fish as it does in mammals.

Adaptive responses involving T and B lymphocytes and specific immunoglobulin production are also critical for antiviral immunity in fish. The structure of the T cell receptor complex (αβ or γδ) 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 (sIgM1 or sIgτ/ξ1)—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. Indeed, original vaccination strategies such as those employed by Arnold Theiler in an effort to prevent African horse sickness, employed the simultaneous inoculation of virulent virus and immune sera to susceptible horses.

Although not commonly used, homologous immunoglobulin is now 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.

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 (HIV-AIDS), influenza, and B- and C-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 nonenzymatic) that are more essential to the replication of the virus than to the survival of the cell. 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 nontoxic for uninfected cells, but very effective in herpesvirusinfected cells. Aciclovir and related analogs (eg, 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 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 activate
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 parvoviruses), 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 Parvoviridae, subfamily Parvovirinae, genus Dependoparvovirus) that can infect both dividing and nondividing 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. Adenoassociated viruses are considered to be avirulent (nonpathogenic), and the capacity for integration is readily abolished by genetic manipulation. Recombinant adenoassociated viruses that express appropriate proteins have been evaluated for the correction of a variety of human genetic disorders, including hemophilia and muscular dystrophy. Adeno-associated viruses have also gained favor as expression vectors of broadly neutralizing antibodies against HIV that may provide preexposure prophylaxis and protection against infection in "vaccinated"
individuals.
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 immunemediated 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.

Veterinary virology – 2019

Lecture 5. Laboratory Diagnosis of Viral Infections

Tests to support or establish a specific diagnosis of a viral infection are of five general types:
(1) those that demonstrate the presence of infectious virus;
(2) those that detect viral antigens;
(3) those that detect viral nucleic acids;
(4) those that demonstrate the presence of an agentspecific antibody response; and
(5) those that directly visualize ("see") the virus. Most available routine tests are agent-dependent—that is, they are designed to detect a specific virus and will give a negative test result even if
other viruses are present in the sample. For this reason, agent-independent tests such as virus isolation and electron microscopy are still used to identify the unexpected or unknown agent in a clinical sample. In addition, highthroughput nucleic acid sequencing, frequently referred to
as next generation sequencing, is capable of identifying unknown and/or noncultivatable viruses.

Traditional methods such as virus isolation are still widely used; however, many may be too slow to have any direct influence on clinical management of an index case. A major thrust of the developments in diagnostic sciences continues to be toward rapid methods that provide a definitive answer in less than 24 hours or, optimally, even during the course of the initial examination of the animal. A second major area of focused effort is the development of multiplexed tests that can screen simultaneously for several pathogens from a single sample. The best of these methods fulfill
five prerequisites: speed, simplicity, diagnostic sensitivity, diagnostic specificity, and low cost. For some economically important viruses: (1) standardized diagnostic tests and reagents of good quality are available commercially; (2) assays have been miniaturized to conserve reagents and decrease costs; (3) instruments have been developed to automate

tests, again often decreasing costs; (4) computerized analyses aid in making the interpretation of results as objective as possible in addition to facilitating reporting, record keeping, and billing.

Although less impressive in veterinary medicine in comparison with human medicine (for reasons of economic return on investment and range of tests required across each species), there has been recent expansion in the number of commercially available rapid diagnostic kits. These tests
detect viral antigens, allowing a diagnosis from a single specimen taken directly from the animal during the acute phase of the illness, or they test for the presence of virusspecific antibody. Solid-phase enzyme immunoassays (EIAs) or enzyme-linked immunosorbent assays (ELISAs), in particular, have revolutionized diagnostic virology for both antigen and antibody detection, and are now methods
of choice in many situations. For laboratory-based diagnosis, polymerase chain reaction (PCR) technology is now widely used to detect viral nucleic acids in clinical specimens, offering a very rapid alternative to other methods of virus detection. Real-time (quantitative) PCR assays, in particular,
facilitate the very rapid, sensitive, and specific identification of many known pathogenic viruses, and automation of these assays allows the processing of large numbers of samples in short periods of time (high sample-throughput). Another major advantage of real-time PCR assays is that they provide an objective estimate of viral load in a clinical sample if properly standardized. Research efforts in PCR
continue to move testing from the laboratory to the field, particularly for high-consequence agents for which rapidity of diagnosis is critically important.

The provision, by a single laboratory, of a comprehensive service for the diagnosis of viral infections
of domesticated animals is a formidable undertaking. Viruses in more than 130 different genera and belonging to more than 30 families cause infections of veterinary significance. Add to these numbers the rapidly expanding array of viruses that occur in wildlife and fish, and it is not surprising that no single laboratory can have the necessary specific reagents available or the skills and experience for the detection and identification of all viruses of all animal species. For this reason, veterinary diagnostic laboratories tend to specialize (eg, in diseases of food animals, companion animals, poultry, fish, or laboratory species, or in diseases caused by exotic viruses (foreign animal diseases)).
Contacting the laboratory to determine its specific capabilities should be a first step in submitting specimens for testing. Table 5.1 provides a general guide to diagnostic tests currently used in veterinary medicine. These will be defined in more detail later in this chapter.

RATIONALE FOR SPECIFIC DIAGNOSIS

Why bother to establish a definitive laboratory diagnosis of a virus infection? In earlier times when laboratory diagnostic testing was in its infancy, diagnosis of diseases related to viral infections was achieved mainly on the basis of clinical history and signs, and/or gross pathology and histopathology; laboratory test results were viewed as confirmatory data. This is no longer the case, for several

reasons: (1) the recent development of rapid test formats for specific and sensitive identification of individual viral infections; (2) many clinical cases occur as disease complexes that cannot be diagnosed on the basis of clinical signs or pathology alone—for example, the canine and
bovine respiratory disease complexes; (3) diagnostic medicine, especially that pertaining to companion animals, increasingly demands reliable and specific antemortem diagnoses; (4) legal/regulatory actions for diseases of production animals and zoonoses can require identification
of the specific agents involved, avian influenza being a relevant contemporary example. Other areas in which laboratory testing data are essential are considered below.

At the Individual Animal or Individual Herd Level. На уровне отдельного животного или отдельного стада

Diseases in which the management of the animal or its prognosis is influenced by the diagnosis. Respiratory diseases (eg, in a broiler facility, acute respiratory disease in a boarding kennel, shipping fever in a cattle feedlot), diarrheal diseases of neonates, and some mucocutaneous diseases may be caused by a variety of different infectious agents, including viruses. Rapid and accurate identification of the causative agent can be the basis for establishing a management plan (biosecurity, vaccination,
antimicrobial treatment) that prevents additional losses in the stable, kennel, flock, or herd.

Certification of freedom from specific infections. For diseases in which there is lifelong infection—such as bovine and feline leukemia virus infection, persistent bovine viral diarrhea virus infection, equine infectious anemia virus infection, and certain herpesvirus infections—a negative test certificate or history of appropriate vaccination is often required as a condition of sale, for exhibition at a state fair or show, for access to competitions, and for international movement.

Artificial insemination, embryo transfer, and blood transfusion. Males used for semen collection and females used in embryo transfer programs, especially in cattle, and blood donors of all species are usually screened for a range of viruses to minimize the risk of virus transmission to recipient animals.

Zoonoses. Viruses such as rabies, Rift Valley fever, Hendra, influenza, eastern, western, and Venezuelan equine encephalitis are all zoonotic, and are of sufficient public health significance as to require veterinary diagnostic laboratories to establish the capability for accurate detection of these agents. Early warning of a potential influenza virus epidemic through diagnosis of infection and/or disease in an individual poultry flock or in affected swine allows the implementation of control programs to eradicate the infection and/or restrict movement of exposed animals. As an example, laboratory identification of rabies virus in a dog, skunk, or bat that has bitten a child provides the basis for treatment decisions.

At the Regional, Country, and International Level На региональном, страновом и международном уровне

Epidemiologic and economic awareness. Provision of quality veterinary service in any region (eg, a state or province) or country depends on knowledge of prevailing diseases, hence epidemiologic studies to determine the prevalence and distribution of particular viral infections are frequently undertaken. Such programs are also directed against specific

zoonotic, food-borne, water-borne, rodentborne, and arthropod-borne viruses. Internationally, the presence of specific livestock diseases in a country or region requires notification to the World Organization for Animal Health (syn. Office Internationale des Epizooties (OIE)), which records the occurrence of these notifiable diseases in the approximately 180 member countries of the organization.

Test and removal programs. For infections caused by viruses, such as equine infectious anemia virus, Marek's disease virus, bovine herpesvirus 1, pseudorabies virus, and bovine viral diarrhea virus, it is possible to reduce substantially the incidence of disease or eliminate the causative virus from herds or flocks by test and removal programs.

