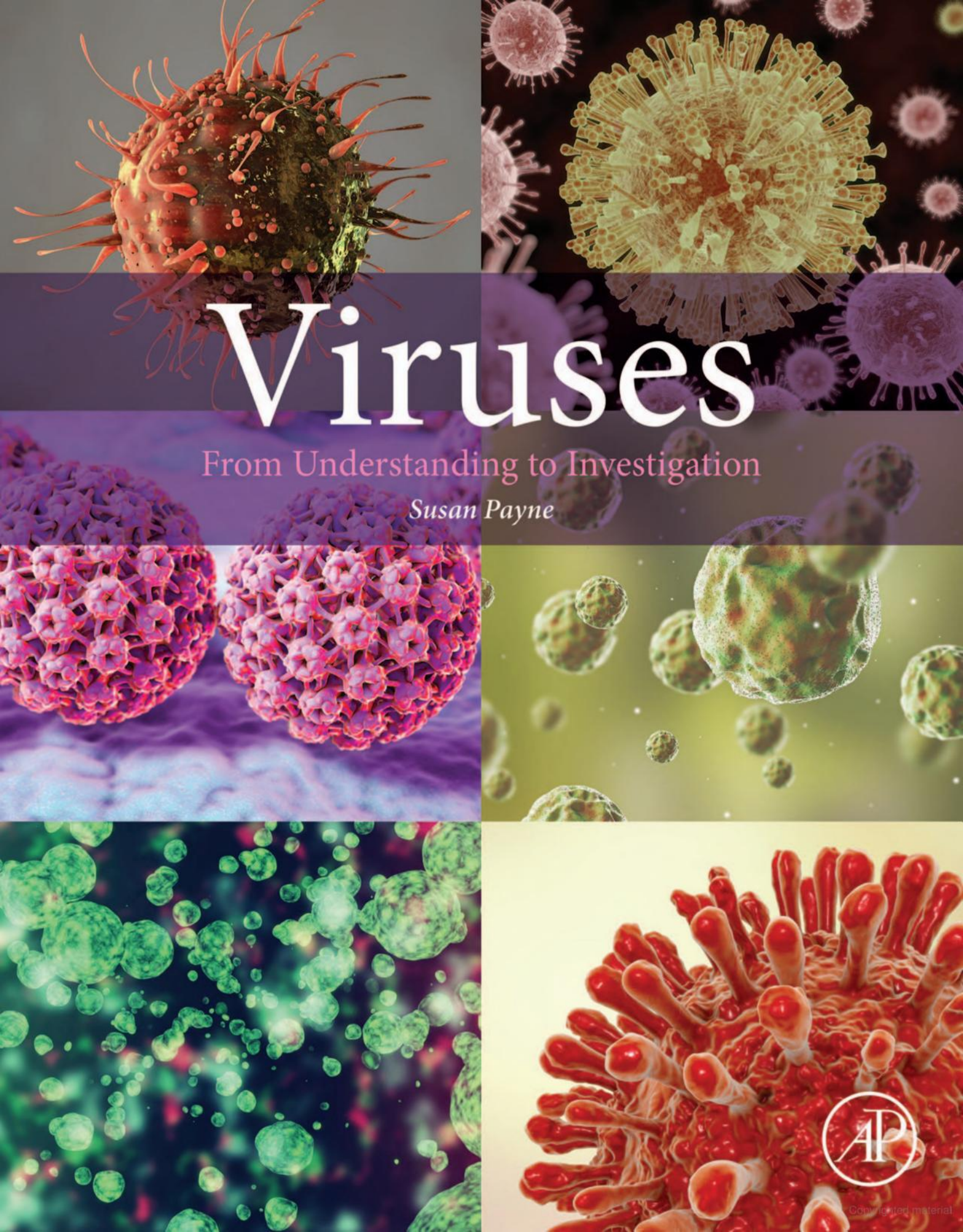

Viruses

From Understanding to Investigation

Susan Payne

VIRUSES

From Understanding to Investigation

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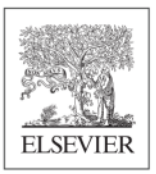

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Contents

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About the Author

Dr. Susan Payne is an associate professor in the Department of Veterinary Pathobiology at the Texas A&M University, United States. During her career, she has mentored graduate and undergraduate students at three universities and has taught virology to undergraduate, graduate, medical, and veterinary students. Those courses are the basis for this textbook. She has also had an active research career and has written over 40 peer reviewed research and review articles. She serves as an ad hoc reviewer for several virology journals. She currently lives in Caldwell, Texas with her husband, mom, five cats, one dog, nine goats, one donkey, eight chickens (if the dog has not eaten one recently), and eight guinea fowls. She is most easily available in email at SPayne@cvm.tamu.edu.

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Preface

This book, *Viruses: From Understanding to Investigation*, was inspired by a long career of teaching and research. My students have included undergraduate, graduate, medical, and veterinary students.

As regards the book title, my intent is to lead students of virology from a basic understanding to an interest in the investigations that have provided the information contained herein. The focus of this textbook is on animal and human viruses, only because these have been the focus of my research and teaching for many years. The viruses of plants, fungi, bacteria, and single-celled organisms are certainly no less interesting.

There is a huge amount of information about viruses available online, in journals, books, websites, and blogs. So why the need for another virology textbook? My intent was to organize and present a thoughtful, understandable, and up-to-date summary of the volumes of information available for consumption elsewhere. While every textbook, including this one, contains many facts, I have tried to emphasize general concepts.

With 38 chapters, this book contains more than enough material for a semester long course in introductory virology. The book is geared toward students with some background in cell biology, microbiology, immunology, and/or biochemistry, and I hope that it will be useful for both undergraduate and beginning graduate students. I also hope that no instructor will try to cover all of the material contained herein during a single semester. The book is organized into two parts, the first nine chapters cover topics including an introduction to viruses (containing information on replication cycle, diversity, taxonomy, and outcomes of virus infection), structure, interactions with the host cell, methods for studying viruses, immunity to viruses, and introductions to viral epidemiology, evolution, and pathogenesis. There are also chapters that serve as introductions to RNA and DNA viruses. I imagine that this will be more than enough information for many instructors and students.

The remaining chapters present viruses by family, with information about structure, genome organization, replication strategies, and disease. I have tried to be up-to-date and include virus families that are relatively new (hence these chapters are short). While each chapter includes basic information about a particular virus family, I am fond of narratives that tie the molecular basis of virus replication to pathogenesis, and have provided examples from a variety of animals, including human animals. The inclusion of "animal diseases" specifically serves as a reminder that companion and food animals play integral roles in human health and well-being. (As do plant and bacterial viruses, but those are subjects for other authors to address.)

I encourage instructors to review the material on virus families and choose a handful of these chapters to use in their courses. Positive-strand RNA viruses are presented first followed by negative and dsRNA viruses. The DNA viruses are presented from the smallest to the largest. Last, but certainly not least, are chapters covering the reverse transcribing retroviruses and hepadnaviruses. I have included some taxonomic information in each chapter, sometimes more, sometimes less. I imagine this to be a reference resource and starting point for students who wish to know more. (And I ask my colleagues not to make these boxes a giant exercise in memorization.)

Throughout the book, I have included brief discussions of both a historical nature (for example, oncogenic retroviruses and an account of the discovery of hepatitis B virus) and current issues such as the recent initiative of the World Health Organization and the World Organization for Animal Health (OIE) to collaborate to reduce human deaths by rabies virus in underdeveloped countries. In the mix are also topics relevant to basic research such as use of vesicular stomatitis virus G protein for pseudotyping and lymphocytic choriomeningitis virus (LCMV) as a model for pathogenesis.

For instructors and colleagues, a final word. You will find the depth of coverage somewhat mixed throughout, and I may have neglected your favorite virus or disease. I am also quite sure that I have presented ideas with which you disagree. Share these with your students, start a conversation, and call me out if necessary. My research manuscripts have always been improved by thoughtful criticism, and if this book is to have a life beyond the first edition, I expect that the same will be true in this case.

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Acknowledgments

I cannot overstate the contributions of my illustrator, and wonderful sister, Marcy Edelstein. As I expected, she went "above and beyond" in assisting with this project. In addition to creating illustrations, she learned virology and gently pushed this project to completion with constant help and advice. Many thanks are also owed to my husband, Ross Payne, and to my mom for their patience and understanding during this project. I also wish to thank Texas A&M University, United States, for providing an incredible work and learning environment and my virology colleagues at the College of Veterinary Medicine and Biomedical Sciences and the Health Science Center for their support and inspiration. Finally, many thanks to my mentors over the years and to the dedicated and imaginative researchers who work to unravel the complex and beautiful world of viruses.

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CHAPTER

1

Introduction to Animal Viruses

OUTLINE

After studying this chapter, you should be able to:

- Provide a meaningful definition of a virus.
- Explain difference between cell division and virus replication.
- Explain the correct usage of "virion" versus "virus."
- Describe the basic steps in a virus replication-cycle.
- Draw, label, and describe each part of a "one-step" growth curve.
- List possible outcomes of a virus infection (1) at the level of the individual cell and (2) at the level of the host animal.
- Define the term "host range" as regards viruses.

WHAT IS A VIRUS?

Most of us are familiar with the term virus and know viruses as disease causing agents, transmitted from one person or animal to another. We are familiar with "cold" and "flu" viruses; we fear a worldwide pandemic of Ebola. We may even be aware that viruses are used to deliver genes to cells for the purposes of gene therapy or genetic engineering. But what are viruses?

- Viruses are infectious agents that are *not* cellular in nature.
- Viruses must enter a living host cell in order to replicate, thus *all* viruses are obligate intracellular parasites. Synthesis of the proteins and nucleic acids (DNA and RNA) for assembly into new virus particles (virions) requires an energy source (ATP), building materials (amino acids and nucleotides), and protein synthesis machinery (ribosomes) supplied by the host cell. The cell also provides scaffolds (microtubules, filaments, membranes) on which virus particles replicate their genomes and assemble. Thus the cell is a factory providing working machinery and raw materials. The infected cell may or may not continue normal cellular processes (host cell mRNA and protein synthesis) during a viral infection.
- Viruses have nucleic acid genomes that are surrounded by and protected by protein coats called capsids. Capsids protect genomes from environmental hazards and are needed for efficient delivery of viral genomes into new host cells. Some viruses have lipid membranes, called envelopes that surround the capsid (Fig. 1.1).
- Viruses are structurally much simpler than cells. Some viruses can be crystalized. Viruses do not increase in number by cell division; instead they assemble from *newly synthesized* protein and nucleic

Viruses. DOI: http://dx.doi.org/10.1016/B978-0-12-803109-4.00001-5

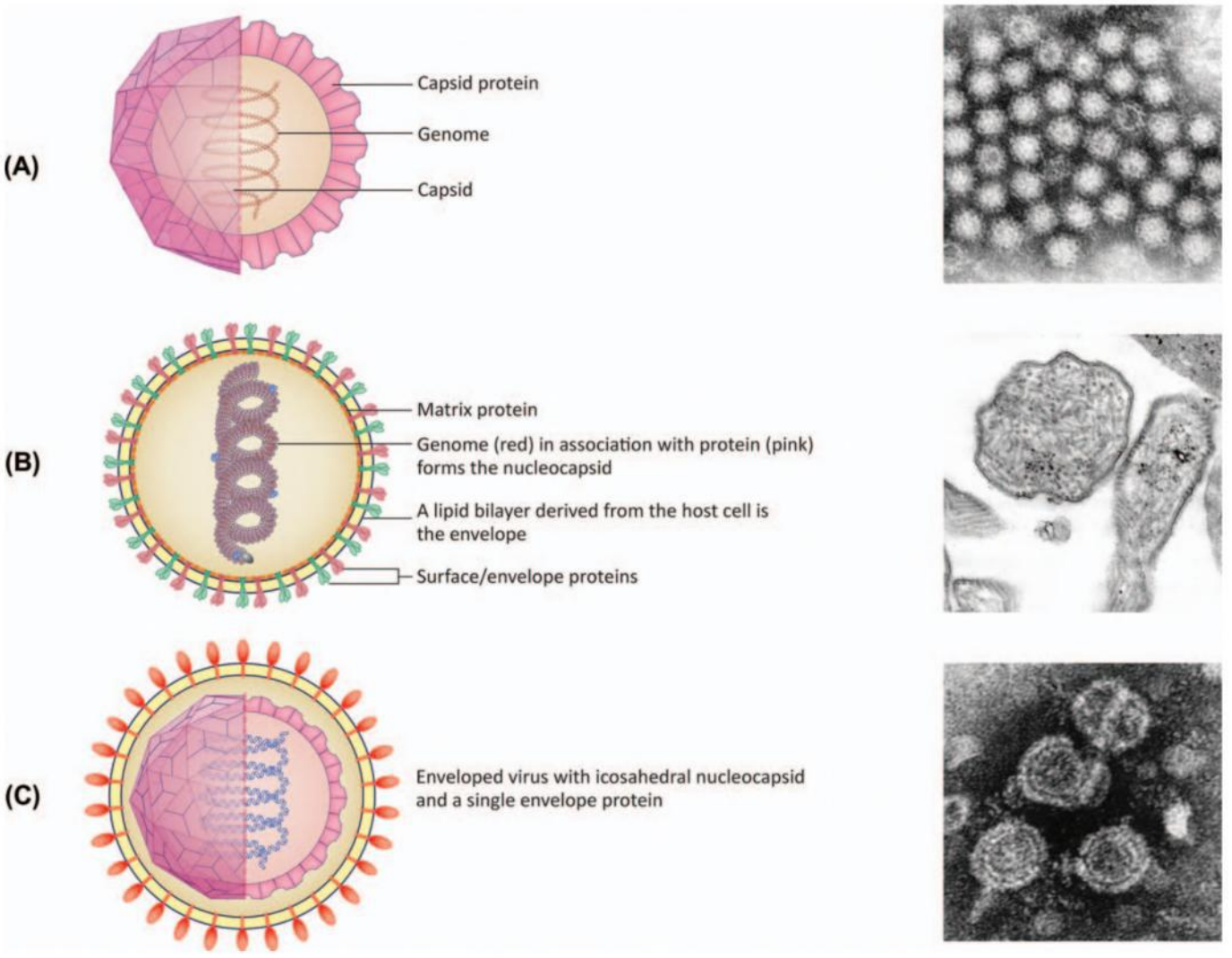

FIGURE 1.1 Basic features of virions. Panel A. On left, simple diagram of an unenveloped virus with icosahedral symmetry; on right, electron micrograph of calicivirus (Chapter 12: Family *Caliciviridae*). Panel B. On left, simple diagram of enveloped virus with a helical nucleocapsid; on right, electron micrograph of measles virus, a paramyxovirus (Chapter 20: Families *Paramyxoviridae* and *Pneumoviridae*). Panel C. On left a simple diagram of an enveloped virus with an icosahedral nucleocapsid; on right, electron micrograph of hepadnavirus (Chapter 38: Family *Hepadnaviridae*).

acid parts (building blocks). As viruses are not cells, they have none the organelles associated with cells. A sample of purified virions has no metabolic activity.
- Viruses are packages designed to deliver nucleic acids to cells; they are excellent examples of "selfish genes."

The preceding description might suggest uninteresting, inanimate particles, but examining virus replication strategies and interactions with host cells provides a diverse and dynamic view into cellular and molecular processes. Viruses are not a homogenous group. They are an extremely diverse group of infectious agents. It is highly unlikely that they arose from a single common ancestor (Box 1.1).

DIVERSITY IN THE WORLD OF VIRUSES

- All viruses have nucleic acid genomes, but some utilize DNA as genetic material, while others have RNA genomes. Viral genomes are not always double-stranded molecules; there are single-stranded viral RNA and DNA genomes. There are viral genomes that consist of a single molecule of nucleic acid, but some genomes are segmented. For example, reoviruses (Chapter 26: Family *Reoviridae*) package 11–12 different pieces of double-stranded RNA and each genome segment encodes a different gene.
- Some viruses have lipid envelopes in addition to a genome and protein coat. Viral envelopes are not

BOX 1.1

WHAT IS IN A DEFINITION?

By the late 19th century, the term "virus" was used to describe infectious agents that could pass through filters designed to remove bacteria from liquids. Thus viruses were "smaller than bacteria" and size became an important part of the definition of a virus. Today we know of a few viruses that are larger than many bacteria, so the trend has been to drop "smallness" from the definition. Another part of the definition of a virus is that they are all obligate intracellular parasites. This is certainly true of all viruses, but there are also bacteria and protozoan parasites that are obligate intracellular parasites. When the biochemical nature of viruses was discovered, it became clear that viruses lack many of the complex structures common to cells. This resulted in definitions of viruses based on comparisons to cells. While these comparisons emphasize the many ways that viruses are different from cells, they do not help us understand these unique infectious agents. So, what are viruses? Very simply, they are genes packaged within a protein coat. Their replication process begins when the virion delivers its genome to a cell. The viral genome encodes proteins required for the synthesis of new viral genomes. New viral proteins plus new viral genomes assemble to form new particles or virions, and so the cycle continues.

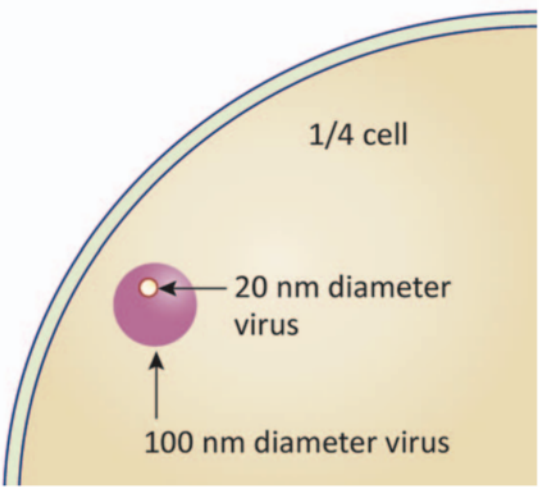

FIGURE 1.2 Relative sizes of an animal cells and virions.

homogenous. Different types of host membranes may be utilized, and their specific lipid and protein components can differ.

- Viruses range in size from 10 to 1000 nm is size (Fig. 1.2).
- Viral genomes range in size from 3000 nucleotides (nt) to over 1,000,000 base pairs.
- Outcomes of viral infections are diverse. Infection does not always result in cell or host death. Some host genes are derived from viruses and have played key roles in evolution. (Some plant viruses are beneficial in extreme environments.)
- Some viruses complete their replication cycles in minutes while others take days. Some viruses are transiently associated with an infected host (days or weeks) while others (for example, herpesviruses) are life-long residents.
- Where did viruses come from? Three general scenarios for virus evolution have been proposed:
  - Retrograde evolution: Intracellular parasites lost the ability for independent metabolism keeping only those genes necessary for replication. Poxviruses are very large complex viruses that *may* have evolved in this manner.
  - Origins from cellular DNA and RNA components: Some DNA genomes resemble plasmids or episomes. Did these DNAs acquire protein coats and the ability to be transferred from cell to cell efficiently?
  - Descendants of primitive precellular life forms: Viruses originated and evolved along with primitive, self-replicating molecules. This is the likeliest origin of the RNA viruses described in this text.

For the most part, names of specific viruses have been omitted in this section, to emphasize the general subject of viral diversity. Throughout this text, the details will be forthcoming. But I hope that now, when reading about any virus, you will want to learn its place in the complex world of viruses. (Big? small? friend? foe? transient visitor? life-long partner?)

ARE VIRUSES ALIVE?

Viruses parasitize every known form of life on this planet and they have both short-term and long-term impacts on their hosts. But are viruses alive? This question is the subject of ongoing debate, but the

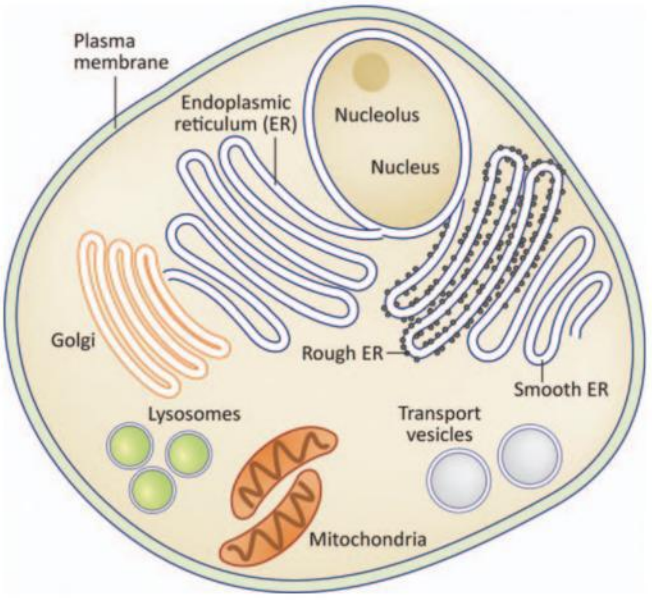

FIGURE 1.3 Simple schematic of a eukaryotic cell identifying some major organelles.

answer does not change the *nature* of the virus. As we discuss and describe viruses it is easy to assume that they are alive. They replicate to increase in number and the terms "virus replication-cycle" and "virus life-cycle" are often used interchangeably. Viruses also evolve (change their genomes), sometimes very rapidly. In this manner they adapt to new hosts and environments.