The elimination of pseudorabies virus from commercial swine facilities in the United States is an example of where differential laboratory tests (the so-called differentiation/ discrimination of infected from vaccinated animals (DIVA) test) were essential to the eradication effort. Surveillance programs in support of enzootic disease research and control activities. Surveillance of viral infections based on laboratory diagnostics is central to all epidemiologic research, whether to determine the significance of a particular virus in a new setting, to unravel the natural history and ecology of a virus in a particular host animal population, to establish priorities and means of control, or to monitor and evaluate control programs. Surveillance programs in support of exotic disease research and control activities. The countries of western Europe, North America, Australia, New Zealand, and Japan are usually free of many devastating diseases of livestock such as foot-and-mouth disease, classical swine fever,
African swine fever, and fowl plague that are still enzootic in other parts of the world. However, periodic incursions of these feared exotic diseases into previously free areas occur with alarming regularity and very substantial adverse economic impact. Thus it is of the utmost importance that
the clinical diagnosis of a suspected high-consequence virus infection be confirmed quickly and accurately. Many countries maintain or share the use of specialized biocontainment laboratories devoted to rapid and accurate diagnosis and research on high-consequence viruses that cause economically devastating "foreign animal diseases."

Prevention of new, emerging, and reemerging viral diseases of animals. Continuous surveillance of animal populations for evidence of new viruses, new diseases, and new epizootics is essential if new threats are to be dealt with rapidly and comprehensively. New viruses and new virusdisease associations continue to be discovered, virtually every year, as domestic species continually interface with wildlife. Vigilance by astute veterinary clinicians as well as by diagnosticians and epidemiologists is essential for early recognition of such occurrences.

COLLECTION, PACKAGING, AND TRANSPORT OF SPECIMENS

The chance of detecting a virus depends critically on the attention given by the attending veterinarian to the collection of specimens. Clearly, such specimens must be taken from the right site, from the most appropriate animal, and at the right time. The right time for virus detection is as soon as possible after the animal first develops clinical signs because maximal amounts (titers) of virus are usually
present at the onset of signs and often then decrease rapidly during the ensuing days. Specimens for virus detection taken as a last resort when days or weeks of empirical

therapy have failed are almost invariably a useless endeavor and a waste of consumer and laboratory resources.
Similarly, the incorrect collection and storage of specimens, and the submission of inappropriate specimens, will diminish the likelihood of a valid diagnostic laboratory result. The site from which the specimen is collected will be influenced by the clinical signs and knowledge of the pathogenesis of the suspected agent(s) (Table 5.2). In respiratory virus infections in cattle, for example, the most important diagnostic specimens that should be collected include nasal or throat swabs or transtracheal wash fluid from live animals, and lung tissue and lymph nodes from dead animals; whole-blood samples from this type of case are often useless because the causative viral agents (bovine respiratory syncytial virus, bovine herpesvirus 1, bovine coronavirus, etc.) may not produce detectable concentrations of virus in blood samples (viremia).

Likewise, for routine enteric cases (diarrhea), feces would be the primary sample in calves with rotavirus, coronavirus, or torovirus infections, with whole-blood being useful only if bovine virus diarrhea virus was a likely cause. Timing of sample collection is also critical, particularly with enteric cases, as detection of rotavirus may not be possible more than 48 hours after the onset of clinical signs. PCR tests do extend the sampling period because of their high analytical sensitivity and their ability to detect viral nucleic acids even if the causative virus is already complexed with neutralizing antibodies, but this longer detection period does not eliminate the need to be attentive to timing of sample collection. Furthermore, the extended detection of viral nucleic acid by PCR assays increases the likelihood of false-positive results, wherein a virus detected by PCR is not the actual cause of the affected animal's disease.

Tissue specimens should always be taken from any part of the body where lesions are observed, either by surgical biopsy or at necropsy of dead animals, as it is critical that laboratory findings be reconciled with lesions that are manifest in the affected animal. Thus, separate samples should be split between material that will be fixed (formalin or other fixative) and material that will remain unfixed for virus detection assays such as immunofluorescence staining, PCR testing, or virus isolation.
Because of the lability of many viruses, specimens intended for virus isolation must always be kept cold and moist, which requires preparation ahead of time. In collection of specimens such as swabs, the discussion immediately turns to viral transport media. The various transport media consist of a buffered salt solution to which has been added protein (eg, gelatin, albumin, or fetal bovine serum) to protect the virus against inactivation and antimicrobials to prevent the multiplication of bacteria and fungi. A transport medium designed for bacteria or mycoplasma should not be used for virus sampling unless it has been proven not to be inhibitory for the intended test. Separate samples should be collected for bacterial testing. An example of a kit containing materials suitable for the collection and transportation of specimens is shown in Fig. 5.1.

Specimens should be forwarded to the testing laboratory as soon as possible. With courier services increasingly available throughout the world, overnight delivery services have greatly decreased the time interval required for agent detection, and also greatly increased the rate of diagnostic success (pathogen detection rate). Specimens should not be frozen but should be kept cold (refrigeration

temperature), if delivery to the laboratory will be within several days. While viability is not necessary for PCR assays and direct antigen detection, maintaining the specimens under optimum condition for virus isolation will also enhance detection by these other techniques.
Specimens should never be sent to the diagnostic laboratory without a detailed clinical history of the animal and/ or herd from which the specimens are derived. Clinical histories assist diagnosticians in selecting the most appropriate tests for the specimens received and permit a dialog with the clinician over additional specimens if needed. Similarly, a detailed and accurate description of the nature
and distribution of the lesions in affected animals is critical if samples are to be submitted for histopathological evaluation, regardless of whether the tissue specimens were obtained at necropsy or at surgical biopsy.

Packaging and specimen labeling and identification may be a mundane topic, but attention to these details maximizes the likelihood of safe arrival of the specimens at the laboratory and prevents legal sanctions over incorrectly shipped hazardous materials. The submitter should have an understanding of local transport regulations, which in most instances mirror international air transport regulations, and
pack diagnostic specimens accordingly. Although specimens may have been dispatched locally by land transport, often shipments will be partially transported by air over even short to moderate distances, without the knowledge of the shipper. The specimens should be protected from breaking in transit, packaged to prevent leakage, and should be sent refrigerated (but not frozen), with "cold packs." Wherever possible, sampling should include specimens that allow the use of several diagnostic tests, as no single test will provide an unambiguous diagnosis in all cases.

DIAGNOSIS OF VIRAL INFECTIONS BY GROSS EVALUATION AND HISTOPATHOLOGY

The gross and histological evaluation of tissues from animals with presumptive viral diseases is still a useful and critical diagnostic method. If biopsy/necropsy samples are collected for possible histopathological diagnosis of viral infections, then the appropriate tissue specimens in the
appropriate fixative—routinely formalin—are required. If special procedures are to be requested, such as electron microscopy or frozen sections for immunohistochemical staining, the receiving laboratory should be consulted for procedural and material details. It is critical that a thorough, accurate history and description of the lesions in affected animals accompany the submitted specimens.

The great benefit of pathology is that it can provide confirmation of specific viral diseases, especially when done in conjunction with appropriate laboratory virological testing such as immunohisto-chemistical staining for viral antigens or nucleic acid detection. In contrast, the mere demonstration of a particular virus (eg, by real-time PCR or next generation sequencing), or seroconversion of
an animal to that virus, is not necessarily proof of disease causality. Thus, laboratory demonstration of a specific virus combined with compatible clinical signs and lesions in the affected animal strongly reinforces confidence in a specific diagnosis. Similarly, the identification of characteristic lesions in an animal without associated detection of the

relevant virus should stimulate additional laboratory efforts to confirm or refute the tentative diagnosis.

METHODS OF DETECTION OF VIRUSES

Detection of Viruses by Electron Microscopy Perhaps the most obvious method of virus detection/ identification is direct visualization of the virus itself (Fig. 5.2).

The morphology of most viruses is sufficiently characteristic to identify the image as a virus and to assign an unknown virus to the correct family. In the context of the particular case (eg, detection of parapoxvirus in a scraping from a pock-like lesion on a cow's teat), the method may provide an immediate definitive diagnosis. Noncultivable viruses may also be detectable by electron microscopy. Beginning in the late 1960s, electron microscopy was the means to the discovery of several new families of previously noncultivable viruses, notably rotaviruses, noroviruses, astroviruses, and toroviruses, and unknown members of recognized families such as adenoviruses and coronaviruses. Even today, noncultivable viruses such as those in the genus Anellovirus (torque teno viruses) have been identified by electron microscopy in samples from humans and a variety of animals.

Two general procedures can be applied to virus detection by electron microscopy: negative-stain electron microscopy and thin-section electron microscopy. For the negative stain procedure, virus particles in a fluid matrix are applied directly to a solid support designed for the procedure. Contrast stains are applied and the virus particles are directly visualized by electron microscopy. Thin-section electron microscopy can be used directly on fixed tissue samples, usually containing "viral" inclusions from the affected animal or on cell cultures growing an unidentified virus. Low sensitivity is the biggest limitation of electron microscopy as a diagnostic tool, followed by the need for expensive equipment and a highly skilled microscopist. To detect virus particles by negativestain electron microscopy, the fluid matrix must contain approximately 10^6 virions/mL. Such concentrations are often surpassed in clinical material such as feces and vesicle fluid, or in virus-infected cell cultures, but not in respiratory mucus, for instance. Aggregation of virus particles by specific antiserum (immunoelectron microscopy) can enhance sensitivity and provide provisional identity of the agent. For thin-section electron microscopy, most of the cells in the tissue sample must contain virus if virions are likely to be visualized. Routine electron microscopy procedures have been largely replaced with more sensitive and less expensive procedures such as antigen-capture tests, immunostaining techniques or PCR tests, but because electron microscopy is an agentindependent test, it still has use in specialized cases and in facilities with the necessary equipment and expertise.

Detection of Viruses by Isolation Обнаружение вирусов по изоляции

Despite the explosion of new techniques for "same-day diagnosis" of viral disease by demonstration of viral antigen or viral nucleic acid in specimens, virus isolation in cell culture remains an important procedure. Theoretically at least, a single viable virion present in a specimen can be grown in cultured cells, thus expanding it to produce enough material to permit further detailed

characterization. Virus isolation remains the “gold standard” against which newer methods must be compared, but nucleic acid detection tests, particularly real-time PCR assays, are challenging that paradigm.
There are several reasons why virus isolation remains as a standard technique in many noncommercial laboratories. Until recently it was the only technique that could detect the unexpected—that is, identify a totally unanticipated virus, or even discover an entirely new agent.
Accordingly, even those laboratories well equipped for rapid diagnosis may also inoculate cell cultures in an attempt to isolate a virus.

Metagenomic and “deep sequencing” techniques can detect unknown agents (so-called pathogen mining), and more laboratories are applying this technology to virus discovery. However, cell culture isolation remains the easiest method of producing a supply of live virus for further examination by molecular methods (genome sequencing, antigenic variation, etc.). Research and reference laboratories, in particular, are always on the lookout for new viruses within
the context of emerging diseases; such viruses require comprehensive characterization, as recently shown by the quickly evolving highly pathogenic H5N1 strain of influenza virus. Moreover, large quantities of virus must be grown in cultured cells to produce diagnostic antigens and reagents such as monoclonal antibodies. Until recently, vaccine development has also been reliant on the
availability of viruses grown in culture, although this may quickly change in the future with the increasing sophistication of recombinant DNA technology.
The choice of cell culture strategy for the primary isolation of an unknown virus from clinical specimens is largely empirical. Primary cells derived from fetal tissues of the same species usually provide the most sensitive cell culture substrates for virus isolation. Continuous cell lines derived from the homologous species are, in many cases, an acceptable alternative. As interest in wildlife
diseases increases, most laboratories are challenged to have the necessary cell cultures to “match” with the affected species. Testing strategies for challenging cases tend to reflect the creativity and bias of the diagnostic virologist and the particular laboratory, although the clinical signs exhibited by the affected animals will often suggest which virus might be present. Most laboratories also select a
cell line that is known to grow many types of viruses, in case an unanticipated agent is present. Arthropod cell cultures are used frequently as a parallel system for isolating “arboviruses.” Even with the best cell culture systems available, many viruses such as papillomaviruses will not grow
in traditional cell culture conditions. Special culture systems such as organ cultures and tissue explants can be of value, but contact should be made with the testing laboratory to determine their capabilities before requesting such specialized and sophisticated diagnostic expertise.

Historically, when standard methods had failed to diagnose what appeared to be an infectious disease, inoculation of the putative natural host animal was used to define the infectious nature of the problem and to aid in the eventual isolation of the agent. This practice has largely been abandoned, as a result of costs and animal welfare concerns. Some specialized laboratories still have the capability to inoculate suckling mice, a system

that has been valuable for isolating arboviruses that resist cultivation in cell cultures. Embryonated hens' eggs are still used for the isolation of influenza A viruses, even though cell cultures (MadinDarby canine kidney (MDCK) cells) are now more commonly used. Many avian viruses also replicate more readily in eggs than in cell cultures derived from chick embryo tissues, and there is a lack of widely available avian cell lines for routine virus isolation procedures. According to the virus of interest, the diagnostic specimen is inoculated into the amniotic cavity, or the allantoic cavity, the yolk sac, onto the chorioallantoic membrane or, in rare instances, intravenously into the vessels of the shell membrane and embryo. Evidence of viral growth may be seen on the chorioallantoic membrane (eg, characteristic pocks caused by poxviruses), but otherwise other means are used to detect viral growth (eg, death of the embryo, hemagglutination, immunofluorescence or immunohistochemical staining of viral antigens, PCR, or antigen-capture ELISA).

Attempts to isolate viruses require stringent attention by the clinician to the details of sample collection and transport, because success depends on the laboratory receiving a specimen containing viable virus. Contact with the testing laboratory before specimen collection is strongly advised in order to clarify the sampling strategy, assess shipping requirements, and alert the laboratory to the number and type of specimens being shipped. Having cell cultures available on the day of arrival of a specimen can enhance the success of isolation. There is no such thing as an emergency ("stat") virus isolation; each virus has its own biological clock and no amount of concern will speed up the replication cycle. For viruses such as the alphaherpesviruses, a successful isolation can be evident as cytopathic effect in the inoculated cell cultures within 23 days, whereas others are considerably slower and require repeated serial passage.

In general, the time for detection will depend on the laboratory's procedures for identifying virus in the culture system. For instance, noncytopathic bovine viral diarrhea virus can be detected by virus isolation as early as 3 days postinoculation or as late as 3 weeks, depending on laboratory
procedures. Procedures for routinely detecting and identifying virus in inoculated cell cultures include immunofluorescence or immunohistochemical staining of the infected monolayer, antigen-capture ELISA, nucleic acid detection tests such as PCR, hemadsorption, or even negative-stain electron microscopy for unknown isolates.

Detection of Viral Antigens

The direct detection of viral antigens in a clinical sample can be achieved in as little as 15 minutes with some immunoassays, or the procedure can take several days if extensive sample preparation and staining is involved. Viable virus is generally not required in the specimen for a positive antigen detection test result, but the timing of sample collection is as important with these assays as it is
for virus isolation. Analytical sensitivity varies across the various test modalities, ranging from detection of a single infected cell to assays that require as much as 10^5 antigen units. The advance that revolutionized this type of testing was the development of monoclonal antibodies. These
reagents are highly specific in their binding to antigen and, once developed, provide a virtually inexhaustible supply of the same material for test consistency.

The downside to antigen detection tests is that many antigens are altered or masked by tissue fixation.
Furthermore, they are agent specific, thus a test for canine parvovirus cannot detect the presence of canine coronavirus in the specimen, which would require a separate and additional agent-specific test.

Immunofluorescence Staining

Immunofluorescence or fluorescent antibody staining is an antigen-detection test that is used primarily on frozen tissue sections, cell "smears," or cultured cells; formalin-fixed tissue samples are generally not useful with this procedure. Antigen is detected through the binding to the sample matrix of specially modified, agent-specific antibodies. The modification is the "tagging" of the antibody with a fluorochrome that absorbs ultraviolet light of a defined wavelength, but emits light at a higher wavelength. The emitted light is detected optically with a special microscope equipped with filters specific for the emission wavelength of the fluorochrome. The fluorochrome can be bound directly to the agent-specific antibody (direct immunofluorescence) or it can be attached to an anti-immunoglobulin molecule that recognizes the agent-specific antibody (indirect immunofluorescence) (Fig. 5.3A). The indirect method enhances the sensitivity of the test, but may also increase background.

Immunofluorescence staining does require specialized equipment, including a cryostat for sectioning frozen tissue along with a fluorescent microscope for detecting the bound antibody. Immunofluorescence has proven to be of great value in the identification of viral antigens in
infected cells taken from animals or in cultured cells inoculated with specimens from infected animals. For certain viral diseases, specimens that include virus-infected cells can easily be collected from the mucous membrane of the upper respiratory tract, genital tract, eye, or skin, simply by swabbing or scraping the infected area with reasonable firmness. Cells are also present in mucus aspirated from the nasopharynx or in fluids from other sites, including tracheal and bronchial lavages, or pleural,
abdominal, or cerebrospinal fluids. Respiratory infections with parainfluenzaviruses, orthomyxoviruses, adenoviruses, and herpesviruses are particularly amenable to rapid diagnosis (less than 2 hours test time) by immunofluorescence staining. The method can also be applied to tissue—for example, biopsies for the diagnosis of herpesvirus diseases, or at necropsy on brain tissue from a raccoon showing neurological signs as a result of infection with canine distemper virus or rabies virus (Fig. 5.4).

Immunohistochemical (Immunoperoxidase) Staining

In principle, immunohistochemical staining is very similar to immunofluorescence staining of viral antigens, but with several key differences (Fig. 5.3B).
The "tag" used in immunohistochemical staining is an enzyme, generally horseradish peroxidase. The enzyme reacts with a substrate to produce a colored product that can be visualized in the infected cells with a standard light microscope. The tissue sample will often be formalin-fixed, which permits testing of the specimen days to weeks after sampling, without the need for low temperature storage. Another major advantage for the immunohistochemical staining technique is that it involves an amplification process wherein the product of the reaction increases with increasing incubation, whereas

immunofluorescence staining generates a real-time signal that does not get stronger with a longer incubation period. Furthermore, immunohistochemically stained slides can be kept for extended periods of time for several observations, whereas the immunofluorescence slides deteriorate more rapidly. Immunofluorescence does have the advantage of speed; immunohistochemical staining
on formalin-fixed tissues requires more than 24 hours to obtain results. Perhaps the greatest benefit of immunohistochemical staining is that it readily facilitates comparison of viral antigen distribution with lesions present in the tissue section (Fig. 5.5). For example, a lesion "consistent with canine parvovirus infection" becomes a "canine parvovirus lesion."