In contrast, the virion (the physical package that we view with an electron microscope) has no metabolic activity. Some viruses can be assembled simply by mixing purified genomes and proteins in a test tube. The genomes may have been synthesized by machine and the viral proteins may have been produced in bacteria. If those component parts combine under suitable conditions, a fully infectious virion can be produced. To avoid the question of living versus nonliving, the term "infectious agent" is both appropriate and descriptive. We can then speak of *infectious* virions that are *capable of entering a cell and initiating a replication-cycle*, or inactivated virions that cannot

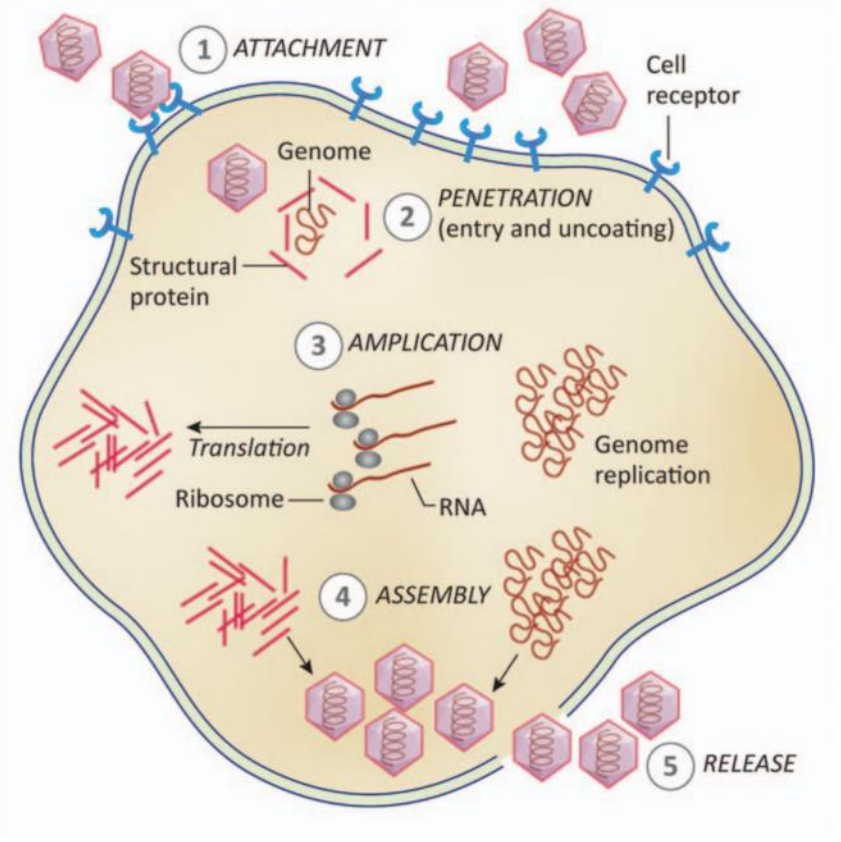

FIGURE 1.4 The basic virus life-cycle is shown in a generic cells. (For simplicity no cell organelles are shown but the processes of virus replication are intimately associated with cell organelles and structures.) The basic virus life-cycle begins with: (1) Attachment of the virion to receptors on a cell. (2) The genome is delivered into cytoplasm (penetration). (3) Viral proteins and nucleic acids are synthesized (amplification). (4) Genomes and proteins assemble to form new virions. (5) Virions are released from the cell.

complete a replication-cycle. As we will see in later chapters, the difference between an infectious and a noninfectious virion may be as small as the cleavage of a single peptide bond.

BASIC STEPS IN THE VIRUS REPLICATION-CYCLE

The first step in a virus life-cycle is attachment (or binding) to the host cell (Figs. 1.3 and 1.4). Attachment results from very specific interactions between viral proteins and molecules on the surface of the host cell. The interactions are usually hydrophobic and ionic, rather than covalent bonds. Thus attachment is influenced by environmental conditions such as pH and salt concentration. Attachment becomes stronger as many copies of a viral surface protein interact with multiple copies of the host cell receptor molecules.

The next step in the virus life-cycle is penetration of the viral genome into the host cell cytoplasm or nucleoplasm. After penetration, there may be a further rearrangement of viral proteins to release the viral genome, a process called uncoating. Penetration and uncoating are two distinct steps for some viruses while for others the viral genome is uncoated during the process of penetration. The processes of penetration and uncoating are irreversible, the infecting virion cannot reassemble.

The next phase in the virus life-cycle is synthesis of the new viral proteins and genomes. This is a complex process that requires transcription (synthesis of mRNA), translation (protein synthesis), and genome replication to generate the parts that will assemble into new virions. Synthesis of viral proteins and genomes occurs in close association with, and depends upon, many host cell proteins and structures. The great diversity among viruses will be evident as we examine processes that regulate transcription, translation, genome replication and the specific virus–host cell interactions that shape these processes.

The next step in the virus replication-cycle is assembly of new virions. New particles assemble from the genome and protein components that accumulate in the infected cell. Viruses are assembled at different sites in host cells; sometime large areas of the cell become virus factories, concentrated regions of viral proteins and genomes from which host cell organelles are excluded.

The final step(s) in the virus replication-cycle are release from the host cell and maturation of the released virions. Virion release may occur upon cell rupture or lysis. Enveloped viruses must acquire their envelopes from cellular membranes in a process called budding. Some enveloped viruses bud through the plasma membrane, but budding can occur at other, intracellular membranes. The budding process can, but does not always, kill the host cell. Other viruses obtain their lipid enveloped by budding into cellular vesicles. These vesicles then fuse with the plasma membrane to release the virions; this is process called exocytosis.

Maturation is the term used to describe changes in virus structure that occur after a virus is released from the host cell. Maturation may be required before a virus is able to infect a new cell; maturation may involve cleavage or rearrangement of viral proteins. Viruses assemble in the cell (under conditions of favorable energy) but when the released virions encounter new cells they must be able to disassemble (uncoating). Maturation events that occur after virus release set the stage for a productive encounter with the next cell. Maturation processes are well understood for several important animal viruses and examples will be presented in future chapters.

It is important to stress that each step in the virus replication-cycle requires specific interactions between viral proteins and host cell proteins. Some viruses can infect many different cell types and organisms because they interact with proteins found on, and in, many cell types. These viruses are said to have a broad host range. Other viruses have a very narrow host range due to their need to interact with specific cellular proteins that are expressed only in a few cell types. Factors that impact virus replication include the presence or absence of receptors, the metabolic state of the cell, the presence or absence of any number of intracellular proteins required to complete the virus replication-cycle.

Another way to view the replication-cycle of a virus is the one-step growth curve (Fig. 1.5). This graph illustrates the concept that penetration of a virus into the host cell is not reversible. During the so-called eclipse phase infectious virions cannot be detected, even if cells are broken open (lysed), there are no infectious particles to be found!

GROWING VIRUSES

Viruses are obligate intracellular parasites; they replicate only within living cells. Thus in the laboratory, susceptible cells or organisms are required to study virus replication. For the virologist, ideal host cells are easily grown and maintained in the laboratory. Animal virologists often use cell and (less often) organ cultures. To culture animal cells, tissues or organs are harvested and disrupted (using mechanical and enzymatic methods) to obtain individual cells. Often cells are derived from tumors that grow robustly in culture. Cells circulating in the blood, such as

lymphocytes, can be obtained directly from animal blood samples. If cells are provided with the appropriate environment (growth media, temperature, pH, and CO_2), they will remain metabolically active and may undergo cell divisions. Cell cultures will be described in more detail in a later chapter (Box 1.2).

Often the best-studied viruses are those that have been adapted for robust growth in a culture system. However, cell or organ cultures may be very different from the natural environment of the human or animal host. The biggest difference is that the cultured cells lack the many antiviral defenses encountered in an organism. Thus it is not uncommon for a virus highly adapted to cell cultures to perform poorly when used to infect an animal. In fact, propagation in culture is a common method for producing attenuated (weakened) live viral vaccines. Attenuated viruses replicate in a host, but do not cause disease. When considering experiments with viruses, it is very important to understand both the host system and the origins of the virus being studied.

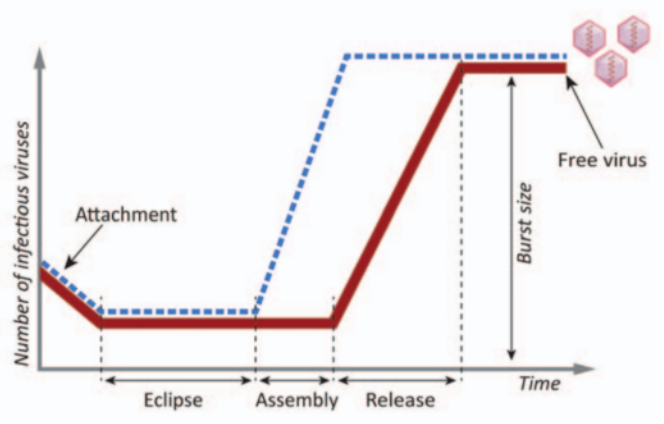

FIGURE 1.5 One-step virus growth curve. The red curve represents infectious virions released from the infected cells. The blue curve represents infectious virions released if the cells are lysed. Key to understanding the one-step growth curve is to note that after attachment, the number of virions detected in media and within cells *decreases*. These virions have penetrated cells and their genomes have uncoated, thus they are no longer "infectious." New virions are detected only after amplification and assembly.

CATEGORIZING VIRUSES (TAXONOMY)

The most widely accepted method to group viruses is by the type of nucleic acid (RNA or DNA) that serves as the viral genome. Within this scheme, there are three overarching groups of viruses:

- DNA viruses: Package DNA genomes synthesized by a DNA-dependent DNA polymerase.
- RNA viruses: Package RNA genomes synthesized by an RNA-dependent RNA polymerase (RdRp).
- The third group of viruses uses the enzyme reverse transcriptase (RT) during the replication-cycle. RT is an RNA-dependent DNA polymerase as it synthesizes a DNA copy of an RNA molecule. Reverse transcribing viruses (examples are the retroviruses and hepadnaviruses) use both RNA and DNA versions of their genomes (at different times) during their replication cycles.

The DNA and RNA viruses are further differentiated by the physical makeup of their genomes (single stranded, double stranded, unsegmented, segmented, linear, circular). The importance of genome type, and how it influences virus replication will be covered in upcoming chapters.

In addition to genome type, other physical traits are used to subdivide viruses into smaller groups. Some viruses have lipid envelopes (enveloped viruses) while others do not (naked viruses). Capsids also come in different shapes and sizes. The goal of viral taxonomy is to categorize viruses using groups of traits. Borrowing nomenclature from the Linnaean classification system, viruses are grouped into orders, families, genera, and species (Fig. 1.6). Orders contain two or more related families, and families can be subdivided into multiple genera. A genus is further subdivided into species (or strains). The family is often called the fundamental unit of viral taxonomy. Viruses in the

BOX 1.2

PERMISSIVE OR NOT?

Some viruses replicate very poorly when first introduced into cultured cells. There may be no visible signs of virus infection, but upon prolonged incubation or "blind passage" (often over a period of weeks or months) the virus will adapt to the new environment. This "cell culture adapted" virus now grows well in cultured cells. Therefore the initial virus infection was permissive, although very poorly so. After becoming adapted to cell culture conditions, the virus may be attenuated (replicate poorly or become incapable of causing disease) in the animal host.

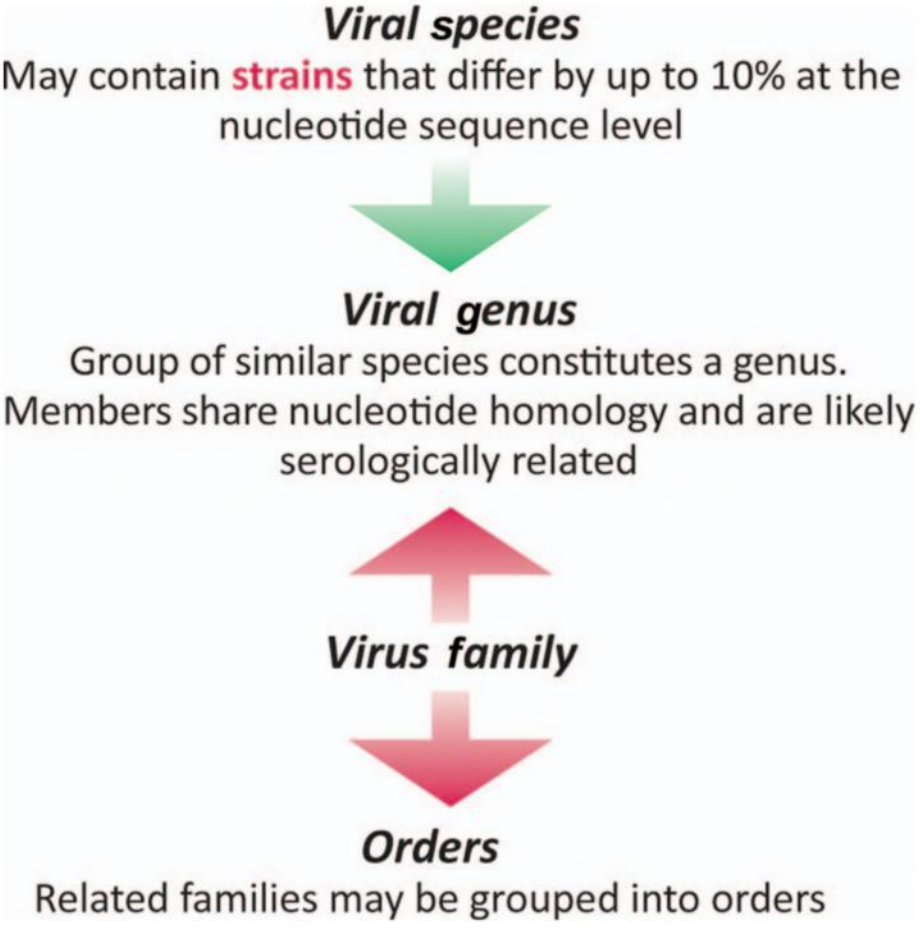

FIGURE 1.6 Viral taxonomy is based on groups of characteristics such as genome type, genome organization, capsid structure, presence/absence of an envelope. The virus family is often considered the focal point of virus taxonomy. Viruses in a family share genome type, overall genome organization, size, and shape. Related families can be group into orders. Families are also subdivided into smaller groups of more closely related viruses (genera) within the family. A genus can contain a number of different species or strains. These may differ by up to 10% at the nucleotide sequence level. Closely related strains may sometimes be quite phenotypically distinct.

same family are considerably more closely related than viruses from different families. Placement of viruses into families is accomplished by examining shared characteristics such as genome type, presence or absence of an envelope, shape of the capsid, arrangement of genes on the viral genome, etc. All viruses within a family share a core set of properties. Thus, if one knows the major characteristics of any single member of the family *Picornaviridae* (for example, poliovirus), one know the genome type, general genome organization, approximate size, and shape of all picornaviruses. One needs only to learn the characteristics of a handful of virus families, rather than thousands of individual viruses.

Viral taxonomy is determined by groups of expert virologists from around the world who volunteer to serve on the International Committee on the Taxonomy of Viruses (ICTV). Visit the ICTV website at http://ictvonline.org/virusTaxonomy.asp to find the most recent virus classification schemes. The site also provides a helpful history of virus names.

Before it was possible to generate genome sequences quickly and cheaply, classifying viruses was often done using phenotypic traits such as host range, or tissue tropism. Now it is standard practice to use genome sequences to categorize or classify viruses. Genome sequences provide detailed and objective criteria to subdivide viruses into related groups. Genome sequences from many different viruses can be compared to generate phylogenies that provide a visual "map" of relationships among viruses (Fig. 1.7). In some cases, many thousands of viral genome sequences are compared in order to generate detailed phylogenies. Such is the case with human immunodeficiency viruses (HIV).

The recent explosion viral in genome sequence data has necessitated extensive taxonomic changes in some virus families. For example, until recently the site of infection (respiratory versus enteric) was used as a

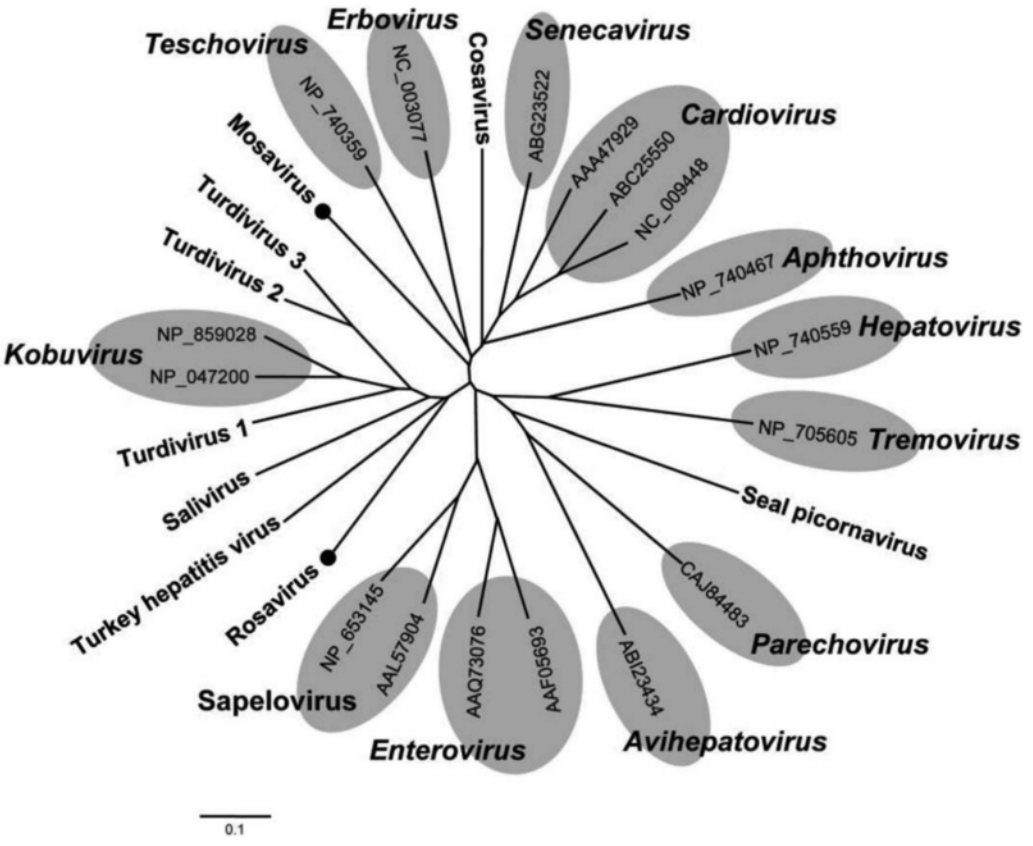

FIGURE 1.7 Phylogeny of the family *Picornaviridae* showing recognized genera. In some cases a genus contains only one virus isolate or strain. *Phan, T. G., Kapusinszky, B., Wang, C., Rose, R., Lipton, H., E. Delaware. 2011. The Fecal Flora of Wild Rodents. PLOS Pathogen 7:e1002218.*

criterion to define genera within the family *Picornaviridae*. However a phylogeny based on *genome sequences* does not split the picornaviruses cleanly along these lines. Thus the family *Picornaviridae* still contains the genus *Enterovirus*, but there is no longer a genus *Rhinovirus*, although you will see frequent reference to it in older literature.

Alternatives to ICTV taxonomy are sometimes used to group viruses that share common phenotypic characteristics. Hepatitis viruses are so named because they share the phenotype of replicating in the liver. However the hepatitis B virus (HBV) and the hepatitis C virus (HCV) are not related, either structurally or genetically, and vaccines and antiviral treatments developed for HCV are not effective for treating, or preventing, HBV infection. Another common phenotypic grouping is use of the term arbovirus (meaning arthropod-borne virus) to describe viruses that are transmitted by insects. Members of many different virus families can properly be called arboviruses; the term does not imply genetic relatedness among the diverse members of this "group".