Enzyme Immunoassay—Enzyme-Linked Immunosorbent Assay

EIAs—often referred to as ELISAs—have revolutionized diagnostic testing procedures. Assays can be designed to detect antigens or antibodies. Although EIAs have moderate sensitivity, samples may still require more than 10^5 virus particles/mL for positive reactions with many tests.
This level of sensitivity still makes these tests highly valuable, particularly in group settings, where any positive animal defines the herd status. Assays may be conducted on a single sample in the veterinarian's clinic or on many hundreds of samples at the same time, using automated systems in centralized laboratories. Some commonly used antigen detection test kits include those specific for feline leukemia virus, canine parvovirus, bovine viral diarrhea virus, rotavirus, and influenza virus.
There are many different types of EIA tests that differ in their geometric properties, detector systems, amplification systems and sensitivity. Not all possible tests will be discussed, as the basic test principles apply to all. Most EIAs are solid-phase enzyme immunoassays; the "capture" antibody is attached to a solid substrate, typically the wells of polystyrene or polyvinyl microtiter plates. The simplest format is a direct EIA (Fig. 5.6).

Virus and/or soluble viral antigens from the specimen are allowed to bind to the capture antibody. After unbound components are washed away, an enzyme-labeled antiviral antibody (the "detector" antibody) is added; various enzymes can be linked to the antibody, but horseradish peroxidase and alkaline phosphatase are the most commonly used. After a washing step, an appropriate organic
substrate for the particular enzyme is added and readout is based on the color change that follows. The colored product of the reaction of the enzyme on the substrate can be detected visually or read by a spectrophotometer to measure the amount of enzyme-conjugated antibody bound to the captured antigen. The product of the enzyme reactions can be modified to produce a fluorescent or chemiluminescent signal to enhance sensitivity. With all such assays, extensive validation testing must be carried out to determine the cut-off values of the test, which define the diagnostic sensitivity and diagnostic specificity of the test.

Indirect EIAs are widely used because of their greater analytical sensitivity, but the increase in sensitivity is usually accompanied by a loss of diagnostic specificity. In this test format, the detector antibody is unlabeled and a second labeled (species-specific) anti-immunoglobulin is added as the "indicator" antibody (Fig. 5.6).

Alternatively, labeled staphylococcal protein A, which binds to the Fc moiety of IgG of many mammalian species, can be used as the indicator in indirect immunoassays. Monoclonal antibodies have especially facilitated the development of EIA tests, because they provide a consistent supply of highly sensitive and specific reagents for commercial tests.
However, any variation (antigenic variation of the virus target) in the specific epitopes recognized by specific monoclonal antibodies can lead to loss of binding and loss of test sensitivity because of false-negative results. EIAs have been adapted to formats for use in veterinary clinics on single animal specimens (Fig. 5.7).

Immunochromatography

Immunochromatography simply refers to the migration of antigen or antigenantibody complexes through a filter matrix or in a lateral flow format—for example, using nitrocellulose strips. In most formats, a labeled antibody binds to the antigen of interest. The antigenantibody complexes
are then immobilized in the support matrix by an unlabeled antibody bound to the matrix. All controls are included in the membrane as well, and results are seen as colored spots or bands, as one of the test reagents is conjugated to colloidal gold or a chromogenic substance. This test format is
especially convenient for point-of-care testing, as the test process is simple and each test unit contains both positive and negative controls to assess test validity.

Detection of Viral Nucleic Acids Обнаружение вирусных нуклеиновых кислот

Developments in the area of nucleic acid technology in the past few years have relegated some (earlier) techniques to the annals of history with respect to their use in the diagnostic testing. For example, classic hybridization techniques are not typically amenable to use for routine testing, especially with the requirement for rigorous quality-control standards. The most dramatic changes in nucleic acid detection technology have been in the evolution of polymerase chain reaction (PCR) testing, and the equally important standardization of nucleic acid extraction procedures. In addition, the rapid advances in nucleotide sequencing technology, oligonucleotide synthesis, and development of genetic databases permit inexpensive sequence analysis that has replaced less rigorous procedures for comparing genetic changes in virus strains and isolates. Current technology permits PCR amplification of virus “populations” with direct sequencing of the amplified products from the clinical specimen without the potential introduction of cell culture selection bias. More recent developments permit the detection and characterization of unknown agents (viral metagenomics). Nucleic acid amplification technology and sample preparation protocols have matured to the point where reliable and relatively inexpensive units can be deployed in the field or the clinician’s office without the need for highly trained personnel.
Data generated by field units can be transmitted anywhere in the world for interpretation and storage.
Nucleic acid detection methods are invaluable when dealing with:
(1) viruses that cannot be cultured readily;

(2) specimens that contain inactivated virus as a result of prolonged storage, fixation of tissue, or transport;
(3) latent infections in which the viral genome lies dormant and infectious virus is absent;
(4) virus complexed with antibody as would be found in the later stages of an acute infection or
during some persistent viral infections;
(5) viruses that have not been previously identified.
However, the added sensitivity provided by amplification of viral nucleic acid can actually create new problems. Unlike the situation with bacterial pathogens, it has usually been the case that merely detecting a pathogenic virus in a lesion, or from a clinically ill animal, has been considered evidence of its etiologic role (causal relationship). As detection methods have become increasingly more sensitive and testing includes more agents, questions of viral "passengers" become more pertinent. Indeed, with viruses such as bluetongue virus, viral nucleic acid can be detected in the blood of previously infected ruminants several months after infectious virus has been cleared. Furthermore, with bovine herpesvirus 1 as an example, detection of viral nucleic acid does not address whether it is present as a consequence of an acute infection, reactivation of a latent infection, or vaccination.

Polymerase Chain Reaction

The PCR assay is an in vitro method for the enzymatic synthesis of specific DNA sequences using two oligonucleotide primers, usually of about 20 residues (20-mers), that hybridize to opposite strands and flank the region of interest in the target DNA; the primer pairs are sometimes referred to as forward and reverse primers (Fig. 5.8).

Primers are necessary to provide the DNA polymerase with a substrate upon which to add new nucleotides, and to direct the reaction to the specific region of the DNA for amplification. Primers can also be designed to provide "tags" or "barcodes" on the amplified products for purposes of detection and sorting in complex reactions. Computer programs are used for the design of optimum primer sets and to predict the parameters (time/temperature) for the reactions, but empirical testing is still
necessary. Where there are either known mismatched bases or anticipated mismatches between the primer and target sequences, the primers can be made to be degenerate—sets of primers with different bases at a given location. This can increase the diagnostic sensitivity of the test, as more genetic variants can be detected. For PCR, reactions are carried out in a thermocycler under
carefully controlled conditions of ionic strength, temperature, primer concentration, and nucleotide concentration. Repetitive cycles involving template denaturation by heating, primer annealing, and extension of the annealed primers by DNA polymerase result in the exponential accumulation of a specific DNA fragment, the termini of which are defined by the 50 ends of the primers.

The primer extension products synthesized in one cycle serve as templates in the next, hence the number of target DNA copies approximately doubles every cycle; 20 cycles yields about a millionfold amplification. Since the introduction of the concept of PCR in 1983, there have been numerous changes to virtually every facet of the process. Incorporation of a thermostable DNA polymerase permitted high temperature denaturation

and strand separation of the synthesized products, which eliminated the need to replenish the polymerase at each cycle.
The use of a thermostable polymerase also increased the specificity of the reaction, as cycling could be done under more stringent annealing conditions; specifically, higher annealing temperatures reduce mismatch base pairing which can lead to false-positive results. In order to increase the sensitivity of the test, a "nested" PCR procedure was developed. In this procedure, one set of primers
was used to do an initial amplification of a target area and the product of the first reaction became the template for a second PCR test in which new primers targeted a region internal to the first set of primers. This amplification of amplified product greatly increased the sensitivity of the test, but greatly increased the chances for falsepositive results through contamination of test materials by the initial amplified product. Further developments in real-time PCR technology have markedly reduced the use
of nested procedures.

The development of reverse transcriptase polymerase chain reaction (RT-PCR) methods to detect RNA sequences was a major advance in cell biology and viral diagnostics. There is some confusion on the use of the term RT-PCR with the advent of the real-time procedure, leading to various designations of the real-time procedure as "qRT-PCR" or "RRT-PCR" or "RT-qPCR" for RNA targets or R-PCR for DNA targets. For RT-PCR, the RNA is first transcribed into cDNA using a DNA polymerase capable of using RNA as a template, such as retrovirus reverse transcriptase. Newer reverse transcriptase enzymes have been developed that permit synthesis of the cDNA strand at higher temperatures, which increases the analytical sensitivity and specificity of the reaction. In single-tube RT-PCR tests, all components for both reactions are placed in the reaction tube at the onset of the testing. The cDNA synthesis step is followed immediately by the PCR reaction. In this test format, there is no opportunity for products of one reaction to cross contaminate another, because the reaction tube is never opened until the end of the testing protocol. Advances such as the single-tube test greatly increased the reliability of PCR test results by virtually eliminating laboratory contamination problems.