You might ask if it is useful to generate or understand, phylogenies of viruses. The answer is a resounding yes. For example, the origins of a disease outbreak can be determined using detailed genetic information. Information from genome sequencing can be used to analyze past outbreaks and track the transmission of viruses from one person or animal to another in order to determine the best methods to curb virus transmission during an epidemic.

OUTCOMES OF VIRAL INFECTION

Virus infection impacts individual cells, and these cellular changes may or may not noticeably influence the health and fitness of the organism. There are four general outcomes when a virus encounters a cell:

- Productive or permissive infection. Viral proteins and nucleic acids are synthesized and virions are assembled and released.
- Nonpermissive infection. The cell is completely resistant to infection.
- Abortive or nonproductive infection. The virus enters the cell, but replication becomes irreversibly blocked at some step before particles are produced.
- Latent infection. Describes a situation where a viral genome is present in the cell, but no or only a few

BOX 1.3

LATENT VERSUS CHRONIC INFECTIONS: WHERE IS THE BOUNDARY?

A latent infection is one in which viral genomes are present in cells but virions are not produced. The term chronic infection describes one where virions can be routinely detected. Thus the sensitivity of the assays used for virus detection becomes an important factor in the distinction. As virus detection methods become more sensitive, the distinction between latency and chronic infection has become blurred. Consider genital herpes, caused by human herpesviruses 1 and 2 (HHV1 and 2). These viruses are abundant in visible lesions but also can be transmitted when there are no visible lesions. So is the infection latent or is it chronic? How often are the HHVs found on the skin in the absence of lesions? How often must a latent virus reactivate before it is considered chronic? From a public health standpoint calling genital herpes, a chronic infection might better convey the fact that herpesvirus can be transmitted in the absence of lesions.

viral proteins are produced. Latency implies that the virus can productively replicate given the right conditions (Box 1.3).

Both productive and nonproductive infections can impact the cell. The effects of infection can range from no apparent change, to cell death, to transformation (immortalization). Productive infection often results in cell death (lytic or cytopathic infection), but this is not always the case. Some viruses can replicate without damaging the cell, resulting in an inapparent infection. Viruses that cause inapparent infections are often produced in small amounts for the life of the cell. Sometimes an inapparent infection results from latency. A much less frequent outcome of infection is transformation or immortalization that allows the cell to divide without restriction. Immortalized cells may be productively infected (virus is released) or the condition may result from a nonproductive infection.

In the preceding paragraphs we learned that cells can be inapparently infected by a virus. Inapparent infection also occurs at the level of the animal host. Some viruses replicate in hosts without causing disease. After all, the "job" of a virus is replicate and infect another host; disease is not a required side effect. Until very recently it was hard to find viruses that caused inapparent infections. But many inapparent infections are now being identified through large-scale sequencing of host nucleic acids. (Methods for virus detection and discovery will be discussed in a later chapter).

Disease is the result of damage to tissues or organs. Many viral infections cause disease, and diseases can be described as acute, chronic, or latent (Fig. 1.8). Acute disease has a rapid onset, lasts from days to months, and the virus is either controlled or cleared, or causes death of the host. There are many examples of acute viral infections, the common cold being one. From a public health standpoint, it is important to know that virus replication and spread may begin well before symptoms develop and virus may be shed for days or weeks after symptoms have resolved. The peak of clinical signs and symptoms may or may not correspond to peak virus titers, or the time of maximum transmissibility.

Chronic viral infections have a slower progression and the time to resolution is years to a lifetime. These viral infections may, but do not always, lead to death of the host. Chronic infections are also called persistent infections. Virus is produced and shed continuously (albeit sometimes at very low levels). Examples of viruses that may cause chronic or persistent infections of humans are hepatitis C virus (HCV), hepatitis B virus (HBV), and human immunodeficiency virus (HIV). It should be noted that a chronic viral infection can be without symptoms (inapparent) for years.

Latent infection describes the maintenance of a viral genome without the production of detectable virus. Herpesviruses are a good example of viruses that cause latent infections. The chickenpox/shingles virus, formerly known as varicella-zoster virus, but recently renamed human herpesvirus 3 (HHV3) is an instructive example. Prior to 1995 chickenpox was a common childhood infection in the United States. Chickenpox infection is usually mild, characterized by blister-like pustules that resolve in about a week. However, HHV3 remains in the body long after the pustules have disappeared. HHV3 genomes are silently maintained in neurons, for decades. Shingles, a very painful and debilitating disease of adults, occurs when HHV3 exits latency and travels down neurons to the skin to produce blister-like lesions. These lesions contain infectious virus, thus a person with shingles can transmit chickenpox to a nonimmune person. HHV3 reactivates (breaks out of latency) when the host's immune

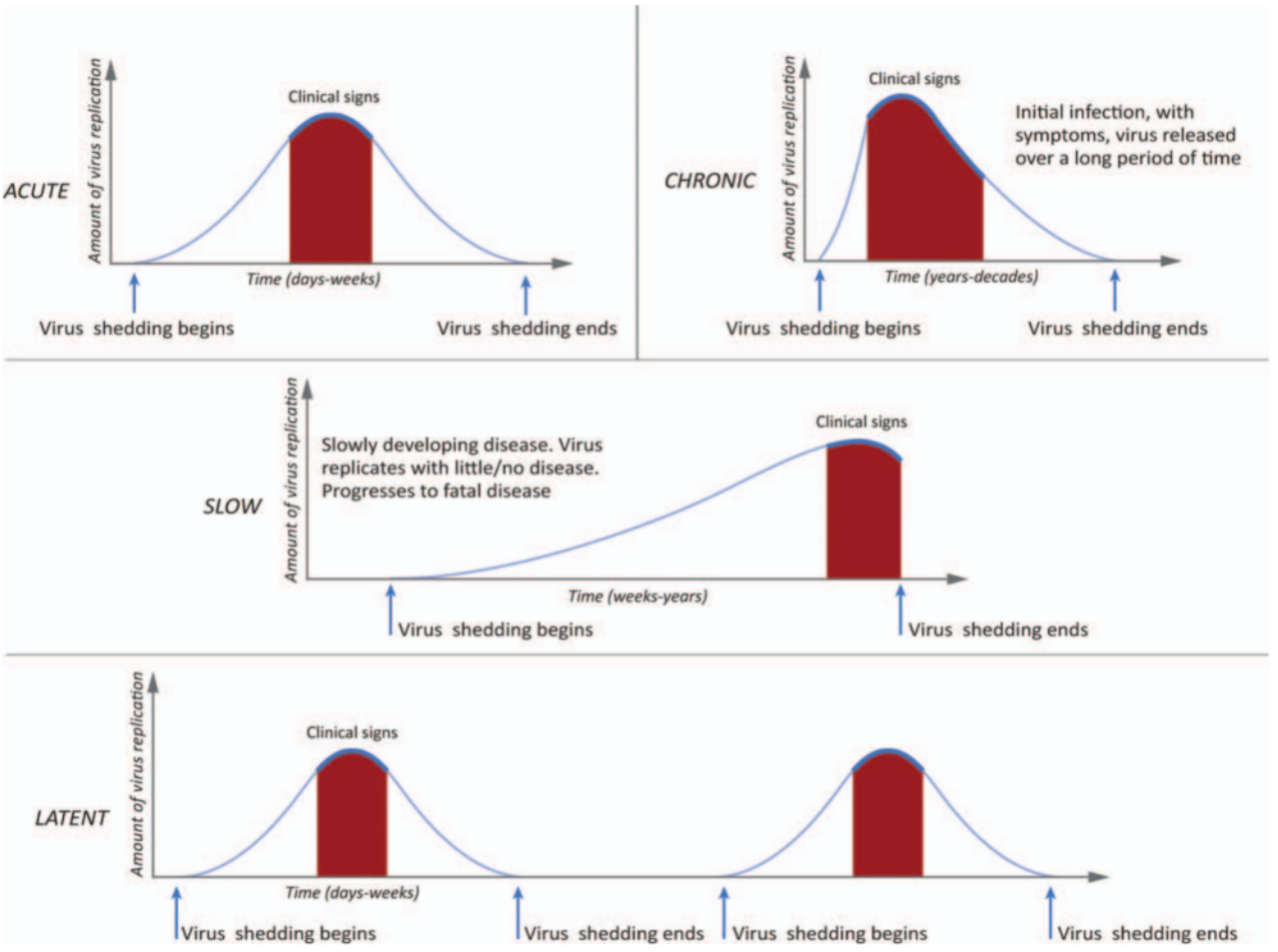

FIGURE 1.8 Outcomes of viral infection at the level of the animal host are quite variable. The red areas under the curves depict periods of clinical disease. In the examples depicted here, virus shedding begins before the onset of symptoms and ends after symptoms have resolved. Note that periods of virus shedding vary. Shedding may begin at the time of onset of clinical symptoms and may end prior to the resolution of disease. During latent infection, there may be intermittent virus shedding without clinical symptoms.

system is impaired (by advancing age or stress, for example). Shingles vaccines boost immune responses to HHV3, reducing the likelihood of virus reactivation.

INTRODUCTION TO VIRAL PATHOGENESIS

Viral pathogenesis is defined as the mechanism by which viruses causes disease. A simple view of viral pathogenesis is that viruses replicate and kill cells, thus causing disease. For example, death of liver cells (hepatocytes) causes hepatitis, death of enterocytes may cause diarrhea, death of respiratory epithelial cells may cause severe respiratory tract disease. However loss of cell function, without death, can also produce disease. During HIV infection, immunodeficiency is not simply caused by cell death; the virus also alters the function of some cells needed to maintain a healthy immune system.

Signs and symptoms of disease can also result from tissue damage caused by host immune responses. Inflammation, killing of virus-infected cells by the immune system, or deposition of immune complexes are examples. Of course, like any biological event, disease is often a complex combination of direct damage by virus in concert with host immune responses. Understanding viral pathogenesis, the mechanism by which disease develops, is an important consideration in developing effective treatments.

INTRODUCTION TO VIRUS TRANSMISSION

How are viruses transmitted from one animal to another? Common routes of infection include:

- fecal-oral,
- respiratory droplets,

- contact with contaminated fomites,
- exchange of infected bodily fluids, tissues, or organs,
- airborne,
- insect vectors.

Fecal-oral transmission occurs via ingestion of contaminated food or water. Virus enters the body through epithelial cells or lymphoid in the gastrointestinal tract. Examples include rotaviruses and the Norwalk-like viruses (noroviruses). Noroviruses have caused notable outbreaks on cruise ships, sickening hundreds of guests and crew in a matter of days. Human hepatitis A virus is also transmitted by the fecal-oral route via contaminated produce or uncooked shellfish. Fomites (objects contaminated with infectious organisms) can also play a role in fecal-oral transmission.

Respiratory transmission occurs when viruses in the respiratory tract are expelled as droplets. The transmission may be directly from one individual to another (please do not cough in my face) or may occur through fomites, hence the advice to wash your hands often! Viruses expelled from the respiratory tract may also be transmitted by contact with mucosal surfaces such as the eye. Health care providers and infectious disease researchers must remember to keep gloved hands away from their eyes. Examples of viruses that can be spread by the respiratory route are influenza viruses, rhinoviruses (one of the common cold viruses), and the severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) coronaviruses.

Transmission of viruses via exchange of bodily fluids can result from blood transfusions, use of dirty needles, trauma (bleeding), organ or tissue transplantation, sexual contact, or artificial insemination. The human viruses HIV, HBV, and HCV are all transmitted via contaminated blood. But these viruses can also be transmitted through contact with other bodily fluids such as semen or saliva. HIV can be transmitted via breast milk. Rabies virus is transmitted by saliva.

A few viruses, such as foot-and-mouth disease virus of livestock, can be transmitted over long distances through the air, a process called airborne transmission. Measles virus is also known for airborne transmission. Simply sitting in a room with a measles-infected individual can lead to infection! It should be noted that airborne transmission is distinct from aerosol transmission. In airborne transmission, particle sizes are very small and remain suspended in the air for long periods. The importance of understanding the distinction between these two types of transmission is exemplified by the 2014 Ebola virus epidemic. Ebola virus is transmitted through contact with body fluids of an infected individual. Transmission occurs when the patient is clearly symptomatic and virus titers are highest. Ebola can be transmitted via respiratory droplets but there is no evidence that the virus is transmitted in the absence of direct contact with respiratory droplets, or other secretions, thus Ebola is not considered to be an "airborne" virus.

Many viruses (West Nile virus, the equine encephalitis viruses, dengue virus, chikungunya virus, and zika virus, for example) are transmitted from one host to another primarily via an *insect* intermediary. Blood-feeding insects such as mosquitos, ticks, and midges are common vectors. Viruses transmitted by insect vectors are collectively called arboviruses.

It should be emphasized that a virus can be transmitted by more than one route. The SARS coronavirus, considered primarily a respiratory virus, is also transmitted by the fecal-oral route. Blood transfusions, dirty needles, and organ transplants may facilitate transmission of viruses usually spread by other routes. Mucosal surfaces, such as the eye, can be entry points for transmission of virus in present in blood or other bodily fluids. Some mosquito-vectored viruses (West Nile, chikungunya, yellow fever, and equine encephalitis viruses) require special precautions to avoid transmission in a research setting, where these viruses can be transmitted via aerosols.

Finally, a discussion of virus transmission should also include brief mention of virus transmissibility. Transmissibility is the ease of virus spread from one host to another. Measles virus is highly transmissible by the airborne route, and outbreaks can quickly become widespread in nonimmune population. Transmissibility is not related to the ability of a virus to cause disease (virulence). A virus may be relatively difficult to transmit, but highly virulent if transmission does occur. It is easy to overestimate the transmissibility of a highly virulent virus.

In this chapter we have learned that:

- Viruses are infectious agents (but are not cells).
- Viruses are obligate intracellular parasites that require host cells for their replication.
- Virions are the packages that contain the viral genome.
- Virions assemble from viral proteins and genomes synthesized within the infected cell.
- In the laboratory viruses are cultured or grown in cell or organ culture.
- Viruses can change or adapt to new growth conditions.
- Viruses have different genome types, capsid types, routes of infection, and diverse interactions with host cells.
- Virus infection may but does not always lead to cell death or host disease.
- Virus infections may be relatively short lived (acute infections) or may be life-long (chronic or persistent).

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CHAPTER

2

Virus Structure

OUTLINE

After studying this chapter, you should be able to:

- Define "capsid" and explain its functions.
- Define "nucleocapsid," "envelope," and "envelope protein."
- Describe the difference between "structural" and "nonstructural" proteins.
- Be able to distinguish between icosahedral and helical capsids.
- Indicate the twofold, threefold, and fivefold axes of symmetry on an icosahedral capsid.
- Describe the activities of envelope glycoproteins.
- Describe the location and a common function of matrix (MA) proteins of enveloped viruses.

ANATOMY OF A VIRUS

The simplest viruses consist of genome packaged in a protein shell or capsid (see Fig. 1.1). Capsids are assembled from many copies of a single, or a few types of capsid proteins. Some viruses surround their capsids with a lipid bilayer called the envelope. Envelopes may be derived from the cell plasma membrane, nuclear membrane, or other intracellular membranes. All enveloped viruses encode proteins that are associated with the lipid bilayer. They are usually glycosylated (thus are envelope *glyco*proteins) and often contain transmembrane anchoring domains. They often project out from the surface of the envelope forming distinct spikes. Many enveloped viruses have a matrix protein positioned inside, and associated with the envelope (via direct membrane interactions or through interactions with the cytosolic tails of envelope glycoproteins). The matrix protein often forms a link between the membrane and the nucleocapsid. The term nucleocapsid refers to a complex of viral nucleic acid and protein. The term is most often used to refer to the assemblage of protein and nucleic acid within an *enveloped* virus. If viral envelopes are gently lysed, the nucleocapsids are released.

The proteins that assemble to form the virion (the extracellular particle) are called structural proteins. Additional proteins may be encoded by a virus, but are not present in the virion. These so-called nonstructural proteins have a variety of functions in the virus replication cycle. For example, nonstructural proteins may modulate cell and host antiviral responses; others are enzymes such as proteases or polymerases. It is important to note: nonstructural does not mean

nonfunctional, or unimportant. Most nonstructural proteins are in fact essential for virus replication.

CAPSID STRUCTURE AND FUNCTION

In the simplest terms, viral capsids are protein packages that protect the genome. However capsids should not be considered static boxes, as they are dynamic structures that have other important functions. In addition to simply providing "packaging," the capsids of unenveloped viruses mediate attachment to, and penetration into, the host cell. Capsids must also be able to assemble, specifically package viral genomes and direct budding or release from cells.

Capsids come in two basic shapes: helical (rod shaped) or icosahedral (spherical). The simplest capsids are assemblies of many copies of a single protein (often called the capsid protein). As capsids assemble, they are stabilized by the repeated interactions (largely electrostatic) of the capsid protein building blocks. It should come as no surprise that there are few covalent bonds between these building blocks, because the genome must be released from the capsid at a later time!

The repeated occurrence of similar protein–protein interfaces leads to construction of a symmetrical capsid. The simple helical capsid of tobacco mosaic virus (TMV), a plant virus, is assembled from many copies of a single capsid protein. Each capsid protein forms identical same side-to-side and top to bottom interactions with its neighbors, as indicated in Fig. 2.1. In addition to interacting with neighboring proteins, each TMV capsid protein interacts with three nucleotides of the viral (RNA) genome. The capsid proteins are tightly packed around the RNA and form a rigid rod whose length is determined by the length of the genome. Not all helical capsids are rigid rods, many enveloped animal viruses have very flexible, helical nucleocapsids surrounded by an envelope.

Viruses with spherical capsids have icosahedral symmetry. An icosahedron is a closed cube with twofold, threefold, and fivefold axes of symmetry (Fig. 2.1). The simplest icosahedron can be assembled from 20 equilateral triangles. More complex, and larger, icosahedra can be built by assembling more than 20 triangular subunits.

Capsids that vary from the definitions of a helix or an icosahedron are sometimes called "complex." For example, there are viruses of bacteria (bacteriophage) with icosahedral "heads," rod like "tails" and long "fibers" extending from the tails.

CAPSIDS ARE BUILT FROM MANY COPIES OF ONE OR A FEW TYPES OF PROTEIN

Biological constraints require that capsids be assembled from multiple copies of one, or a few, small (usually in the range of 20–60 kDa) proteins. A key consideration of capsid assembly is the relative size of an amino acid and the triplet codon for that amino acid. The average size of an amino acid is 110 daltons (Da) while the average size of a triplet codon is ~330 Da × 3, or nearly 1000 Da. The codon is considerably larger than the amino acid, so it requires many copies of any single amino acid to package it.

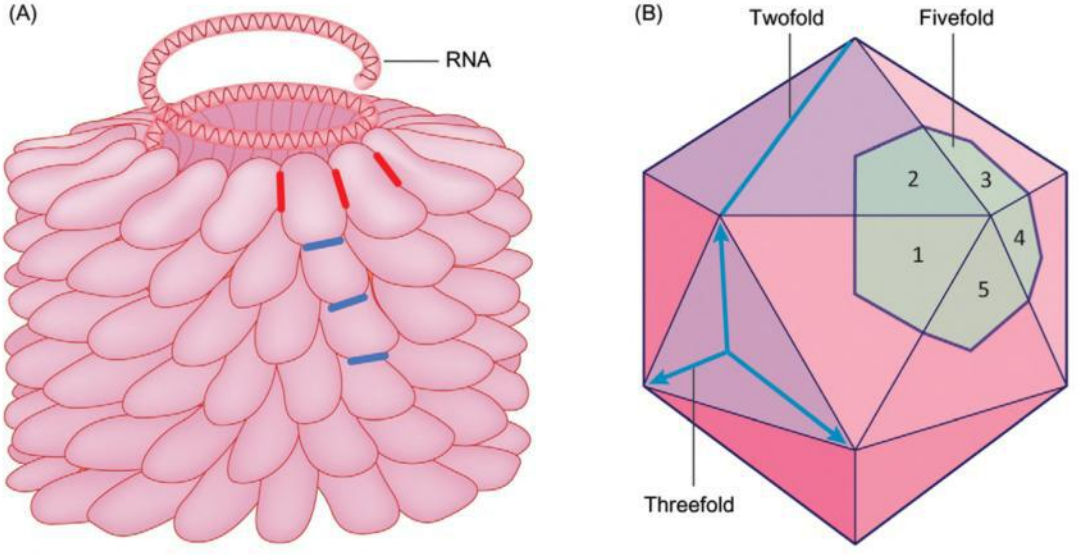

FIGURE 2.1 Capsids come in two basic shapes helices (rods shown on left) and spherical particles with icosahedral symmetry (shown on right). (A) The red and blue bars on the helical virus indicate sites of repeating contacts between subunits. Red bars indicate side-to-side contacts. Blue bars indicated top to bottom contacts. (B) The two, three and five fold axes symmetry are indicated for this simple icosahedral capsid.