Methods for Detection of Amplified Products увеличенные продукты

In the initial era of PCR testing, the amplified products were detected by analyzing the reaction products by gel filtration to visualize the amplified product. Amplified products of a defined sized were visualized by using fluorescent dyes that bound to the oligonucleotides separated in agarose gels. A "band" at the appropriate size was taken as a positive test for the presence of an agent in a sample. Methods were developed to increase the sensitivity of detecting bands in the gels, but even with enhanced sensitivity, this detection procedure had one major flaw—the reaction tube had to be opened in order to assess the status of the sample. Many laboratory areas became contaminated with the amplified reaction products, with false-positive results frequently obtained from subsequent samples run in the facility. Heroic efforts were made to avoid the false-positive problem, but suspicion of positive test results became prevalent and still linger.
Fortunately, technology provided an answer that has come to dominate PCR testing: real-time PCR testing (Fig. 5.9).

This major technology advance was facilitated by the development of a thermocycler with a fluorimeter
that could accurately measure (quantify) the accumulation of PCR product (amplicons) in the reaction tube as it was being made—that is, in real time. Product is measured by increases in fluorescence intensity generated by several different fluorescent reporter molecules, including nonspecific DNA binding dyes (SYBR Green I), TaqMan probes (Fig. 5.9A), and molecular beacons as examples.
Once reactants are added to the reaction tubes, the tubes need never to be opened again, thus preventing any opportunity for laboratory contamination. The real-time detection systems are also more sensitive than standard gel systems, and added assay specificity is achieved through the use of reaction detection probes, because signal is generated only if the probe sequence is also able
to bind to the amplified target sequence.

Another advantage of the real-time system is that the process can be quantitative. Under optimized conditions, the amount of the amplicon increases by a factor of 10 with each 3.3 amplification cycle (Fig. 5.9B). With realtime systems, the generation of product is recorded at each cycle. The amount of product generated in a test reaction can be compared with a copy number control and, with proper extraction controls in the system, a direct measure of the amount of starting sequence can be
determined. In humans, for example, this feature has particular value in monitoring responses over time to drug treatments for infections with hepatitis C and human immunodeficiency (HIV) viruses.
A further variation in PCR testing that is becoming more commonly used is multiplex PCR. In this method, two or more primer pairs specific for different target sequences are included in the same amplification reaction.
In this manner, testing can be done for several agents at the same time and in the same assay tube, thereby saving time and costs. With real-time, multiplex PCR assays, several probes with different fluorescent molecules can be detected simultaneously. This type of application is useful in evaluation of samples from disease complexes, such as acute respiratory disease in dogs. Issues of test sensitivity must be addressed in this format, because several reactions must compete for common reagents in the reaction, thus an agent in high copy number might mask the presence of one at low copy number.
A newer technology that expands on the concept of multiplexing is the new “OpenArray” platform available on the QuantStudio 12K Flex machine offered by Thermo-Fisher/Life Technologies. The “platform” is a microscope slide-sized plate with 3072 wells of 33 nL each. In one configuration, 48 subarrays are produced with 64 wells/subarray. In this format, 48 samples can be tested on each plate. Primers and probes are printed on the plate in a customized format. To ensure reliability, multiple wells can contain a specific primer/probe combination. For example, in a canine respiratory PCR panel, the canine herpesvirus test can be located in three wells of the subarray. For a given sample, there will be three PCR reactions assessing the presence of canine herpesvirus DNA in the test sample. The same would be true for all of the other targets in the subarray. This type of format also permits multiple primer/probe reactions for the same agent in order to accommodate the genetic
variation found in field samples. The cost associated with this type of platform with automated sample

handling is such that it will not be economical to run a single agent PCR in those instances where the panel format is available.

Advantages and Limitations of the Polymerase Chain Reaction Technology

Given the explosion in use and availability of PCR assays in virological testing, consideration should be given to the potential benefits and limitations of these assays. The PCR assay is especially useful in the detection of viruses that are difficult to grow in culture, such as certain enteric adenoviruses, papillomaviruses, astroviruses, coronaviruses, noroviruses (family Caliciviridae), and rotaviruses. PCR can be used on any sample that is appropriate for virus isolation; the decision to do PCR as opposed to other virus detection tests is based on speed, cost, and laboratory capability. PCR tests also may be preferred for the initial identification of zoonotic viruses, such as rabies virus, certain poxviruses, filoviruses, or influenza viruses, to minimize the risk of exposure for laboratory personnel as amplification of infectious virus is not necessary for detection.

A limitation of PCR or any nucleic acid amplification technique can be the matrix in which the target sample is embedded. Material in the sample matrix can inhibit the enzymes on which the assay is based, which has been a constant source of concern when dealing with fecal samples and, to some extent, milk samples. Extraction controls need to be included in these types of sample in order to detect problems with the amplification process itself (rather than lack of specific template).
Standard PCR assays should be validated for the matrix in which the target agent is embedded. Furthermore, PCR and simple nucleic acid amplification tests are agent specific, thus no signal will be generated if the primers do not match the sequence of any virus contained in the sample. With earlier direct PCR assays, and especially with nested PCR assays, false-positive test results were a very significant concern as a result of the ease of laboratory contamination with amplified product. With the availability of single-tube real-time PCR testing formats and real-time PCR tests, this problem has largely been eliminated, although correct performance of PCR assays remains a technically challenging process.

Performance of real-time PCR assays is being continually improved with standardized reagent kits, robust instrumentation, standardized extraction protocols, and defined laboratory operating procedures, and this nucleic acid detection test format has become the mainstay of testing laboratories. However, test interpretation still requires evaluation of whether or not a particular test result (either positive or negative) is biologically relevant, which in turn requires a global assessment of history, clinical signs, and lesions in the particular animal from which the sample was obtained. As a final precaution, all PCR tests for the same agent are not created "equal" and variation between laboratories can affect the outcome of the testing.

Microarray (Microchip) Techniques

Another technological advance that is impacting the field of diagnostics is the advent of microarrays or microchips. The microchip for nucleic acid detection is a solid support matrix onto which have been "printed" spots, each containing one of several hundred to

several thousand oligonucleotides. Increasingly, these oligonucleotides can represent conserved sequences from virtually all viruses
represented in the various genetic databases, or can be customized to represent only viruses from a given species involved in a specific disease syndrome, such as acute respiratory disease in cattle. The basis of the test is the capture by these oligonucleotides of randomly amplified labeled nucleic acid sequences from clinical specimens.

The binding of a labeled sequence is detected by laser scanning of the chip and software programs assess the strength of the binding. From the map position of the reacting oligonucleotides, the software identifies the species of virus in the clinical sample. This type of test was used to determine that the virus responsible for severe acute respiratory syndrome (SARS) was a coronavirus.
With knowledge of the oligonucleotide sequences that bound the unknown agent, primers can be made to eventually determine the entire nucleotide sequence of a new species of virus. The low cost of oligonucleotides synthesis, development of laser scanning devices, nucleic acid amplification techniques, and software development have made this technology one of the methods by which newly
emerging viruses can be identified quickly in outbreak situations or in surveillance programs. Instruments and trained personnel have been deployed to various parts of the world where "emergence" of new viral pathogens might be anticipated. In the standard format, this technique would probably not detect a new virus family not represented in a current database, because oligonucleotides for the new agent would not be included on the microchip. Also, the current microarray systems lack the analytical sensitivity of real-time PCR testing even with pre-amplification of the target nucleic acid and are not routinely used for agent specific diagnostic testing.

Gene Amplification by Isothermal Amplification

For nucleic acid amplification, it is necessary to continually displace the newly synthesized product so that another copy of the sequence can be made. With PCR, the strand displacement is achieved with temperature: the 95C temperature maximum melts (separates) the DNA strands, permitting binding of new primers that provide the polymerase starting point. Isothermal amplification is a technique that does not require the temperature cycling and accompanying equipment used in PCR. There are at least six different strategies that have been developed to amplify DNA targets in an isothermal format. One of these, loopmediated isothermal amplification (LAMP), is receiving more attention with the advent of in-office testing with microfluidic devices. The test requires four types of primers that initiate two types of elongation reactions, which provides high specificity for the test. The DNA polymerase
used for amplification is not as sensitive to inhibitors as the TAQ-type polymerases thus the nucleic acid extraction processes can be simplified and less rigorous. The high quantity of amplified product produced permits either qualitative or quantitative detection modalities. The isothermal feature of the test eliminates the need for a thermocycler.

This feature along with the simplified extraction process makes this type of testing more compatible for a microfluidic environment. An isothermal-based test for influenza virus A or B was approved by the FDA for point-of-care use. The downside currently for the

isothermal amplification tests is that they are not readily formatted for multiplex testing or for nontargeted agent detection.