A single polypeptide is always physically smaller than the gene that encodes it. Luckily, translation produces many copies of a protein from each mRNA.

Another biological constraint to the size of a capsid protein is its ability to fold. Small polypeptides can fold tightly while larger ones are unable to fold without leaving gaps in the structure; gaps could render the genome susceptible to environmental damage. Larger proteins also need chaperones to help them fold correctly. Indeed, there are some viral capsids that assemble with the help of chaperones.

Fidelity of protein synthesis is another issue that constrains the size of a capsid protein. The observed error rate of protein synthesis is $\sim 10^{-4}$, or 1 mistake per 1000 amino acids polymerized. Thus on average, a 1000 amino acid long protein would contain one mistake. In contrast, synthesis of 10 copies of a 100 amino acid protein would produce, on average, nine perfect proteins and one with a mistake.

SIMPLE ICOSAHEDRAL CAPSIDS

In the previous section the icosahedron was described as an assembly of equilateral triangles. However, capsids are not assembled from "triangular" or even symmetrical, proteins. Among animal viruses, the most common type of capsid protein structure is called an eight-stranded jelly roll β-barrel motif. This structure consists of four pairs of antiparallel β sheets (Fig. 2.2).

The most common icosahdral capsids can be envisioned as an arrangement of three capsid proteins into a triangle. Thus, as shown in Fig. 2.2, the simplest icosahedral capsid would contain 60 capsid proteins (20 triangular faces, each having three capsid proteins). This structure is called a $T = 1$ capsid, and T is the triangulation number (Box 2.1). Without reviewing the mathematics involved, a $T = 1$ icosahedron is an assembly of 20 triangular faces, a $T = 3$ icosahedron has 3×20 or 60 triangular faces, a $T = 4$ icosahedron has 4×20 or 80 triangular faces and so on. The T number can be used to determine the theoretical number of capsid proteins required to assemble a shell, if each triangular face is constructed from three capsid proteins. A $T = 1$ virus (for example, a parvovirus) has a shell assembled from 60 capsid proteins. Caliciviruses have $T = 3$ capsids, assembled from 180 (3×60) molecules of a capsid protein. Togaviruses have $T = 4$ capsids that are assembled from 240 (3×80) molecules of capsid protein (Table 2.1). Thus larger capsids are assembled by using more building blocks (Fig. 2.3).

How do capsid proteins assemble to form a simple icosahedral shell? The process is stepwise and sometimes the substructures can be identified. $T = 1$ viruses assemble from 60 capsid proteins. A general model is that three capsid proteins assemble to form a capsomere. Five capsomers form a pentamer and 12 pentamers assemble to form the closed shell. $T = 3$ and $T = 4$ capsids are built with pentamers and hexamers (hexamers are assemblies of six capsid proteins). $T = 3$ capsids assemble from 12 pentamers and 20 hexamers while $T = 4$ viruses assemble from 12 pentamers and 30 hexamers.

The surfaces of icosahedral capsids can be quite variable. Some capsids are relatively smooth, while others have prominent projections and/or deep canyons or pits (Fig. 2.4). Electron micrographs of unenveloped icosahedral viruses are shown in Fig. 2.5. In the case of unenveloped viruses, attachment proteins may

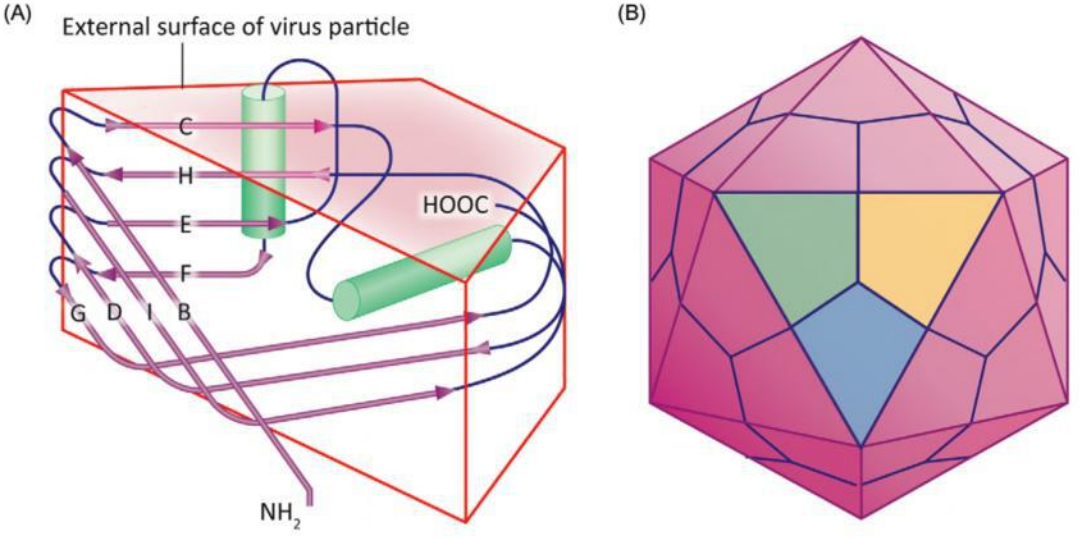

FIGURE 2.2 (A) The icosahedral capsids of most animal viruses are assembled from proteins that have a common shape: an eight-stranded jelly roll β-barrel motif. (B) Each triangular 'face' of the capsid is assembled from three copies of an eight-stranded jelly roll β-barrel motif.

BOX 2.1

TRIANGULATION NUMBERS AND STRUCTURAL SUBUNITS

The *T* number was first described by Caspar and Klug in 1962 to explain the structural basis for icosahedral capsids of different sizes. In their models, the *T* number predicted the number of capsid proteins in a structure, and their predictions proved true for many small viruses. However, as more icosahedral capsids were analyzed it became apparent that *T* numbers more often predicted numbers of "structural subunits" rather than numbers of polypeptides in a capsid. The most common structural subunits of icosahedral animal virus capsids are β-barrels (eight-stranded jelly roll β-barrel motifs). This common structure is shown in Fig. 2.3. Capsid proteins of different viruses do not have conserved amino acid sequences; however, they do assume this conserved structure. The distinction between a structural subunit and a polypeptide in building capsids can be demonstrated by comparing the capsids of caliciviruses, picornaviruses, and cowpea mosaic virus. All three virus groups have $T = 3$ capsid architecture. The calicivirus capsid is assembled from 180 copies of a single capsid (C) protein while picornaviruses are assembled from 60 copies each, of three capsid proteins (VP1, VP2, and VP3). The three picornavirus capsid proteins have different primary amino acid sequences but fold to assume similar structures. Thus the capsids of picornaviruses are assembled from 180 equivalent structural subunits. The $T = 3$ capsids of picornaviruses are sometimes called pseudo $T = 3$ or $P = 3$ capsids as they do not strictly adhere to the original predictions put forth by Caspar and Klug. In the case of cowpea mosaic virus the capsid is assembled from two different polypeptides, 60 copies each of a large (L) and a small (S) capsid protein. However, a close look at capsid architecture shows that L folds into two independent β-barrels connected by a hinge region. Thus the cowpea mosaic virus capsid is assembled from 180 "structural" domains, where the structural unit is the β-barrel.

TABLE 2.1 Construction of Simple Icosahedral Capsids

T number	Triangular faces/ icosahedron	Number of proteins (structural subunits)/capsid	Number of pentamers + number of hexamers
1	20	60	12 + 0
3	60	180	12 + 20
4	80	240	12 + 30

project out from the capsid to engage a receptor, while in other cases attachment sites are inside a canyon or pit.

LARGER ICOSAHEDRAL CAPSIDS

Large icosahedral capsids can also be envisioned as assemblages of triangular faces. For example, one could construct a $T = 7$ icosahedron using 140 equilateral triangles (420 structural subunits) or a $T = 13$ icosahedron from 260 equilateral triangles (780 structural subunits). However cryo-electron microscopic visualization of viruses with larger icosahedral capsids reveals that some of these structures (described below) are not simply larger versions of a $T = 1$ or $T = 3$ virus.

Polyomavirus and Papillomavirus Capsids

Based on the predictions of Caspar and Klug, these $T = 7$ capsids (~50 nm diameter for polyomavirus and ~60 nm diameter for papillomavirus) would contain 420 structural subunits assembled into 12 pentamers and 80 hexamers, but this is not exactly the case. Instead the capsids of polyomaviruses and papillomaviruses are assembled from 360 structural subunits organized as 72 pentamers (Fig. 2.3). These capsids structures are referred to at $T = 7d$ (rather than $T = 7$). Papillomavirus capsids are assembled from two structural proteins, L1 and L2. Each pentamer assembles from five molecules of L1 and a single molecule of L2.

Adenovirus Capsids

The large (~95 nm diameter) icosahedral capsids of adenoviruses contain 12 different polypeptides. Their capsid structure is described as pseudo $T = 25$. The major capsid protein (MCP) is called the hexon protein and 720 copies of hexon protein assemble to form 240 trimers. Each trimer forms a hexamer subunit. But how can a hexamer be assembled from a trimer of capsid proteins? It turns out that hexon protein is a large capsid protein that contains two structural domains. Adenovirus capsids also contain 12 pentamers or vertices (Fig. 2.3, Panel E) assembled from three additional structural proteins, the penton base protein

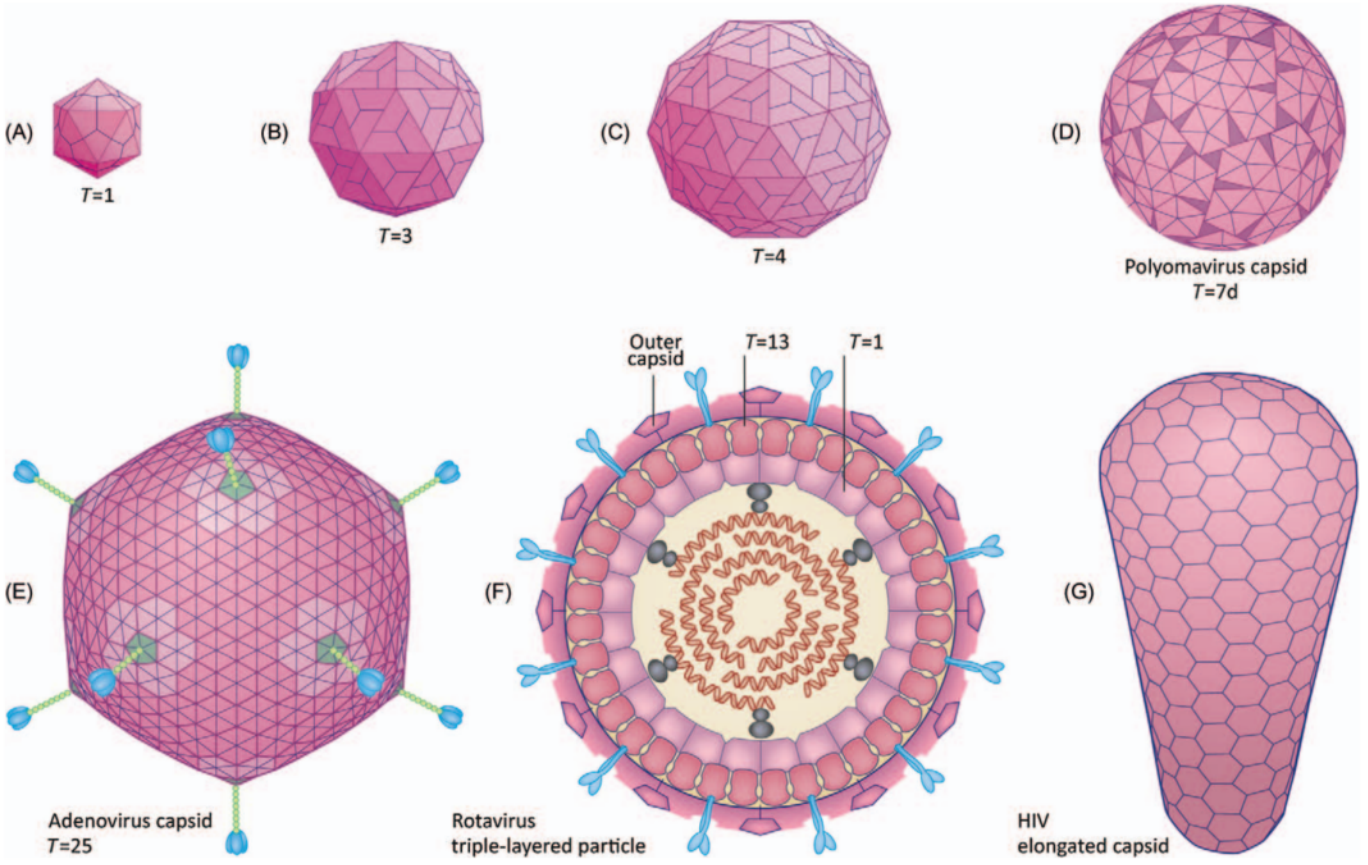

FIGURE 2.3 Examples of large and small icosahedral capsids. (A) T = 1 icosahedral capsid; (B) T = 3 icosahedral capsid; (C) T = 4 icosahedral capsid; (D) T = 7d polyomavirus capsid; (E) T = 25 adenovirus capsid; (F) Triple layer particle of rotavirus contains a T = 1 core surrounded by T = 13 shells. (G) HIV capsid. Elongated icosahedral capsids are formed by adding rows of hexamers to the middle of the structure.

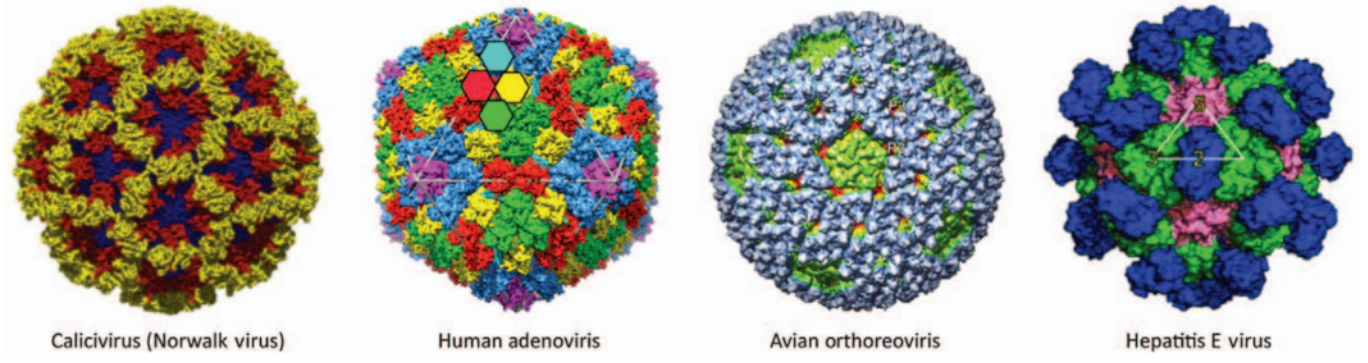

FIGURE 2.4 Molecular models of icosahedral viruses.

(polypeptide III), the fiber protein (polypeptide IV), and polypeptide IIIa. The long spikes that extend from each vertex are homotrimers of the fiber protein. They are anchored in place by the penton protein. Five copies of polypeptide IIIa are arranged in a ring beneath each vertex. Thus pentons and hexons are assembled from different types of capsid proteins.

Reoviruses

Reoviruses have multilayered icosahedral capsids. Viruses in the genus *Rotavirus* have triple-layered particles (Fig. 2.3, Panel F). The smallest, innermost layer is a $T = 1$ capsid formed from 60 copies of viral protein (VP) 2. The middle layer is a $T = 13$ capsid assembled

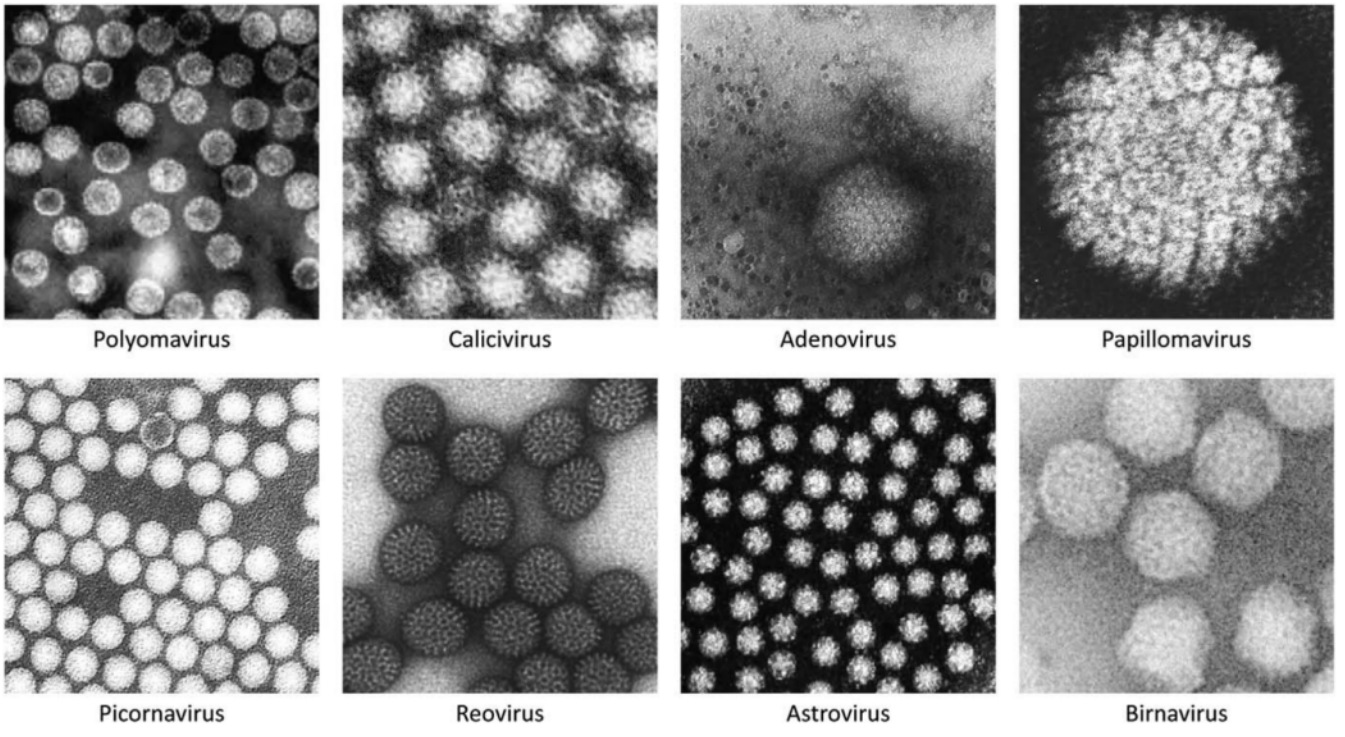

FIGURE 2.5 Examples of unenveloped viruses.

from the ~45 kDa VP6. The outmost layer of the rotavirus capsid is assembled from two additional structural proteins, VP4 and VP7. Members of the genus *Reovirus* have two capsids layers, an inner $T = 1$ core surrounded by an outer $T = 13$ shell.

Herpesvirus Capsids

Herpesviruses are large, enveloped viruses with icosahedral capsids. The major capsid protein or MCP is quite large (~1300 amino acids) and folds to assume a structure that is distinct from the eight-stranded jelly roll β-barrel motif of other animal viruses. MCP assumes a shape very similar to the capsid proteins of double-stranded (ds) DNA bacteriophage such as P22 or HK97. Herpesviral capsids are assembled from a total of 162 capsomers (150 hexons and 12 pentons). MCP forms all of the hexons and 11 of 12 pentons. The 12th penton is formed from a different herpesvirus protein called the portal protein to form the portal "complex." The portal complex is cylindrical and contains a channel. Bacterio phage portal complexes actively transport DNA into and out of bacteriophage capsids. Herpesvirus portals likely serve a similar function. The structure of herpesvirus capsids suggests an evolutionary relationship between herpesviruses and dsDNA phage.

Poxvirus Structure

Poxviruses are among the largest of the animal viruses. Virions are brick shaped or ovoid. They are enveloped and range in size from 140 to 260 nm in height and 220 to 450 nm in length. The envelope surrounds a dumb bell-shaped core (capsid) and two globular protein structures called lateral bodies. Morphogenesis of poxvirions is described in Chapter 35, Family *Poxviridae*.

VIRAL ENVELOPES

Many important human and animal pathogens are enveloped viruses. Their helical or icosahedral capsids are enclosed within a lipid bilayer. Enveloped viruses come in a variety of shapes, ranging from the long, filamentous ebolaviruses (Chapter 21: Family *Filoviridae*) to the icosahedral togaviruses (Chapter 16: Family *Togaviridae*). Most enveloped viruses acquire their lipid bilayer by budding through a cellular membrane (for example, plasma membrane, endoplasmic reticulum (ER), Golgi, or nuclear membranes). However some large viruses, such as poxviruses apparently build their membranes from crescent-shaped lipid fragments associated with protein scaffolds (Chapter 35: Family *Poxviridae*).