In Situ Hybridization

With the explosion in the identification of "new" viruses in virtually any animal species examined, the diagnostic dilemma becomes linking the presence of a virus in a clinical specimen with having caused the clinical disease under investigation. As previously noted this can be done using either immunofluorescence or immunohistochemistry. An issue with these techniques particularly with a
newly discovered agent is having a validated antibody reagent available. The alternative to the antibody detection systems is the use of nucleic acid probes (FISH—fluorescence in situ hybridization). Small (2550 nucleotides) DNA probes corresponding to conserved regions of
the genome are synthesized with a fluorescent tag at the 50 end (6-carboxyfluorescein as an example). From the histopathological assessment of tissue samples, sections can be selected that show a characteristic lesion associated with the clinical disease (for example, see Chapter 27:
Caliciviridae and Astroviridae, Figs. 27.9 and 27.10).

Applying the virus-specific probe to the tissue section permits the determination of whether the agent is specifically associated with the lesions as opposed to the nonspecific positive PCR signal from a tissue extract. This type of assessment is becoming critical as the multiplexed PCR panels detect multiple pathogens in clinical samples.

NUCLEIC ACID SEQUENCING

Perhaps no area in molecular biology has advanced so rapidly as nucleic acid sequencing. With speed and capacity has come low cost, so that direct sequencing of complete viral genomes is now commonplace. Older techniques such as restriction mapping and oligonucleotide fingerprinting that were used to detect genetic differences among virus isolates have been displaced by sequencing
methodology. In the area of diagnostics, new viruses are being discovered by techniques that take advantage of random nucleic acid amplification and low-cost sequencing (high-throughput sequencing also known as next generation sequencing). Sequencing technology is used in several areas
of diagnostic virology from confirmation of a PCR targeted sequence to the discovery of unknown agents. The intended use of the sequence data will dictate the type of technology used and the level of bioinformatics supported needed to analyze the sequence.
Three relevant examples will illustrate the range of complexity and utility of this approach: 1. An RT-PCR test for bovine viral diarrhea virus (BVDV) detected a positive response on tissue from an aborted fetus. Amplicons for diagnostic PCR tests are best if relatively short (80120 nucleotides) and therefore of limited value in typing virus isolates. A second PCR reaction is done using "typing" primers that span a 400600 nucleotide region previously used to classify virus isolates. The amplification reaction is analyzed on a gel and the "band" representing the PCR product is extracted and sent to a sequencing center. Usually within 24 hours the sequence file is returned. This sequence is then compared with the other bovine viral diarrhea virus sequences in shared databases. Software programs assist in aligning the sequence and in constructing a phylogenetic tree

if desired (see Chapter 1: The Nature of Viruses). The virus that was present in the aborted tissue was a BVDV type 1c, inconsistent with the suspicion that a live-attenuated vaccine virus caused the abortion.

2. An outbreak of respiratory disease in dogs is determined to involve an influenza virus with a different HA type than had previously been documented in dogs. A question of interest, therefore, is the identity of the other seven genes in this type A influenza virus. Sequencing primers with unique barcodes for all eight genes were used to amplify all genetic material in the virus' genome, and a single sequencing reaction determined the sequences of all amplified products. Barcodes
segregate these sequences by gene and computer software aligns the overlapping sequences into one continuous gene. Each gene sequence is then compared to comparable gene sequences found in shared databases. The results quickly confirm that the new canine influenza virus is a reassortant between an avian virus (six genes) and a contemporary swine virus (two genes).
Thus, the influenza virus involved in this outbreak in dogs is unique, possibly indicating a need for a new canine influenza virus vaccine.

3. An acute episode of disease occurs in an equine stable, with test results indicating affected horses have acute hepatitis. Standard diagnostic testing does not identify any etiological agent. Sera from several of the horses are submitted for high throughput sequencing and bioinformatic analysis ("metagenomics"). With no knowledge of the nature of the potential virus in the sample, sera
were passed through a 0.2 um filter to enrich for any virus present, the filtrate was treated with RNase and DNase to reduce nonviral nucleic acid targets, and the extracted nucleic acid was subjected to random primer amplification. The amplified products were sequenced and clustered using de novo assembly. Unique "reads" are compared against sequences of known viruses available in a public database (GenBank). A previously undescribed, noncultivatable hepatitis C-like virus is identified in the serum samples (see Chapter 30; Other Viruses: Hepeviridae, Hepadnaviridae, Deltaviruses,
Nodaviridae, and Unclassified Viruses). The role of this agent in the acute disease episode cannot be inferred from this finding alone.

Clearly, these types of nucleic acid detection protocols can be used to discover and characterize previously unknown viruses, without the requirement that they first be propagated in cell culture.

DETECTION AND QUANTITATION OF VIRUS-SPECIFIC ANTIBODIES (SEROLOGIC DIAGNOSIS) ОБНАРУЖЕНИЕ И КОЛИЧЕСТВО ВИРУСНО-СПЕЦИФИЧЕСКИХ АНТИТЕЛ (СЕРОЛОГИЧЕСКАЯ ДИАГНОСТИКА)

The detection of an immune response to an infectious agent has, for the most part, relied on determining the antibody response of the host to the agent of interest. This approach measures only one limb of the adaptive immune response (humoral immunity); techniques for reliably measuring the cell-mediated responses have not been routinely available or cost-effective. For many situations,
measurement of antibody responses remains a valuable technique for defining the infection status of animals.

Serological tests can be used to: (1) define whether an animal has ever been infected by a particular virus; (2) determine if a specific virus (or other pathogen) is linked to a clinical event; (3) determine if an animal has responded to a vaccination. For the serologic diagnosis of an acute viral disease in an individual animal, the classic approach has been to test paired sera—that is, an acute and a convalescent serum from the same animal, for a change in titer (fourfold or greater) of virus-specific antibody. The acute-phase serum sample is taken as early as possible in the illness; the convalescent-phase sample usually at least 2 weeks later. Given this time line, diagnosis based on this approach is said to be "retrospective."

In recent years this approach has been complemented by serologic methods for detecting virus-specific IgM antibodies—in many viral diseases a presumptive diagnosis may be made on the basis of detecting IgM antibody in a single acute-phase serum specimen—for example, West Nile virus infection of horses. To assess whether an animal has ever been infected with certain viruses, serological testing can be more reliable than efforts to detect the virus itself. For example,
serological testing is used to screen horses for exposure to equine infectious anemia virus, cattle for bovine leukemia virus, and goats for caprine arthritis encephalitis virus.

In these instances, the number of infected cells in chronically infected animals may be too low for even PCR detection, but infection generally stimulates an antibody response that is readily detected by various tests. Serological testing is also widely used both during virus eradication programs and in the certification of animals for movement and trade. Use of serological tests to assess vaccine efficacy can be an important aspect of an infectious disease management program. In many countries, purchase of vaccine can be done by the animal owner. Antibody testing of selected animals can provide the practitioner with valuable insight as to whether the immunization program of
the producer is being performed correctly.

As eradication programs expand for diseases of production animals, marker vaccines are more frequently being used and so-called DIVA serological assays can distinguish whether a given antibody response is caused by vaccine or natural infection. For herpesvirus infections such as bovine herpesvirus 1, it is essential to determine whether an antibody response is the result of infection, because infection invariably leads to latency. Movement of a latently infected animal into a negative herd can result in an outbreak of disease, thus gene deletion "marker" vaccines were developed to facilitate differentiation of vaccinated and naturally infected cattle.

Serum Specimens for Serologic Assays

For most serological tests, serum is the sample of choice. However, some tests have been validated using plasma as well as serum. Communication with the testing laboratory is necessary when fluids other than serum are being collected, in order to avoid having to re-sample the animal when serum is the only acceptable test material. Antibodies in serum are very stable in moderate environmental conditions. Standard protocols call for serum to be kept cold, but freezing of the sample is not necessary unless several weeks will elapse between collection and testing. Antibodies can even be detected from blood samples dried onto filter paper and stored for months at room temperature before testing.

As with other aspects of diagnostic testing, technological advances continue to modify how antibodies to specific viruses are detected. In most cases, the newer technologies are applied to those tests that have some commercial potential. In veterinary medicine, there are many tests for agents that may be of minor importance but useful in certain situations. Tests available for these agents may be the first ones developed with older testing technology. As viruses of wildlife species assume greater importance through public awareness, it will be necessary to develop additional serological tests, because species-specific tests for domestic species cannot be used. All serological test types will not be discussed in detail (below), but readers should be aware that other test formats may become available and continuing communication with their testing laboratory is the most efficient way to learn about the tests available for each species and for each virus.

Enzyme Immunoassay—Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme immunoassays (EIAs, ELISA) are the serologic assays of choice for the qualitative (positive or negative) or quantitative determination of viral antibodies because they are rapid, relatively cost-effective, and may not require the production of infectious virus for antigen if recombinant antigens are used. In the EIA test format for antibody detection, viral antigen is bound to a solid matrix. Serum is added and, if antibodies to the antigen are present in the sample, they bind to it. In direct EIA tests, the bound antibody is detected by an anti-species antibody tagged with an enzyme. With addition of the enzyme substrate, a color reaction develops that can be assessed either visually or with a spectrophotometer.