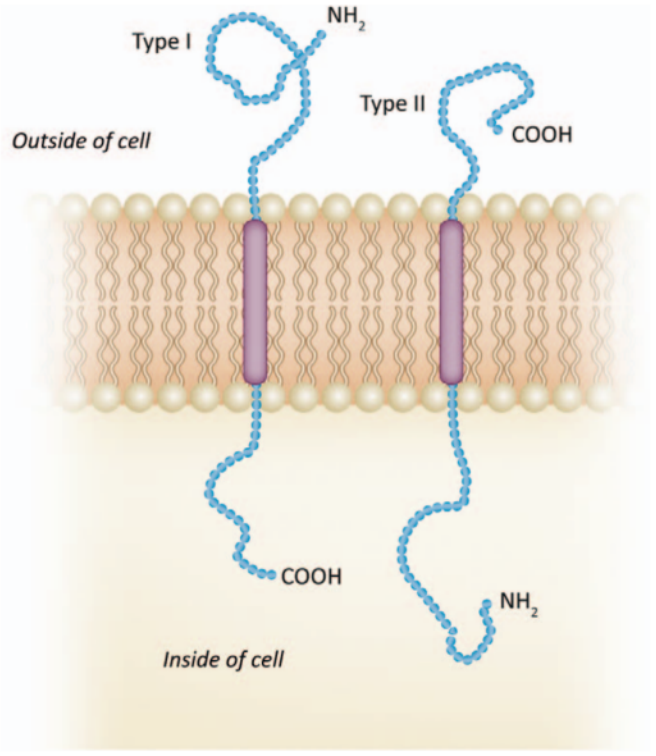

FIGURE 2.6 Arrangement of types I and II membrane proteins.

Enveloped viruses (with the exception of poxviruses) have proteins that are anchored into or across the lipid bilayer. Thus they are membrane proteins and can be categorized as type I or type II depending on their orientation in the membrane (Fig. 2.6). Types I and II membrane proteins span the lipid bilayer a single time, via an α-helical TM domain. Type I proteins are inserted into membranes with their amino-terminal domain to the outside and their carboxyl-terminal domain to the inside of the cell or virion. Type II membrane proteins have the opposite polarity with respect to the membrane Envelope proteins often take the form of long spikes. Spikes are usually homo- or heterodimers or trimers and many have distinct globular heads. Examples of viral type I membrane proteins include: influenza virus HA, rabies virus G protein, paramyxovirus fusion proteins, and hepatitis C virus E1 and E2. Paramyxovirus attachment proteins are type II membrane proteins.

Virus envelope proteins must carry out at least two functions: receptor binding and fusion. These activities may be carried out by a single protein (influenza virus HA, rabies virus G) or by two distinct proteins in the case of the paramyxoviruses. In the case of influenza virus, the HA glycoprotein is cleaved by cellular proteases to generate a fusion-active version of the protein but the two halves of HA (HA1 and HA2) remain associated via disulfide bonds. Rabies virus G protein also has both attachment and fusion activities; however, rabies virus G remains uncleaved. Human immunodeficiency virus (HIV), a retrovirus, also encodes a single glycoprotein precursor, but it is cleaved during synthesis to produce two distinct structural proteins. The amino-terminal domain of the envelope precursor is the surface unit (SU) protein, which functions in attachment. The carboxyl-terminal half of the precursor is the transmembrane (TM) protein. As its name implies, it has a membrane-spanning domain, anchoring it into the envelope. TM is the fusion protein. SU and TM are not covalently linked. SU is on the outside of the virion, held thereby noncovalent interactions with ectodomain of TM (Chapter 37: Replication and Pathogenesis of Human Immunodeficiency Virus). Viruses in the family *Paramyxoviridae* (see Chapter 20: Families Paramyxoviridae and Pneumoviridae) produce two envelope glycoproteins using distinct open reading frames. Both are membrane-anchored proteins. The attachment protein is a type II integral membrane protein while the fusion protein is a type I integral membrane protein.

Glycosylation

The ectodomains of envelope proteins are usually glycosylated (they have polysaccharides linked to the protein backbone). Carbohydrates can be linked to the peptide backbone via the nitrogen atom on an asparagine side chain (N)-linked or to the oxygen atoms of serine or threonine side chains (O-linked). Glycosylation occurs during transit of envelope proteins through the ER and Golgi. The amount of carbohydrate decorating envelope proteins varies. Some viral envelope proteins have one molecule of polysaccharide per polypeptide chain. In the case of the HIV SU, the molecular mass of carbohydrate equals that of the polypeptide backbone. HIV SU protein has an apparent molecular weight of 120 kDa (as determined by SDS-PAGE) but the calculated mass of the amino acid backbone is <50 kDa.

OTHER MEMBRANE PROTEINS

A few enveloped viruses also have membrane-associated proteins with other roles in the replication cycle. Influenza viruses have a receptor-destroying protein called neuraminidase (NA) that is required for efficient release of virions from the surface of the infected cell. Drugs that block the enzymatic activity of NA do not block virion formation, but virions remain tightly associated with the infected cell, thereby

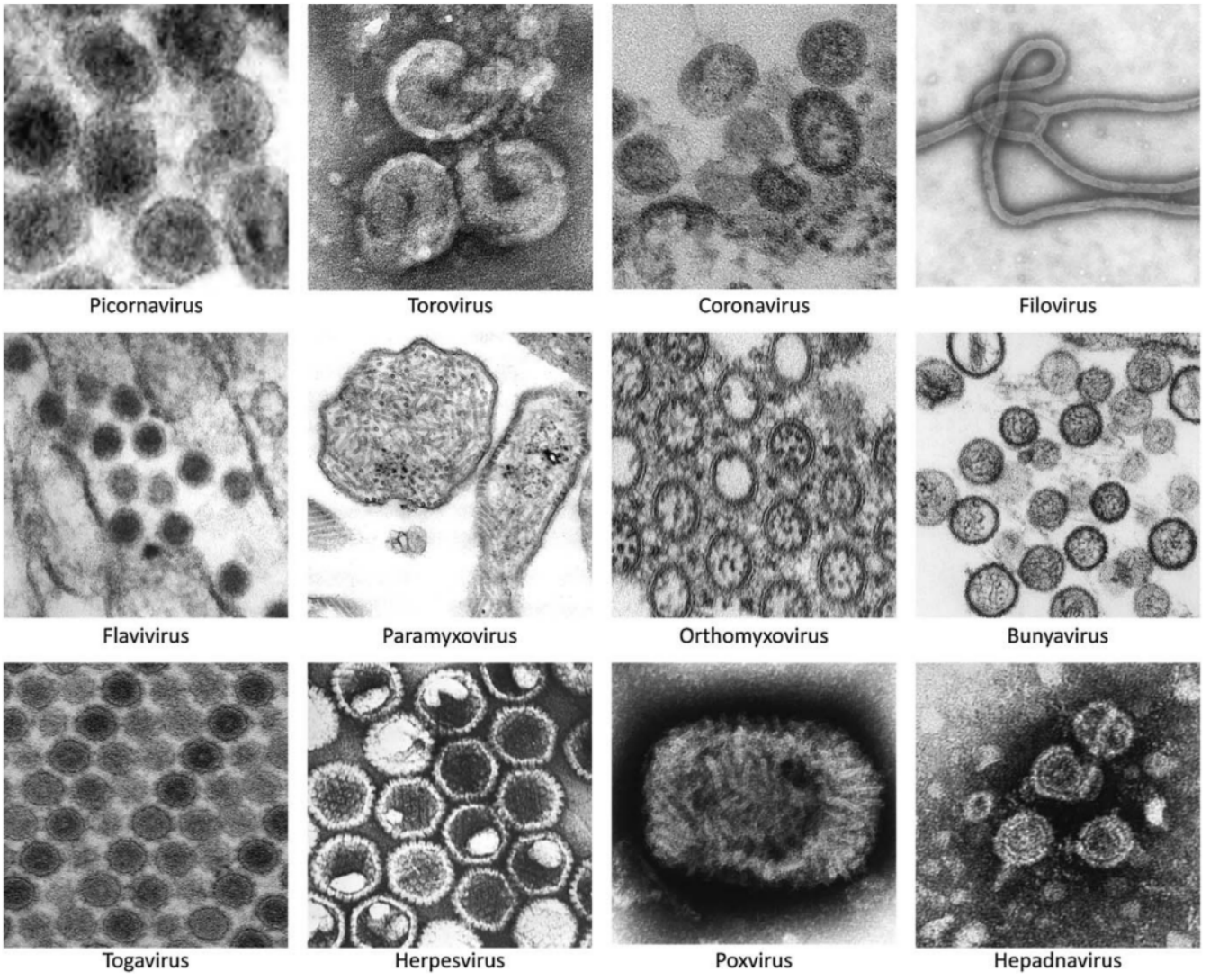

FIGURE 2.7 Examples of enveloped viruses.

limiting their ability to infect other cells (Chapter 23: Family *Orthomyxovirdae*).

Another role for viral membrane-associated proteins is ion transport. Influenza viruses encode a small integral membrane protein called M2. The M2 ion channel is a low pH-activated homotetramer that facilitates transport of H^+ ions across the viral envelope to acidify the core during virus entry (Chapter 23: Family *Orthomyxovirdae*). Fig. 2.7 shows micrographs of some enveloped viruses.

MATRIX PROTEINS

Matrix proteins are not conserved across virus families. The term matrix protein is used to describe a protein that forms layer on the inside of the viral envelope. Matrix proteins play important roles in virus assembly, as they form links or bridge between nucleocapsids/cores and the envelope. Retroviral matrix(MA) proteins are fatty-acylated, allowing them to interact tightly with cellular membranes to form budding sites. This interaction anchors the remainder of uncleaved retroviral GAG polyprotein to the membrane (Chapter 36: Family *Retroviridae*). The influenza virus matrix protein (M or M1) first makes contact with nucleocapsids in the nucleus of the infected cell and may participate in nucleocapsid assembly; M is required for transport of nuclecapsids from the nucleus. Additional M is found at the plasma membrane where it associates with the cytoplasmic domains of influenza virus envelope glycoproteins. Thus M organizes both glycoproteins and the nucleocapsids. The matrix (M) proteins of the paramyxoviruses

are highly basic proteins and paramyxovirus M is the most abundant protein in the virion. M associates with nucleocapsids and the plasma membrane (probably via interactions with cytosolic domains envelope glycoproteins) and is likely the driving force in budding.

NUCLEOCAPSID STRUCTURE

When discussing enveloped viruses, the term nucleocapsid is commonly used instead of capsid. The nucleocapsid refers to the assembly of protein and nucleic acid (the genome) that remains after the viral envelope is removed. In the case of the negative-strand RNA viruses, the main structural protein of the nucleocapsid is an RNA-binding nucleoprotein (N) but it also contains several molecules of the viral RNA-dependent RNA polymerase (Chapter 10: Introduction to RNA Viruses). Hepadnaviruses and retroviruses have icosahedral capsids surrounded by an envelope. These structures are often called "cores."

In this chapter we learned that:

- Capsids are protein coats that package viral genomes. Simple capsids are either rods (helical) or spheres. Spherical viruses have icosahedral symmetry. An icosahedron is a shell defined by its symmetry. An icosahedral shell has twofold, threefold, and fivefold axes of symmetry.
- Capsids are not static packages. They have important functions such as genome packaging, attachment, and entry. Structural flexibility of capsids allows them to carry out multiple functions in a highly regulated manner.
- Virions are either naked (unenveloped) or have a lipid envelope. Naked virions use capsid proteins to mediate attachment and entry.
- Enveloped viruses obtain their lipid membranes during budding. Viral envelopes may be derived from plasma membranes, nuclear membranes, or other internal membranes such as Golgi, ER, or transport vesicles.
- Enveloped viruses encode one or more envelope proteins with transmembrane-domain anchors. Envelope proteins often form spikes extending from the surface of the virion and most are glycosylated. Envelope proteins mediate attachment and fusion.
- The assembly of genome (nucleic acid) and capsid proteins found inside of the viral envelope is often called the nucleocapsid (particularly in the case of negative-strand RNA viruses). In the case of retroviruses, hepandaviruses, and more complex viruses, the structure is often called the core. Gently lysing the envelope will release nucleocapsids or cores. Viral polymerases may also be present in nucleocapsids/cores.
- Matrix proteins are found beneath the lipid membrane of enveloped viruses. They often function to drive virion assembly and budding.
- The group of proteins associated with the extracellular virus particle or virion are collectively called *structural proteins*. All viruses encode one or more structural proteins.
- The term *nonstructural protein* describes any virus proteins produced in the infected cell, but not packaged in the virion. Nonstructural proteins have many critical roles in the virus replication cycle.

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CHAPTER

3

Virus Interactions With the Cell

OUTLINE

After studying this chapter, you should be able to:

- List conditions that impact virus attachment.
- Explain how to set up a synchronized infection in the laboratory.
- Define "penetration" and "uncoating" as regards the virus replication cycle.
- Describe the cellular machinery that viruses use to move through the cell.
- Describe the general structure of a eukaryotic gene, including definitions of promoter, intron, and exon.
- Explain the differences between DNA-dependent DNA polymerases, RNA-dependent RNA polymerases (RdRp), and reverse transcriptase (RT).
- Explain why DNA virus replication is often linked to cell cycle.

This chapter examines the major steps in virus replication within the context of cellular structures and processes. The emphasis is on virus interactions with eukaryotic, primarily animal, cells. Recall that the major steps in virus replication are: (1) attachment, (2) penetration and uncoating, (3) synthesis of viral genomes and proteins, (4) assembly of new virions, (5) virion release and maturation.

VIRUS INTERACTIONS WITH THE CELL

The Extracellular Space

Most animal cells are embedded in an extracellular matrix that helps form the architecture of tissues and organs. Much of the extracellular matrix is proteinaceous in nature; however, in the case of epithelial cells, the plasma membrane (PM) is surrounded by a coat of polysaccharides called the glycocalyx. Thus there are molecules outside of the cell that can interact with infectious agents such as viruses, before they reach the plasma membrane (PM). For example, the glycocalyx can serve as a barrier, but some viruses use the glycocalyx to their advantage by binding to its polysaccharides.

The Plasma Membrane

The PM is both a barrier to, and required for, virus attachment and entry. The PM is a selectively permeable lipid bilayer that contains many proteins; it is a complex and dynamic structure. Small molecules such as carbon dioxide and oxygen cross the PM by diffusion. Sugars and amino acids cross the PM using protein channels or transporters. Larger molecules must be endocytosed in order to enter the cell. PM-associated proteins include receptors, signaling molecules, enzymes, and adherence proteins. As shown in Fig. 3.1, some proteins associated with the PM have cytoplasmic, membrane, and extracellular domains while others are embedded entirely with the bilayer. The proteins in the PM are mobile; they move laterally through the membrane, can reorganize and form complexes as the result of signaling. PM proteins are often glycosylated (carbohydrate chains are attached to the protein backbone). The lipids of the outer leaflet of the PM can also be glycosylated. Finally, the lipids within the PM not homogeneous; cholesterol can be found in discrete regions or microdomains called "rafts." Specific proteins are associated with these lipid rafts.

Attachment Occurs at the Plasma Membrane

The first step in the virus replication cycle is attachment to PM-associated molecules. The process requires the interactions of receptor molecules on the host cell with attachment proteins on the infecting virus. Receptors are often proteins, but viruses also bind to the sugar residues found on glycoproteins or glycolipids on the cell surface. Influenza virus is an example of a virus that attaches to carbohydrate receptors. Naked (unenveloped) virions use capsid proteins for attachment while enveloped viruses use an envelope-associated protein.

Attachment is achieved by interactions of small subdomains of molecules. Interaction faces usually comprise just a few amino acids or sugar residues, and the interactions are usually electrostatic in nature. Thus the initial contacts between virus and receptor are weak and reversible. However, as multiple viral attachment proteins interact with multiple receptor molecules, binding becomes strong and irreversible. Thus it follows that cells with a higher density or number of receptors are more readily infected (Fig. 3.2). Because attachment is electrostatic, it can be affected by pH, ion concentration, and types of ions in the extracellular space. When we propagate viruses in the laboratory, the type of media used, and its pH, are important. Attachment does not require energy and can take place in the cold (4°C). A virus infection can be synchronized by allowing attachment to occur in the cold. Once sufficient time has passed the culture is quickly warmed up and the attached virions penetrate at the same time.

The presence or absence of receptors is a major *host range* determinant, as absence of receptors excludes viruses from a cell. Different cells within an organism display different surface molecules, thus one cell or tissue type may be permissive for virus attachment while others are not. Epithelial cells are an example of polarized cells. They display different molecules on their apical (facing the lumen) and basolateral (facing the inside) surfaces (Fig. 3.3). Receptor molecules may be expressed on only one surface of the polarized cell, such that passage through those cells is a one-way process.

The receptors for many human and animal pathogens have been identified, but it is important to note that viruses adapted to cell cultures may use different receptors than those used during a natural infection. Even within an organism, a virus may utilize a variety of receptors. Attachment can also require interaction of

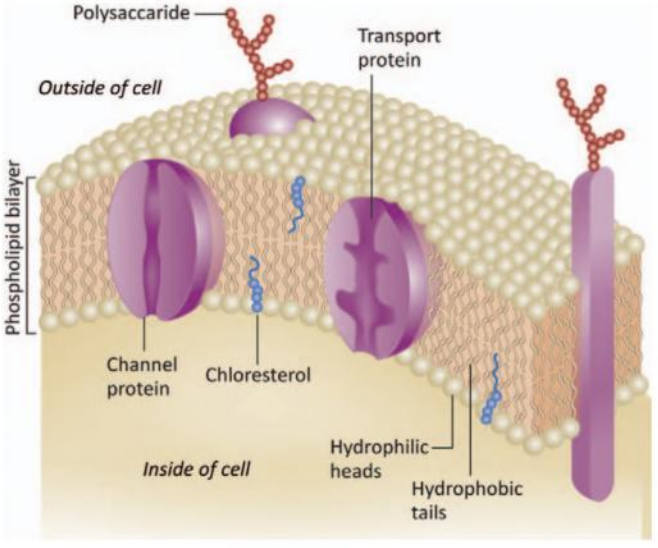

FIGURE 3.1 The PM is a selectively permeable lipid bilayer. Proteins associated with the PM include receptors, signaling molecules, enzymes, and adherence proteins.

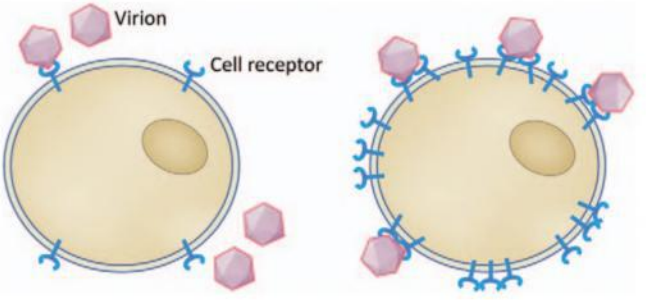

FIGURE 3.2 Receptor density and attachment. Cells with a higher density or number of receptors are more readily infected.

the virus with more than one type of receptor molecule. An example is human immunodeficiency virus (HIV). The surface unit (SU) glycoprotein of HIV (also called gp120) attaches to the CD4 protein present on helper T-lymphocytes, macrophages, and dendritic cells. After initial interactions between SU and CD4, SU then binds to a second receptor, one of several chemokine receptors on the cell surface. The chemokine receptors are the coreceptors for HIV (Chapter 37: Replication and Pathogenesis of Human Immunodeficiency Virus).

Virus Penetration and Uncoating at the Plasma Membrane

Once a virus attaches to a cell, the next critical step is delivery of the viral genome into the cytoplasm or nucleoplasm. Different viruses exploit different strategies to accomplish this. The RNA genome of a picornavirus crosses the PM through a channel or pore formed by capsid proteins (Fig. 3.4). In the case of the reoviruses, the whole capsid crosses a channel through the PM.

Enveloped viruses must transport their genomes across two sets of membranes, both the viral envelope and a cell membrane. The process sometimes occurs at the PM (Fig. 3.5). Unfavorable energetic barriers must be overcome to bring the two membranes into close enough proximity to allow formation of a pore (Box 3.1). If fusion occurs at the PM, viral envelope proteins remain on the cell surface.