Controls run with the sample define whether the test is acceptable and which samples in the test are positive. Kinetics-based EIAs offer the advantage that quantitative assays can be based on a single dilution of serum. The product of the enzyme reaction is determined several times over a short interval. Software programs convert the rate of product development to the amount of antibody bound to the antigen. A disadvantage of direct EIA tests is that they are species specific. A test developed for canine distemper virus antibodies in a dog cannot be used to determine the presence or absence of antibodies to the same virus in a lion. To obviate this problem, competitive or blocking EIA tests have been developed. In this test format, an antibody that binds to the antigen of interest (usually a monoclonal antibody) is tagged with the enzyme.

Unlabeled antibody that can bind to the same site as the monoclonal antibody will compete with the labeled monoclonal antibody for that site. A reduction in the binding of the labeled monoclonal antibody indicates that the sample did contain antibody (Fig. 5.10). In this test format, the species of the unlabeled antibody is not a factor. The diagnostic sensitivity and specificity of EIA tests, whether direct or indirect, have been greatly enhanced by the development of monoclonal antibodies and the production of recombinant antigens.

In a widely used format for test kits that can be run in a practitioner's office, the test serum flows through a membrane filter that has three circular areas impregnated with antigen,

two of which have already interacted with a positive and a negative serum, respectively (Fig. 5.7). After the test serum flows through the membrane and a washing step is completed, a second antispecies antibody with an enzyme linked to it is added and the membrane is again rinsed before the addition of the enzyme substrate. The result is read as a color change in the test sample circle, which is compared against the color change in the positive control and no change in the negative control. Such single-patient tests are relatively expensive compared with the economies of testing hundreds of sera in a single run in a fully automated laboratory. The great savings in time and effort to send samples to the laboratory, in addition to the fact that decisions can be made while both client and patient are still in the consulting room, make single tests attractive and useful in the immediate clinical management of critically ill animals.

Serum (Virus) Neutralization Assay

As virus isolation is considered the gold standard for the detection of virus against which other assays must be compared, the serum (virus) neutralization test has historically been the gold standard, when available, for the detection and quantitation of virus-specific antibodies. Neutralizing antibody also attracts great interest because it is considered a direct correlate of protective antibody in vivo. For the assay of neutralizing antibody, two general procedures are available: the constant-serum variable-virus method and the constant-virus variable-serum method. Although the constant-serum variable-virus method may be a more sensitive assay, it is rarely used because it utilizes relatively large amounts of serum, which may not be readily available. The basis of the neutralization assay is the binding of antibody to infectious virus, thus preventing the virus from initiating an infection in a susceptible cell. The growth of the virus is detected by its ability to kill the cell (cytopathic effect) or by its ability to produce antigen in the infected cells that is detected by immunofluorescence or immunohistochemistry.

The amount of antibody in a sample is determined by serial dilution of the sample and "challenging" each of these dilutions with a standard amount of virus (constant-virus variable-serum method). The last dilution that shows neutralization of the virus is defined as the endpoint and the titer of the serum is the reciprocal of the endpoint dilution; for example, an endpoint of 1:160 equates to a titer of 160. The disadvantages of serum neutralization tests are that they are relatively slow to generate a result, require production of infectious virus for the test, and have a constant high overhead cost in maintaining cell culture facilities for the test. These assays have the benefit of being species independent and, as such, are very useful in wildlife studies. With new agents, a serum neutralization test can be operational within several weeks of isolating the virus, whereas EIA test development may take months or even years to validate.

Immunoblotting (Western Blotting)

Western blotting tests simultaneously but independently measure antibodies against several proteins of the agent of interest. There are four key steps to western blotting. First, concentrated virus is solubilized and the constituent proteins are separated into discrete bands according to their molecular mass (Mr), by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE). Secondly, the separated proteins are transferred electrophoretically ("blotted") onto nitrocellulose to immobilize them. Thirdly, the test

serum is allowed to bind to the viral proteins on the membrane.

Fourthly, their presence is demonstrated using a radio-labeled or, most commonly, an enzyme-labeled antispecies antibody. Thus immunoblotting permits demonstration of antibodies to some or all of the proteins of any given virus, and can be used to monitor the presence of antibodies to different antigens at different stages of infection. Although this procedure is not routinely used in a diagnostic setting with viruses, western blots were central to the identification of immunogenic proteins in a variety of viruses. Similarly, the assay is used in the analysis of samples for the presence of prion proteins in ruminant tissues. Western blots are more of a qualitative test than a quantitative one, and are not easily standardized from laboratory to laboratory. For this reason, ELISAs and bead-based assays are preferred test formats.

Indirect Immunofluorescence Assay

Indirect immunofluorescence assays are used for the detection and quantitation of antibody; specifically, these are tests that use virus-infected cells (usually on glass microscope slides) as a matrix to capture antibodies specific for that virus. Serial dilutions of test serum are applied to individual wells of the cell substrate and usually an antispecies antibody with a fluorescent tag is then added as the detector of antibody binding. Slides are read with a fluorescent microscope and scored as positive if the infected cell shows a fluorescent pattern consistent with the antigen distribution of the virus used. This test is rapid (less than 2 hours) and can be used to determine the isotype of the reacting antibody if one uses an antiisotype-specific serum such as an anticanine IgM.

Nonspecific fluorescence can be an issue, particularly with animals that have been heavily vaccinated as they may contain anticell antibodies that will bind to uninfected cells and mask specific antivirus fluorescence. Test slides for some agents can be purchased, so that laboratories offering this test need not have infectious virus or a cell culture facility.

Hemagglutination-Inhibition Assay

For those viruses that hemagglutinate red blood cells of one or another species, such as many of the arthropodborne viruses, influenza viruses, and parainfluenza viruses, hemagglutination-inhibition assays have been widely used. For detecting and quantitating antibodies in the serum of animals, the methods are sensitive, specific, simple, reliable, and quite inexpensive. In spite of all of the technological advances, hemagglutination inhibition assays remain the mainstay for determining antibody responses to specific influenza A viruses. The principle of the assay is simple—virus binds to red blood cells through receptors on their surface (see Chapter 3: Pathogenesis of Viral Infections and Diseases, Fig. 3.11). Antiviral antibodies bind to these receptors and block hemagglutination. Serum is diluted serially in the wells of the microtiter plate, usually in twofold steps, and to each well a constant amount of virus, usually four or eight hemagglutinating units, is added. The reciprocal of the highest dilution of serum that inhibits the agglutination of the red blood cells by the standardized amount of virus represents the hemagglutination-inhibition titer of the serum (Fig. 5.11).

Care should be taken in interpreting many prior sero-surveys based on results of hemagglutination inhibition tests, particularly for paramyxoviruses, as nonspecific inhibitors of agglutination produced many false-positive test results in some of those studies.

Immunodiffusion

Historically, agar gel immunodiffusion (AGID) assays were used for the specific diagnosis of a number of viral infections and diseases, including bluetongue, hog cholera, influenza, equine infectious anemia (the so-called "Coggins test" after its inventor, LeRoy Coggins), and bovine leukemia. These assays are very simple to perform, they utilize inexpensive materials, and they do not require production of infectious material by the testing laboratory. Often crude cell extracts or even tissue extracts from infected animals can be used as the test antigen. AGID tests are relatively fast, easily controlled, but lacked sensitivity as compared with later developed EIA tests. Furthermore, they are strictly qualitative (providing a simple yes/no answer) and cannot be automated

IgM Class-Specific Antibody Assay

A rapid antibody-based diagnosis of a viral infection or disease can be made on the basis of a single acute-phase serum by demonstrating virus-specific antibody of the IgM class. Because IgM antibodies appear early after infection but drop to low levels within 12 months and generally disappear altogether within 3 months, they are usually indicative of recent (or chronic) infection.

The most common method used is the IgM antibody capture assay, in which the viral antigen is bound on a solid-phase substrate such as a microtiter well. The test serum is allowed to react with this substrate and the IgM antibodies "captured" by the antigen are then detected with labeled anti-IgM antibody matched to the species from which the specimen was obtained. A downside to the IgM assays is that they are generally not suitable for use in animals that have been vaccinated as the IgM responses to that antigen has already occurred in response to the vaccine.

New Generation Technologies

Flow Cytometry Platform

As with nucleic acid technologies, technological developments for analyte detection are rapidly evolving, and a substantial number of potentially novel platforms for serological assays have been developed that have not yet been fully validated for routine diagnostic use. It is beyond the scope of this text to provide an exhaustive listing of these technologies, many of which will never find their way into routine diagnostic use. However, one technology that has demonstrated particular promise in both the clinical and research arena is XMAP, developed by Luminex. The success of this testing platform probably reflects the maturity of existing technologies that were combined to provide a versatile analyte detection system. XMAP combines a flow cytometry platform, uniquely labeled microspheres, digital signal processing, and standard chemical coupling reactions to provide a system that can be used to detect either proteins or nucleic acids (Fig. 5.13).

The microspheres carry unique dyes (up to 100 different ones) that emit fluorescent signals that identify the individual beads coupled with a specific ligand. For antibody detection tests, the antigen of interest is coupled to a specific bead. The beads are exposed to the test serum and the bound antibody is detected with an antispecies antibody tagged with a reported dye. The microspheres are analyzed in a flow cytometer in which lasers excite both the bead dyes and the reporter dyes. Multiple beads for each antigen are analyzed in each test, providing independent readings of the reaction.
One distinct advantage of this system is its multiplex capability. Theoretically, 100 or more different antigens can be assessed for antibody reactivity in a single assay. For maximum sensitivity and specificity, recombinant antigens are needed to eliminate extraneous proteins that would reduce specific antigen density on the beads and increase nonspecific background reactivity that can confuse test interpretation. Advantages of this bead-based system are: (1) it utilizes small sample volumes; (2) it can be multiplexed; (3) it has been reported to be more sensitive than standard ELISA tests; (4) it can be less expensive than many serology tests; (5) it can be more rapid than ELISA tests, particularly when testing for antibodies to several antigens.

As an example, this test platform is ideal for the antibody screening tests that are necessary for maintaining research rodent colonies in which antibody responses to several agents are monitored and for which sample volumes are often limiting. This platform can also provide DIVA testing as would be applied for control of important regulatory disease such as foot-andmouth disease. As an example, recombinant antigens representing the capsid proteins present in inactivated foot-and-mouth disease virus vaccines along with nonstructural viral protein can be coupled to different beads to analyze the antibody profile of a suspect animal. In asingle assay, the test can provide evidence of vaccination—response to capsid antigen only—or of a natural infection—response to both types of proteins. One could envision this type of bead-based assay as a quantitative western blot, in that reactivity to several antigens can be assessed. As eradication programs progress for viral diseases of production animals, it is very likely that the requirement for this type of DIVA testing will only increase. The disadvantage for antibody detection is the need for recombinant antigens to achieve acceptable sensitivity, and high validation costs associated with multiplex reactions.

Protein Microarrays

Another potential solution to the issue of simultaneous multiple epitope screening is the protein microarray. This type of test has become feasible as technology is now permitting production of high quality antigens or peptides in unlimited quantities. Protein microarrays of virtually any size can be used to interrogate serum samples for the presence of antibodies to the range of peptides on the array. The output can simply provide a positive versus negative answer, or can be a quantitative
output with serial dilution of the test samples. A practical example of the use of this technology is the screening of serum samples for reactivity to any influenza A virus. Typically this is challenging in that there are now 18 HA types. For example, to answer the question of which influenza A viruses are capable of infecting a given species (eg, bats) entails an 18-HA antigen screen with μL quantities of test serum. Recombinant-generated HA1 antigens are spotted onto nitrocellulose coated slides at multiple locations within a defined array well.

Dilutions of test sera are applied to the protein arrays and the binding of antibody is detected with a
fluorescent-tagged antispecies antibody. Slides are scanned for the intensity of the fluorescent signal and the positive signals are mapped to the particular antigen in the array. This type of antibody detection system can define the presence of antigen-specific antibodies in serum that represent exposure to any number of viruses.

INTERPRETATION OF LABORATORY FINDINGS

As with any laboratory data, the significance of specific results obtained from the virology laboratory must be interpreted in light of the clinical history of the animal from which the sample was collected. To some extent, the significance of any result is also influenced by the type of virus that was detected. A fluorescent-antibody positive test for rabies virus on a bat found in a child's bedroom will elicit a public health response in the absence of clinical data, whereas a positive serological test for bovine
leukemia virus from the dam of an aborted fetus is likely to be an irrelevant finding if the animal is from an enzootic region. With multiplex PCR testing, it may be possible to detect several different viruses, bacteria, and mycoplasma species in a single dog with acute respiratory disease, raising the obvious question, "what is significant?" Are the virus signals due to a recent vaccination, reactivation of a herpesvirus, or "footprints" of the etiological agent? Clearly, several sources of data must be
integrated by the clinician to arrive at a coherent treatment strategy. However, it is also clear that the speed, the number, and the reliability of virus detection tests have changed the way in which clinicians use laboratory test results, and these results are having greater impact on treatment and management decisions. When attempting to interpret the significance of the detection of a specific virus in a clinical specimen, one may be guided by the following considerations.

The site from which the virus was isolated. For example, one would be quite confident about the etiological significance of equine herpesvirus 1 detected in the tissues of a 9-month-old aborted equine fetus with typical gross and microscopic lesions. However, recovery of an enterovirus from the feces of a young pig may not necessarily be significant, because such viruses are often associated with inapparent infections. The epidemiologic circumstances under which the virus was isolated. Interpretation of the significance of a virus isolation result is much more meaningful if the same virus is isolated from several cases of the same illness in the same place and time.

The pathogenetic character of the virus detected.
Knowledge that the virus detected is nearly always etiologically associated with frank disease—that is, rarely is found as a "passenger"—engenders confidence that the finding is significant.
The identity of the specific virus. The detection of foot-and-mouth disease virus in any ruminant in a virusfree country would, in and of itself, be the cause for great alarm, whereas the detection of the related bovine rhinitis virus would not. Similarly, the identification of mouse hepatitis virus in a free colony, or koi herpesvirus amongst highly valuable ornamental fish, would trigger a substantial response.

Interpretation of Serologic Laboratory Findings Интерпретация результатов серологической лаборатории

A significant (conventionally, fourfold or greater) increase in antibody titer between acute and convalescent samples is the basis, albeit in retrospect, for linking a specific virus with a clinical case of a particular disease. However, one must always be aware of the vaccination status of the animal, as sero-responses to vaccines, especially live attenuated virus vaccines, may be indistinguishable from
those that occur after natural infections. The demonstration of antibody in a single serum sample can be diagnostic of current infection in an unvaccinated animal (eg, with retroviruses and herpesviruses), because these viruses establish life-long infections. However, in such circumstances there is no assurance that the persistent virus was responsible for the disease under consideration. Assays designed to detect IgM antibody provide evidence of recent or current infection. A summary of the major strengths and limitations of the several alternative approaches to the serological
diagnosis of viral infections is given in Table 5.1.

Detection of antiviral antibody in pre-suckle newborn cord or venous blood provides a basis for specific diagnosis of in utero infections. This approach was used, for example, to show that Akabane virus was the cause of arthrogryposis-hydranencephaly in calves. Because transplacental transfer of immunoglobulins does not occur in most domestic animals, the presence of either IgG or IgM
antibodies in pre-suckle blood is indicative of infection of the fetus.

Sensitivity and Specificity Чувствительность и Специфика

The interpretation and value of a particular serologic test is critically dependent on an understanding of two key parameters: diagnostic sensitivity and diagnostic specificity. The diagnostic sensitivity of a given test is expressed as a percentage and is the number of animals with the disease (or infection) in question that are identified as positive by that test, divided by the total number of the animals that have the disease (or infection)

For example, a particular EIA used to screen a population of cattle for antibody to bovine leukemia virus may have a diagnostic sensitivity of 98%—that is, of every 100 infected cattle tested, 98 will be diagnosed correctly and two will be missed (the false-negative rate 5 2%). In contrast, the diagnostic specificity of a test is a measure of the percentage of those without the disease (or infection) who yield a negative result. For example, the same EIA for bovine leukemia virus antibody may have a diagnostic specificity of 97%—that is, of every 100 uninfected cattle, 97 will be diagnosed correctly as negative, but 3 will be diagnosed incorrectly as infected (the falsepositive rate 5 3%). Whereas diagnostic sensitivity and diagnostic specificity are fixed percentages intrinsic to the particular diagnostic assay and the population of animals used to validate the test, the predictive value of an assay is affected greatly by the prevalence of the disease (or infection) in the test population.

Thus, if the same EIA is used to screen a high-risk population with a known bovine leukemia prevalence of 50%, the predictive value of the assay will be high, but if it is used to screen a population with a known prevalence of 0.1%, the great majority of the 3.1% of

animals that test positive will in fact be false-positives and will require follow-up with a confirmatory test of much higher specificity. This striking illustration draws attention to the importance of selecting diagnostic assays with a particular objective in mind. An assay with high diagnostic sensitivity is required when the aim is to screen for a serious infection or when eradication of the disease is the aim, in which case positive cases must not be missed.

An assay (usually based on an independent technology) with very high diagnostic specificity is required for confirmation that the diagnosis is correct. The analytic sensitivity of a given immunoassay is a measure of its ability to detect small amounts of antibody (or antigen). For instance, EIAs and serum neutralization assays generally display substantially higher analytical sensitivity than AGID tests. Improvements in analytical sensitivity may be obtained by the use of purified reagents and sensitive instrumentation. However, the analytical specificity of an immunoassay is a measure of its capacity to discriminate the presence of antibody directed against one virus versus another. This quality is influenced mainly by the purity of the key reagents, especially the antigen when testing for antibody and the antibody when testing for antigen.