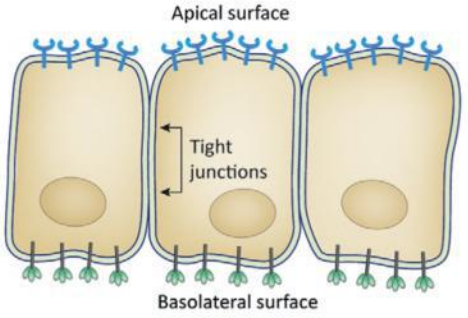

FIGURE 3.3 Polarized cells have discrete apical and basolateral surfaces. Apical surfaces face the outside (for example, the airspace in the lung or the lumen of the intestine) while basolateral surfaces face the inside of the body. Polarized cells display distinct proteins on their apical versus basolateral sides. Materials, such as viruses, can be transported directionally through polarized cells.

Virus Penetration and Uncoating From Membrane Bound Vesicles

Many viruses exploit cell processes designed to bring cargo into cells via membrane bound vesicles. These processes include phagocytosis, macropinocytosis, and receptor-mediated endocytosis. There are different mechanisms by which endocytosis can occur, such as clatherin-dependent or caveolin-1-dependent pathways (Fig. 3.6). The environment inside of endosomes is distinct from the cell cytosol. Of importance to our discussion of viruses, endosomes become acidified as they mature and low pH often triggers penetration. As mentioned above, some picornaviruses form membrane channels at the PM upon attachment. However other picornaviruses are endocytosed, and low pH triggers the capsid rearrangements that form membrane channels. Enveloped viruses often use low pH to trigger rearrangement of their surface proteins, with the effect of releasing previously hidden hydrophobic (fusion) domains.

Membrane Fusion

Membrane fusion results in lipid mixing or formation of a pore through both the viral envelope and a host cell membrane (Fig. 3.7). Key to the process, the viral envelope must be in very close proximity to the

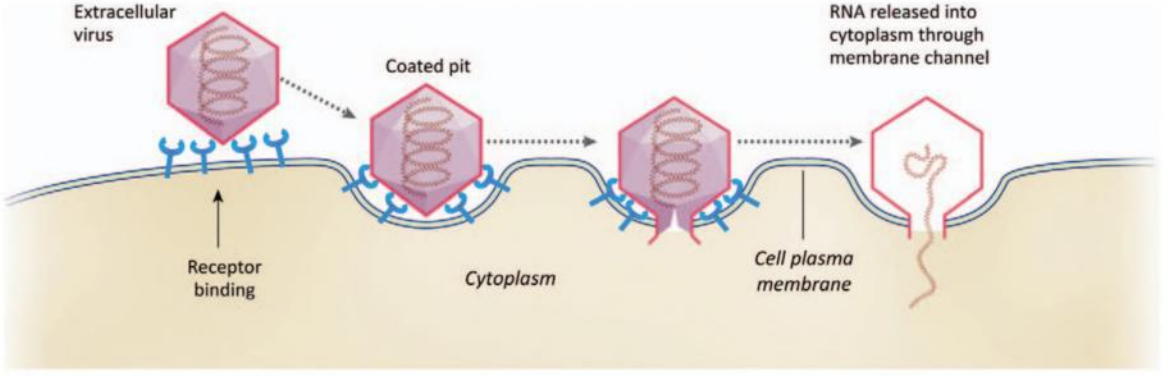

FIGURE 3.4 Illustration depicting the penetration of a viral genome across the PM. In this example the RNA genome of a picornavirus crosses the PM through a channel or pore formed by capsid proteins.

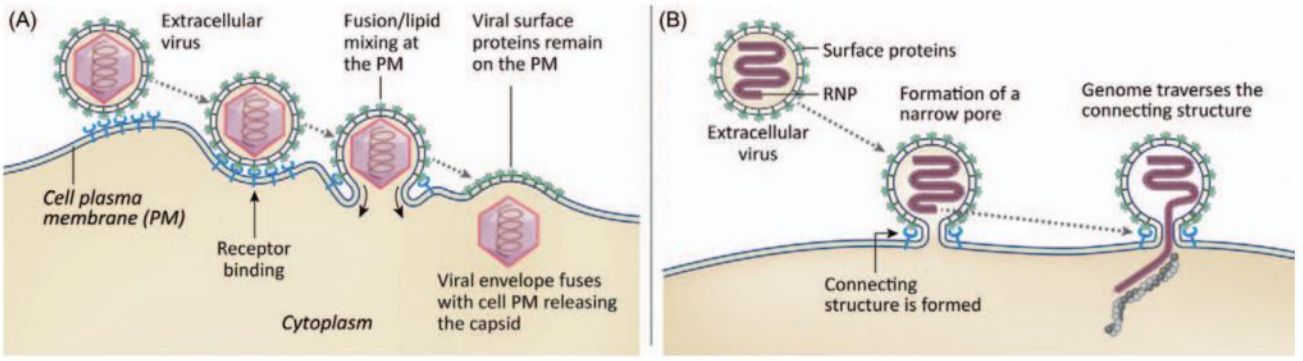

FIGURE 3.5 Illustration depicting the penetration of a viral genome after fusion of an enveloped virus with the PM. Panel A shows a process in which the viral envelope and PM become contiguous. Panel B shows a process in which a small pore is formed through the PM.

BOX 3.1

FUSION PROTEINS

Fusion of a viral membrane to a cellular membrane requires that the high kinetic barrier to membrane fusion be breached. Fusion proteins serve as the catalysts in this process. A hallmark of fusion proteins is that they undergo structural changes as a result of attachment and/or changes in pH (during endocytosis). Often these structural changes expose a hydrophobic segment called the fusion loop or fusion peptide whose function is to engage the target cell membrane. The fusion protein becomes a bridge between the two membranes, drawing them together (see Fig. 36.3). Viral fusion proteins are suicide enzymes that function only once.

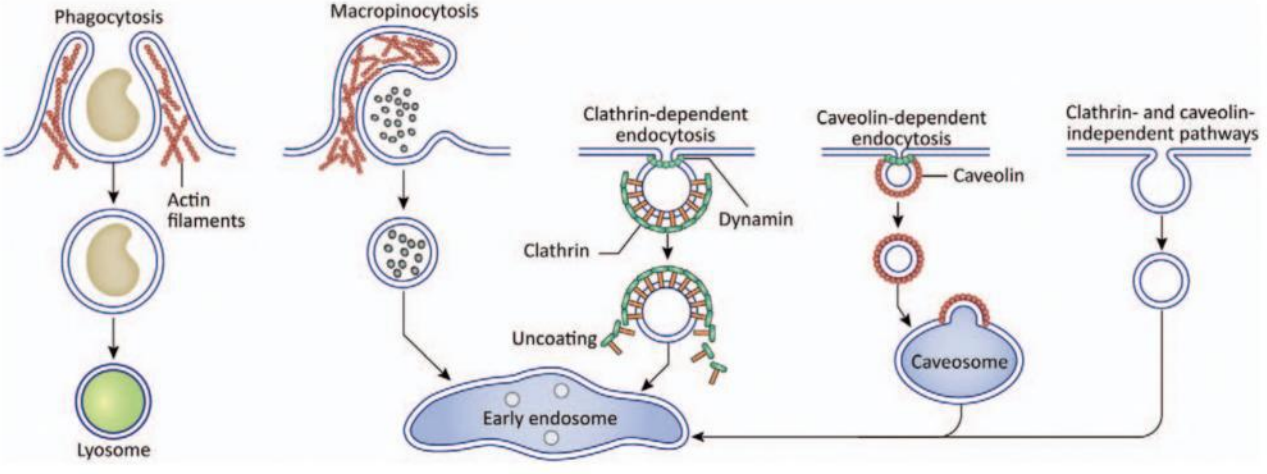

FIGURE 3.6 Cellular uptake of macromolecules by phagocytosis, macropinocytosis, and various endocytic pathways.

target cell membrane. Viruses use attachment and fusion proteins to accomplish this. To initiate fusion, hydrophobic portions of a viral protein are inserted into the target cell membrane. This triggers protein rearrangements that pull the two membranes into close proximity (within a few Å). Some viruses use discrete domains of a single protein to accomplish attachment and fusion. Influenza viruses have attachment and hydrophobic fusion domains in different regions of a single molecule, the hemagglutinin (HA) protein. Other viruses (for example, the paramyxoviruses) encode distinct attachment and fusion proteins. After fusion the viral nucleocapsids or cores are released into the cytosol or nucleoplasm (Fig. 3.8).

Uncoating the Genome

Uncoating is the removal of viral proteins from the genome to allow for translation, transcription, and replication. For some viruses the processes of penetration

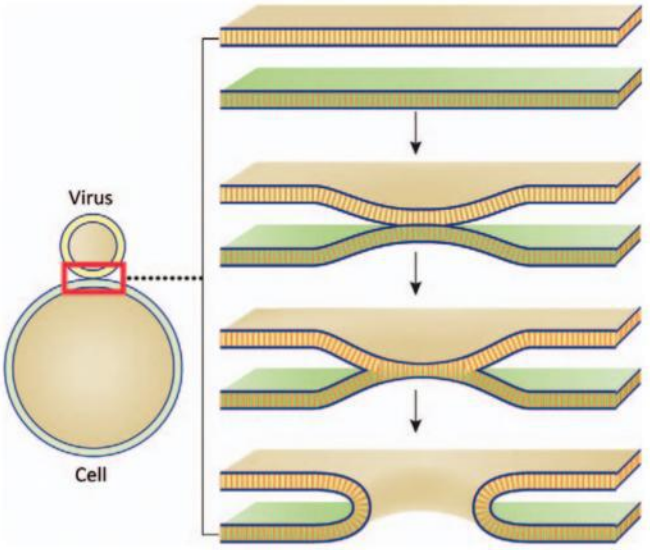

FIGURE 3.7 General illustration of the membrane fusion process. Membrane fusion or pore formation require membranes to be in very close proximity.

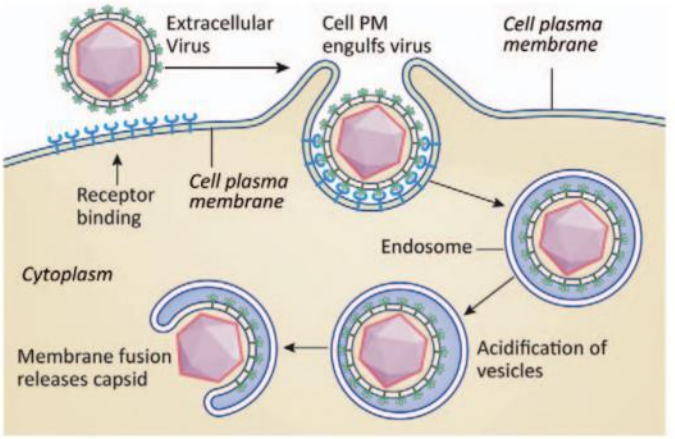

FIGURE 3.8 Many viruses, both enveloped and unenveloped, are brought into cells by endocytosis. The low pH environment in the endosome triggers molecular rearrangements of capsid or envelope proteins. In this example an enveloped virus is fusing with an endosomal membrane to release the capsid into the cytosol.

BOX 3.2

CELL CYTOSKELETON

The cytoskeleton is a dynamic and interconnected network of filaments. There are three major types of filaments. Actin filaments control cell shape, locomotion, and cytokinesis (separation of daughter cells after cell division). Intermediate filaments provide mechanical strength. Microtubules function in intracellular transport and chromosome segregation. Microtubules also control the position of organelles. The cytoskeleton is not static. It is constantly reorganizing via the assembly and disassembly of filaments. Regulation of the cytoskeleton is quite complex, requiring many molecular players. Molecular motors are key to moving materials along filaments. Members of the myosin superfamily of proteins are actin-based motors. For example, myosins facilitate movement of cargo along tracks of actin filaments and also move actin filaments relative to the PM.

and uncoating cannot be separated, but in other cases, uncoating requires additional steps. Some viral genomes are uncoated within the cytosol while in other cases uncoating occurs in the nucleus. Reoviruses are an interesting exception, their genomes are never uncoated but instead are transcribed within the capsid; mRNAs are released to the cytosol through pores in the capsid (Chapter 26: Family *Reoviridae*).

CYTOSKELETON

Movement Through the Cell, Interactions With the Cytoskeleton

The cytosol is a dense network of filaments, organelles, and molecular assemblies (see Box 3.2). It is a highly viscous environment that restricts diffusion of molecules larger than 500 kDa or particles larger than 20 nm. (Recall that most viruses are larger than 20 nm.) Cells have evolved highly regulated processes to move and sort cargo in order to maintain the highly organized and complex environment of the cell. These processes can potentially inhibit virus movement and replication but are often exploited by viruses to enhance their replication. For example, viruses use actin filaments and microtubules to move through the cell as well as from cell to cell. To aid in their movement, viruses highjack the cellular molecular motors associated with actin filaments and microtubules. In fact specific interactions with the cell cytoskeleton are important in every step of the virus replication cycle, from attachment to replication to assembly to release.

VIRUS ASSEMBLY

Viruses assemble in the infected cell when the local concentrations of structural polypeptides and genomes are sufficiently high. Sometimes assembly takes place in discrete regions of the cell called "virus factories" containing high concentrations of capsid proteins and genomes. Cytoskeletal components and cellular organelles are often intimately involved in both virus assembly and release, although some very simple viruses can be assembled (albeit inefficiently) in the test tube.

There are two general ways to build a simple icosahedral capsid. In one scenario, capsid proteins form an empty shell *into which* the genome is inserted. Picornaviruses use this strategy and empty capsids can be seen in virus preparations. Empty particles are less dense than complete virions and can be separated from them by density centrifugation. In the second scenario, an encapsidation signal on the genome interacts with one or more capsid proteins, followed by recruitment of additional capsid proteins.

Most animal viruses with helical nucleocapsids are RNA viruses. They usually encode a basic protein (often called the nucleocapsid (N) protein) that interacts with viral RNA. Initial interactions are specific such that cellular RNAs are not packaged indiscriminately. Genome packaging signals often include sequences at both the 5′ and 3′ ends of the viral genomic RNA. This is a mechanism whereby genomic RNA can be "distinguished" from subgenomic mRNAs. In some cases the N protein surrounds the viral RNA, but in others (i.e., influenza viruses) it appears that the RNA winds around a protein core.

VIRION RELEASE

Mechanisms for virus release from cells include cell death (lysis), budding, and exocytosis. The cytoskeleton can present a barrier to release and some unenveloped viruses encode proteins that disrupt the cytoskeleton to allow dispersal of newly assembled virions. Enveloped viruses obtain their envelope by a budding process. A viral nucleocapsid interacts with a region of host cell membrane into which glycosylated viral envelope proteins have been inserted. The nucleocapsid "finds" the proper place to bud from the cell by forming specific interactions with the cytoplasmic tail(s) of the envelope proteins. Cell membranes that can serve as sites of budding include the PM, endosomal, and nuclear membranes. Viruses released by budding from the PM (for example, the HIV) are released individually (Fig. 3.9). Viruses with envelopes derived from endosomal or nuclear membranes may bud into vesicles that traffic to the PM and fuse, releasing their cargo of virions by a process called exocytosis (Fig. 3.10).

It is easy to assume that release always results in free virions escaping into the extracellular environment. And while this certainly does happen, virions can also be transmitted directly from one cell to another. Vaccinia virus (family *Poxviridae*) uses actin tails to move between cells. We can visualize this process using

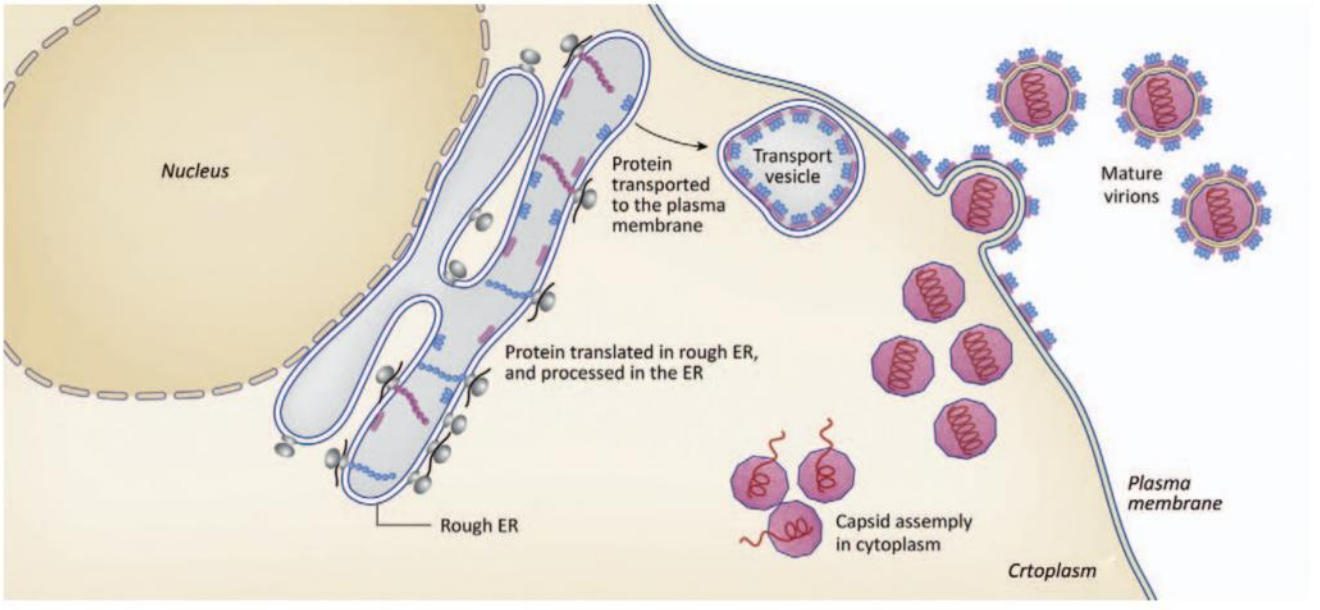

FIGURE 3.9 Illustration showing the process of assembly and budding of a virus particle from the PM.

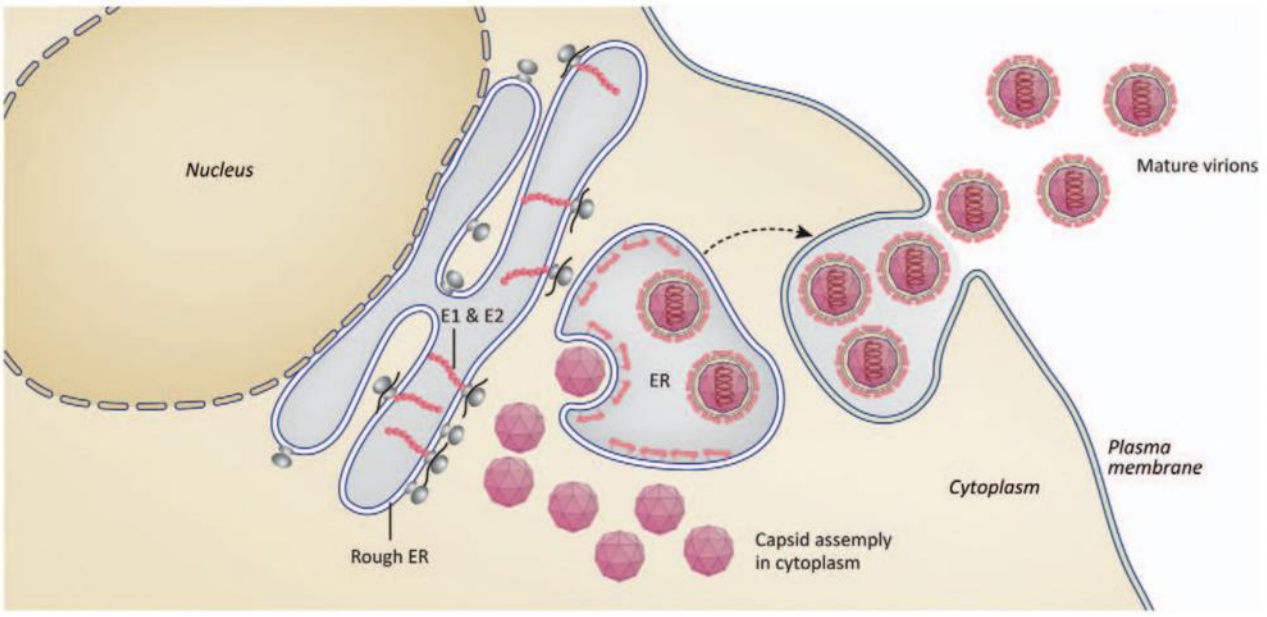

FIGURE 3.10 Illustration showing release of enveloped virions by exocytosis.

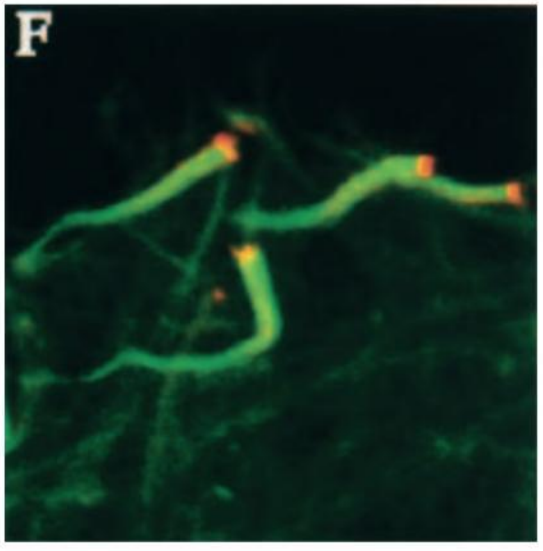

FIGURE 3.11 Actin projections (green) extending from a vaccinia virus infected cell. There is virus particle (red) at the tip of each projection. *From Figure 6 (panel F) of Cudmore, S., Reckmann, I., Griffiths, G., Way, M. 1996. Vaccinia virus: a model system for actin-membrane interactions. J. Cell Sci. 109, 1739–1747.*

cells with fluorescent-tagged actin and virions tagged with a different fluorescent tag (Fig. 3.11).

VIRION MATURATION

Maturation cleavages of virion proteins may occur after particle release, and these may be required to produce infectious virus (Fig. 3.9). Why do some viruses mature after release from a cell? The virion has two very different roles in the replication cycle: To assemble under conditions of favorable energy and to disassemble during the processes of attachment and penetration into a new cell. Maturation cleavages prepare the newly released virion to attach to a new cell and disassemble. For example, picornaviruses include in their capsids, an uncleaved precursor protein. The precursor is cleaved within the assembled capsid. If this cleavage is blocked, the capsid is unable to deliver the genome to a new cell. Retroviruses require a set of cleavages that convert long precursor polyproteins into individual structural proteins. The cleavages are made by a retroviral protease (Chapter 36: Family *Retroviridae*) and inhibitors of HIV protease activity are powerful antiviral drugs. Protease inhibitors are small molecules that interact with the HIV protease to prevent it from cleaving the polyproteins in the immature particle. For many enveloped viruses, maturation involves cleavage of glycoproteins that fuse the viral envelope to a host cell membrane.

AMPLIFICATION OF VIRAL PROTEINS AND NUCLEIC ACIDS IN THE CONTEXT OF THE INFECTED CELL

After penetration and uncoating have been achieved, the next events in the viral replication cycle are the synthesis (amplification) of viral proteins and genomes. In the absence of successful amplification, the proteins and genomes needed to assemble new virions are not made. To better appreciate the processes

used by viruses to synthesize mRNAs, genomes, and proteins let us review some of the most basic aspects of these processes in the host cell.

A Short Review of Transcription in the Eukaryotic Host Cell

Synthesis of cellular mRNAs (transcription) occurs in the nucleus of the eukaryotic cell. The enzyme that synthesizes mRNAs is RNA polymerase II (RNA polII). RNA polII is a DNA-dependent RNA polymerase (an enzyme that uses DNA as the template for synthesis of an RNA product). RNA polII forms protein complexes with other cellular proteins in order to be directed to specific genes. The regions of a eukaryotic gene that interact with the transcription complex are the promoter/enhancer sequences (Fig. 3.12). These can be quite long and complex as they allow graded cell responses to a variety of stimuli. The promoter/enhancer region of a gene defines the conditions under which a gene product will be synthesized. The promoter-enhancer sequences themselves are not transcribed.

Shortly after initiation of a transcript, the RNA is modified by addition of a 5′-methyl guanosine "cap." The cap protects the 5′ end of the mRNA from degradation and also serves as the assembly site of the ribosome. Most eukaryotic genes have long regions, called introns that are not found in the mature mRNA. Instead, they are removed by a process called *RNA splicing*. Splicing is accomplished by an assemblies of small RNAs and proteins called the spliceosomes. Recall that the regions of the mRNA that are not removed (the protein coding regions) are called exons.

An additional modification to the eukaryotic transcript is addition of nontemplated adenosines near the 3′ end, to produce the poly(A) tail. A short RNA sequence defines the polyA addition site. The transcript is cleaved at polyA addition site, followed by addition of the polyA tail (by an enzyme called polyA polymerase). The polyA tail controls degradation of the mRNA from the 3′ end and is also involved in initiating translation. The mature mRNA is now ready to be transported from the nucleus. In summary (Fig. 3.12):

- Many eukaryotic genes have nontranscribed promoter/enhancer sequences.
- The 5′ end of the mRNA is capped.
- Many eukaryotic genes have introns that are removed from RNA by splicing.
- The 3′ ends of mRNAs are cleaved and polyadenylated.

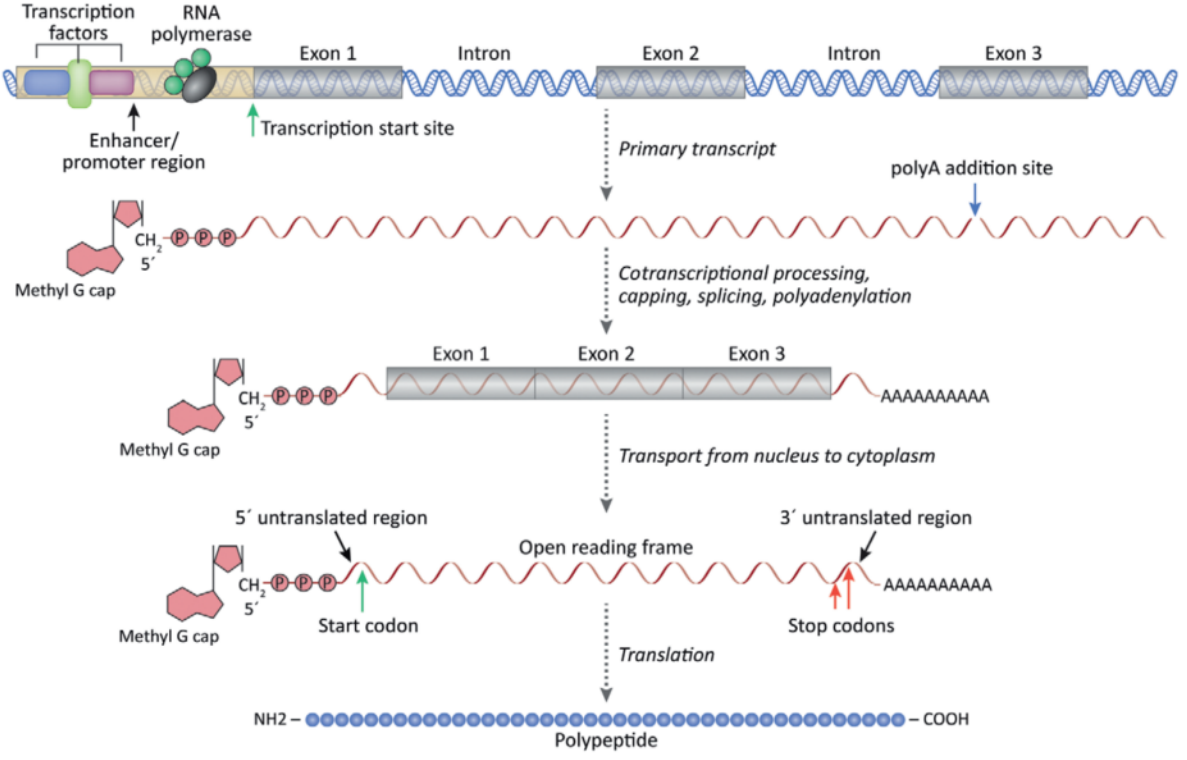

FIGURE 3.12 Organization of a eukaryotic gene showing a promoter region followed by introns and exons. The primary transcript is produced, followed by splicing to remove introns. After export to the cytoplasm, the capped and polyadenylated transcript is translated to produce the protein product of the gene.

- Capped, polyadenylated, spliced mRNAs are transported out of the nucleus.
- The open reading frame or coding region of the mRNA is usually flanked by 5′ and 3′ nontranslated regions.
- For the most part, one mRNA encodes one protein.

Transcription of Viral mRNAs

Do viruses follow the rules of gene organization, transcription and RNA processing used by the eukaryotic cell? Some do, but many do not. As we will see throughout this text, viruses use a variety of unique strategies to control synthesis of their mRNAs, as the abundance of an mRNA can directly impact the amount of protein product produced. Why have some viruses adopted unique strategies?

- The coding capacity of many viruses is small because their genomes are small (compared to the host cell genome), thus they cannot have long complex promoter/enhancer regions and/or long nontranscribed introns.
- Most RNA viruses transcribe their mRNAs in the cell cytoplasm, therefore have no access to spliceosomes or cellular RNA polII.
- Many viruses inhibit host cell transcription and/or translation, therefore must use alternatives to "normal" cellular processes to produce viral proteins.
- An infecting virus usually brings one copy of its genome into a very crowded cell. Viral mRNA synthesis and protein synthesis must be very efficient in order to compete for building materials.

Thus the organization and expression strategies of viral genes may differ from host genes:

- Many viral genes have no introns.
- Some viral mRNAs do not have 5′ caps.
- Some viral mRNAs do not have poly(A) tails.
- Some viral mRNAs have overlapping open reading frames such that more than one type of protein can be produced from a single transcript.
- Viral transcripts with introns can be *alternatively* spliced to generate multiple, different mRNAs. (Obviously these must be viruses that are replicating in cell nucleus where the splicing machinery is present.)
- Some viral mRNAs are not exact copies of the genome. In a process called *RNA editing* or *pseudotemplated transcription*, the polymerases of a few RNA viruses add nucleotides not present in the genome sequence. The term pseudotemplated suggests that there are some specific signals in the genome that instruct the addition of these extra residues.

A Short Review of Translation in the Eukaryotic Host Cell

Let us review a few basics of translation in the cell, focusing on initial interactions of the translation apparatus with the mRNA. Both the 5′ cap and the 3′ poly(A) tail are involved in translation initiation. The poly(A) tail is bound by polyA-binding protein (PAPB). A complex of initiation proteins (eIF4F complex) binds to the 5′ cap (Fig. 3.13) and interacts with PAPB. This is followed by association with a preinitiation complex that includes the 40S ribosomal subunit, $tRNA^{met}$, and other initiation factors. The preinitiation complex moves or scans along the untranslated region of the mRNA until it encounters an AUG codon with the appropriate surrounding sequences (Kozac sequence). Now the 60S subunit binds at the AUG codon to generate the 80S initiation complex with $tRNA^{met}$ in the A site. A second tRNA enters the P site and a peptide bond is formed. The ribosome moves along the mRNA (translocates) one codon (3 nt) at a time. At the end of an open reading frame, a ribosome will encounter stop codons that trigger termination. In eukaryotes transcription termination is facilitated by two release factors (eRF1 and eRF3). eRF1 recognizes stop codons (UAA, UGA, or UAG) in the A site.

Translation of Viral Proteins

We noted above that most eukaryotic mRNAs code for a single protein. But one hallmark of viruses is their ability to efficiently exploit a small genome. So it is not uncommon for individual viral mRNAs to encode different versions of a protein, or two or more completely different proteins. Common strategies are illustrated in Fig. 3.14 and include:

- Use of alternative start codons, a process often called leaky scanning.
- Suppression of stop codons to produce a longer protein product.
- Frame-shifting moves a ribosome to another reading frame to produce a longer protein product.
- Termination-reinitiation or stop-start.

These mechanisms all function at the level of the ribosome and serve to control relative amounts of protein products. For example, use of alternate start codons will result in production of a greater amount of the protein with the best Kozak sequence and lesser amounts of proteins that initiate at alternative start codons. In the case of ribosomal frame-shifting, folding

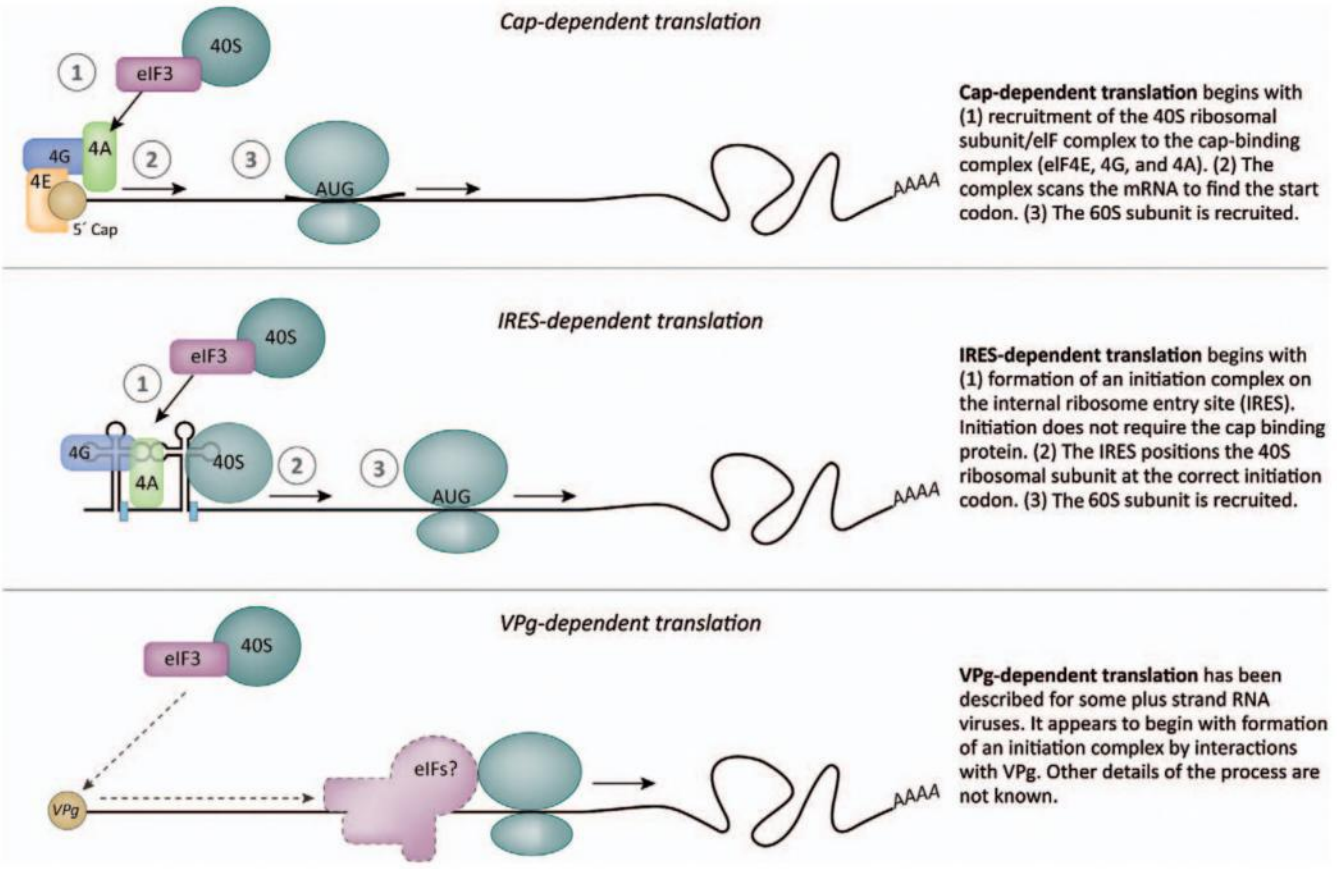

FIGURE 3.13 Methods of translation initiation. Most cellular transcripts have a 5′ cap and a 3′ poly(A) sequence that are key players in ribosome assembly. Many types of viruses adhere to this host cell strategy. However, several positive strand RNA viruses use cap-independent translation. Picornaviruses and some flaviviruses use an RNA structure called the internal ribosome entry site (IRES) to direct ribosome assembly. Another strategy is ribosome assembly directed by a viral protein (VpG) covalently linked to an RNA transcript (see Ch. 12: Family Caliciviridae).

of the mRNA into structures called pseudoknots modifies the translation process (Fig. 3.15) (See Box 3.3).

Many RNA viruses produce polyproteins that are cleaved by viral proteases to generate smaller, functional proteins (Fig. 3.16). The process is exemplified by picornaviruses. The picornaviral genome contains a single, long open reading frame that is translated to produce a large precursor polyprotein. Protease domains within the polyprotein are enzymatically active immediately after being translated and work to quickly cleave the large precursor into mature products. The proteolytic cleavages occur in an ordered sequence. Intermediate cleavage products may have activities distinct from those of the final cleavage products.

As viruses must use the host cell translational apparatus, their viral mRNAs must complete with cellular mRNAs for ribosomes and amino acids. One way to compete is simply to inhibit synthesis of host cell transcription and/or translation. (This also limits the ability of the cell to respond to infection.) Another strategy is to efficiently compete for the translational machinery. Some viruses use a combination of both methods. But how can a virus inhibit translation of host proteins without effecting viral protein synthesis? An example is provided in Box 3.4.

Synthesis of Viral Genomes

As noted in Chapter 1, Introduction to Animal Viruses, viruses can be placed into one of three major groups based on genome type/genome replication strategy. The groups are (1) DNA viruses, (2) RNA viruses, and (3) viruses that use RT.

There are many families of DNA viruses. The genomes of DNA viruses range from 3000 nt to well over 1 million base pairs. Some DNA viruses have circular genomes, others linear. But all DNA viruses use a DNA-dependent DNA polymerase to synthesize additional genome copies. Some DNA viruses use a cellular DNA polymerase but others encode their own DNA polymerases for genome synthesis. There is also obviously a need for a sufficient pool of dNTPs. Eukaryotic DNA synthesis is a highly regulated process. Some

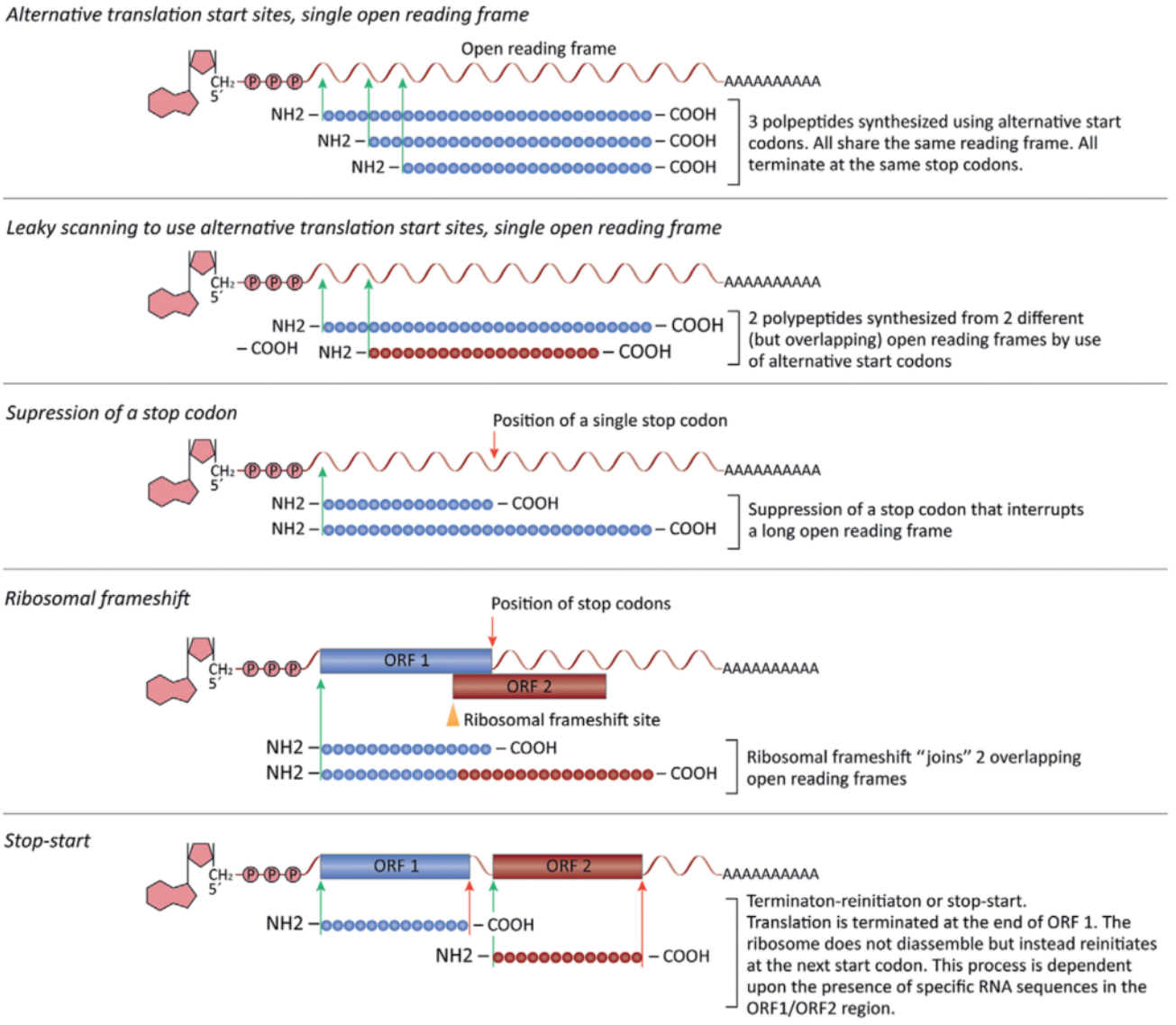

FIGURE 3.14 Viruses use a number of strategies to produce more than one protein from a transcript.

types of animal cells divide regularly (for example, epithelial cells) but others are quiescent, seldom dividing except in response to damage (for example, hepatocytes (liver cells)). Nondividing cells have limited DNA replication machinery and very limited dNTP pools. Strict regulation of cell division causes a problem for some DNA viruses. Some can only replicate in mitotically active cells. But others can stimulate quiescent cells to divide (see Chapter 28: Introduction to DNA Viruses). Some large DNA viruses encode enzymes required for dNTP synthesis, thereby increasing the cellular pools of these building blocks in nondividing cells. Animal DNA viruses (with the exception of the poxviruses) replicate their genomes in the nucleus.

RNA viruses were originally defined as viruses with an RNA genome packaged within the virion. But more critical to the definition is that the RNA genome found in the virion is used as the template for the synthesis of additional RNA genomes. Animal RNA viruses encode an RdRp for genome synthesis. The RdRp is also called the "replicase." The RdRp is used both for genome synthesis and transcription. The RNA viruses never use a host-encoded RNA polymerase to replicate their genomes. As all living cells continuously produce RNAs for "housekeeping" purposes, the synthesis of viral RNA genomes can occur in dividing or nondividing cells.

Members of the families *Retroviridae* and *Hepadnaviridae* use a polymerase called reverse transcriptase (RT) to synthesize their genomes. Retroviruses package an RNA genome while hepadnaviruses package a DNA genome. Both virus families use the enzyme RT to make a DNA copy of an RNA molecule. The RNA genome of a reverse transcribing

virus is an mRNA, transcribed from the viral DNA genome by host cell RNA polII. (In the case of retroviruses the DNA form of the viral genome is integrated into the cell DNA)

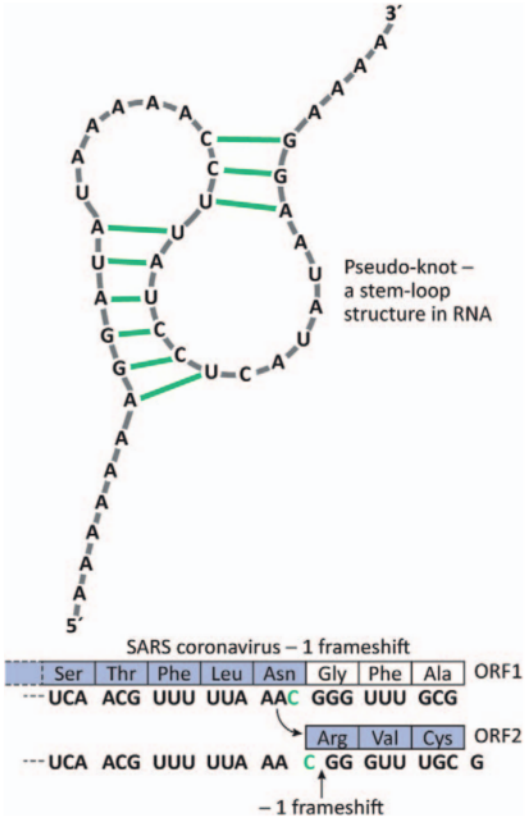

FIGURE 3.15 An RNA pseudo-knot directs ribosomal frame shifting.

In this chapter we have learned that:

- Virus attachment requires specific host receptors and the process is influenced by receptor density, pH, and ions.
- Attachment does not require energy, thus can occur in the cold. Adding virions to cells in the cold is a way to synchronize a viral infection. Penetration is an energy-requiring step that occurs only after the culture has been warmed to physiologic temperature (37°C for most animal cells).
- In order to penetrate into the host cell, a virion or genome must cross a lipid membrane. This can occur at the PM or after endocytosis.
- Uncoating is a process that can occur during or after penetration. Uncoating provides the viral genome access to cytoplasm or nucleoplasm.
- Enveloped viruses use fusion proteins to overcome the kinetic barriers to membrane fusion. Fusion proteins are suicide enzymes that catalyze membrane fusion.
- The cell has a complex and dynamic cytoskeletal system that is often highjacked by viruses.
- Eukaryotic genes are large. They are preceded by untranscribed promoter regions and most have large introns. RNA polII transcribes cellular mRNAs. Cellular mRNAs are highly processed in the nucleus, undergoing capping, splicing (removal of introns), and polyadenylation.
- Viruses maximize use of small genomes by minimizing the size of promoters and encoding multiple proteins from a single mRNA.
  Some viruses make extensive use of alternative splicing. Some viruses modulate the process of translation by inducing ribosomal frame-shifting and suppression of stop codons.

BOX 3.3

RIBOSOMAL FRAME-SHIFTING AND STOP CODON SUPPRESSION

Ribosomal frame-shifting is common among retroviruses and coronaviruses. It is a process whereby the ribosome moves from one reading frame (protein 1) to a second reading frame (protein 2). Ribosomes frame-shift when they stall during protein synthesis. They stall at specific sites on viral mRNAs called "pseudoknots." The pseudoknot forms as a result of base pairing within the mRNA. In most cases the pseudoknot causes frame-shifting about 20% of the time. A surprisingly efficient process!

Suppression of a stop codon is a process whereby a ribosome fails to terminate protein synthesis at a stop codon. Most eukaryotic genes terminate with multiple stop codons, but if there is a single stop codon, an amino acid can be inserted into the growing polypeptide and translation continues. This is estimated to occur as often as 20% of the time. Thus a protein coding sequence downstream of a pseudoknot or a single stop codon will be synthesized at a lower quantity (~20%) than the protein encoded when the signal is ignored (~80% of the time).

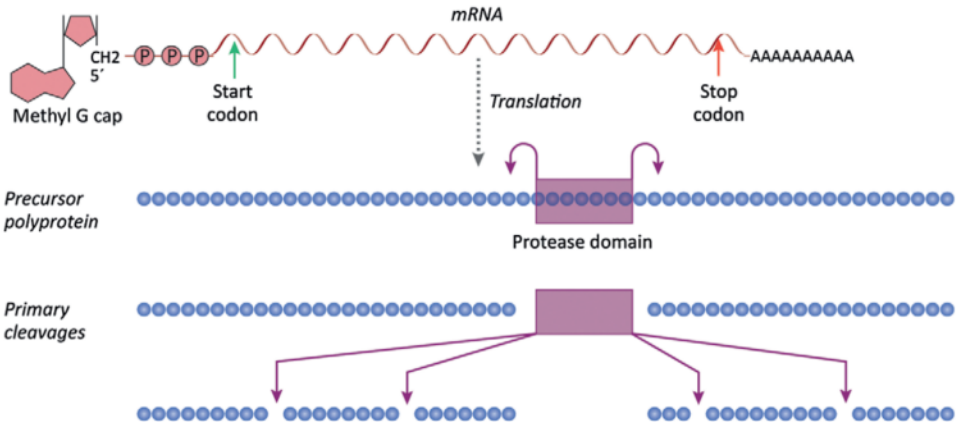

FIGURE 3.16 Proteolytic cleavage of a viral polyprotein.

BOX 3.4

POLIOVIRUS (PV) AND CAP-INDEPENDENT TRANSLATION

PV inhibits cap-dependent translation in the infected host cell. It does so by cleaving the eukaryotic initiation factor 4G (eIF-4G). A function of eIF-4G is to interact with both the 5′-cap on the mRNA and the 40S ribosomal subunit, thereby bringing mRNA and ribosome together. A protease encoded by PV cuts eIF-4G into two pieces. One piece binds to eIF-3 on the 40S subunit and the other piece binds to the cap-binding protein eIF-4E. As the two business ends of eIF-4G are now separate polypeptides, the 40S subunit cannot bind the cap. PV RNA is not capped so the cleavage does not interfere with viral protein synthesis. PV uses a cap-independent mechanism for ribosome assembly onto its mRNA.

- DNA viruses use cellular or virally encoded DNA-dependent DNA polymerases for genome replication. Most DNA viruses require that the cellular DNA synthesis machinery be active to provide adequate dNTP pools.
- RNA viruses use virally encoded RdRp to transcribe mRNAs and to replicate genomes. RNA viruses can replicate in dividing or nondividing cells.

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CHAPTER

4

Methods to Study Viruses

OUTLINE

This chapter describes methods for growing, purifying, counting, and characterizing viruses. It also presents general principles of diagnostic virology. After studying this chapter, you should be able to:

- Describe general requirements for culturing cells and tissues.
- Describe differences between cultures of primary and transformed cells.
- Describe how centrifugation is used to purify viruses.
- Understand the types of information provided by negative staining electron microscopy (EM), thin sectioning EM, cryo-EM, and confocal microscopy.
- Understand what is being measured by each of the following techniques: plaque assays, PCR, ELISA, hemagglutination, and hemagglutination inhibition assays.

GROWING VIRUSES

Viruses replicate only within living cells, thus many early studies of viruses were done in bacteria or plants. Tobacco mosaic virus (TMV) was an early "model virus" as it replicates in a variety of plants, at levels sufficient for biochemical analysis and imaging. Growing TMV is as simple as applying virus to abraded leaves of a susceptible plant. The earliest studies of animal viruses were limited to using whole animals. When possible animal pathogens were adapted to small animals such as mice, rats, and rabbits. These small animal models provided a means to study viral pathogenesis and vaccine efficacy. Fertile chicken and duck eggs were, and continue to be, widely used for propagating viruses. In the 1940s and 1950s development of robust cell culture techniques revolutionized the study of animal viruses. Today, most animal viruses are grown in cultured cells.

Generating Cell Cultures

The following steps describe an overall strategy for generating primary cell cultures. It is of utmost importance that all work is done under sterile conditions (Fig. 4.1):

1. The desired tissue is removed from the animal and is chopped or minced.

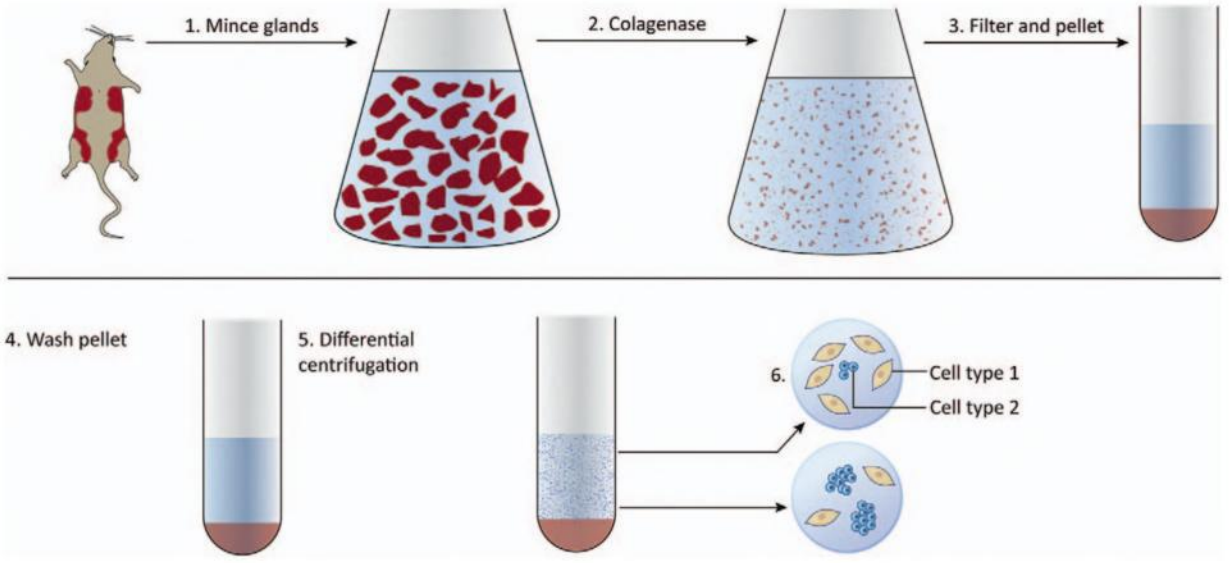

FIGURE 4.1 Generating cell cultures begins with removing tissues (normal or tumor) from an animal. Tissues are minced and treated with enzymes to degrade the extracellular matrix. Centrifugation is used to pellet the cells. Cells are resuspended in media and placed in culture vessels.

2. Tissue fragments are treated with enzymes such as collagenase to degrade the extracellular matrix and release single cells and small aggregates of cells.
3. Cells are pelleted by centrifugation and are resuspended in buffered saline or cell culture media.
4. Additional centrifugation steps may be performed to separate single cells from cell aggregates.
5. Cells and growth media are added to culture dishes and are maintained in a humidified incubator (37°C, 5% CO_2).
6. Cells attach to the bottom of the dish where they grow and divide to form a monolayer.
7. The cells can be removed with trypsin, washed, and divided among new culture plates or dishes. This is called a passage, and is done to increase cell number.

Primary cells can be propagated for only a limited number of passages before the cells undergo a crisis and the culture dies. Embryonic cells can be passaged many more times than cells taken from adults. Some types of cells (for example, fibroblasts) divide more readily than do cells that are normally nondividing in the adult animal (for example, neurons). Tumors provide another source of cells for virus culture. Tumor-derived cells can often be passaged indefinitely. These immortalized cells are excellent tools for the virologist. They are relatively easy to culture, many types are commercially available and they can be genetically modified. Multiple genes can be introduced, mutated, or deleted to generate an unlimited supply of "designer" cells.

PURIFYING VIRUSES

Viruses grown in cultured cells can be purified, quantitated, imaged, and biochemically analyzed. The higher the initial virus concentration the easier it is to purify virus away from cell debris and media components. If a virus is cytopathic it is present amongst the cell debris and media from the dish or plate. In contrast, if the virus is highly cell-associated, the cells must be gently lysed to release the virus. Mixtures of virus and cell debris are subjected to low-speed ($\sim 5000 \times g$) centrifugation to pellet cell debris, but not the much smaller virions. At the end of the low-speed centrifugation, the liquid supernatant, containing the virus, is saved and the pellet (containing the cell debris) is discarded. Separating larger from smaller molecules is called differential centrifugation (Fig. 4.2).

The virus-containing supernatant can then be recentrifuging at a much higher speed ($\sim 30{,}000-100{,}000 \times g$) to pellet the virus. After the centrifugation is completed, the supernatant is discarded and the virus pellet is saved. If desired, the virus can be further purified by centrifugation through a density gradient. Sucrose and glycerol gradients are commonly used; a sucrose gradient might range from 40% sucrose at the bottom of the tube to 5% sucrose at the top of the tube. The gradient is prepared and the virus sample layered carefully onto the top. During centrifugation, the components of the sample will separate into layers depending on their buoyant density. By using a gradient, one can achieve a finer separation of the different macromolecules within a sample. If sufficient virus is present in the sample, a

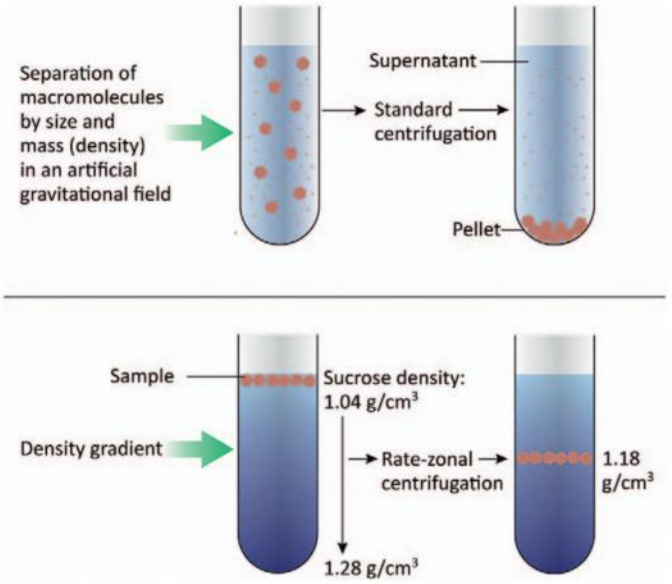

FIGURE 4.2 Centrifugation is a common technique used to purify viruses away from cell debris and other small molecules. Low-speed centrifugation ($\sim 5000 \times g$) is used to remove cells and large cell debris, leaving virions in the supernatant. Virus can be further purified and concentrated by centrifugation at higher speeds. A gradient of sucrose or glycerol can be used to achieve further purification as materials in the sample will separate into layers depending on their buoyant density.

visible band forms, and can be carefully removed from the tube.

VISUALIZING VIRUSES

Optical Microscopy

Light Microscopes

Light microscopes use visible light (400–700 nm wavelengths) to image objects. They are seldom used to visualize viruses, as they lack sufficient magnifying or resolving power to do so. However, the largest known viruses can be seen with a light microscope. Examples of these very large viruses include Mimivirus, Pithovirus, Megavirus, and Pandoravirus, all of which infect amoebas.

Even though many viruses are far too small to be seen with the light microscope one can often observe virally induced changes to infected cells. A classic example is the Negri body, which was once used as a diagnostic test for rabies virus infection. The Negri body is not "a virus" but is a structure (a type of inclusion body) that is seen in rabies virus-infected neurons. Some inclusion bodies are in fact densely packed virus factories that form at discrete locations in the infected cell.

Fluorescence Microscopy

Fluorescence is the emission of light by a substance that has absorbed light (or other electromagnetic radiation). The basic function of a fluorescence microscope is to irradiate a specimen with high-energy excitation light and detect the much weaker emitted fluorescence. The microscope is designed so that only the emission light (fluorescence) reaches the eye or detector, allowing fluorescent structures to be seen with high contrast against a dark background. This is achieved by use of filters of specific wavelength. The advantage of fluorescence microscopy is that a single fluorescein molecule can emit as many as 300,000 photons before it is destroyed, thus making it theoretically possible to detect a single molecule in a field of view.

Fluorochromes (also termed fluorophores) are stains used in fluorescence microscopy. As in light microscopy, the most useful of these stains attach themselves to specific targets in the cell. For example, DAPI (4′,6-diamidino-2-phenylindole) is a fluorescent stain that binds strongly to A-T rich regions in DNA. DAPI (Fig. 4.3) can pass through an intact cell membrane thus can be used to stain both live and fixed cells. It is used extensively in fluorescence microscopy to label the cell nucleus because it binds to ds DNA. Different fluorochromes are excited by specific wavelengths of irradiating light and emit light of defined wavelength and intensity. Thus multiple fluorochromes can be used simultaneously to identify different target molecules within a cell.

Alternatively, fluorochromes can be chemically attached to molecules, such as antibodies, to specifically label their ligands. Alexa dyes (Fig. 4.4) are a series of fluorescent molecules that are widely used as fluorochromes. Among the Alexa dyes, there are choices of molecules with a variety of excitation and emission spectra. If different Alexa dyes are attached to different molecules, cell images can be collected using filters specific for each color. The relationship of one macromolecule to another can be determined by overlaying the images. For example, if a green image and a red image are superimposed, yellow pixels are seen where the tagged macromolecules colocalize in the cell.

A viral diagnostic assay called an immunofluorescence assay (IFA) uses tagged antibodies to detect viral proteins in infected cells. IFAs often include DAPI to stain cell nuclei. The result is that all cells have blue DAPI-stained nuclei and infected cells glow green. If cells have fused together due to virus infection, one may see a large cell (syncytium) containing many blue nuclei.

Another way to add a fluorescent tag to a protein is to use genetic engineering (cloning) to add the coding

sequence for a fluorescent protein to the coding sequence of a protein of interest. Green fluorescent protein (GFP), isolated from a jellyfish, is a 238 amino acid protein (Fig. 4.5) that folds to create a fluorescent center that emits green light. Genes encoding cellular or viral proteins can be modified by addition of the GFP gene to create a "tagged" protein. If the engineered gene is introduced into a cell, the protein product is visible when cells are exposed to UV light. This is a very powerful imaging technique; however, one drawback is that the addition of a relatively large protein tag can alter the trafficking, localization, or enzymatic activity of the protein of interest.

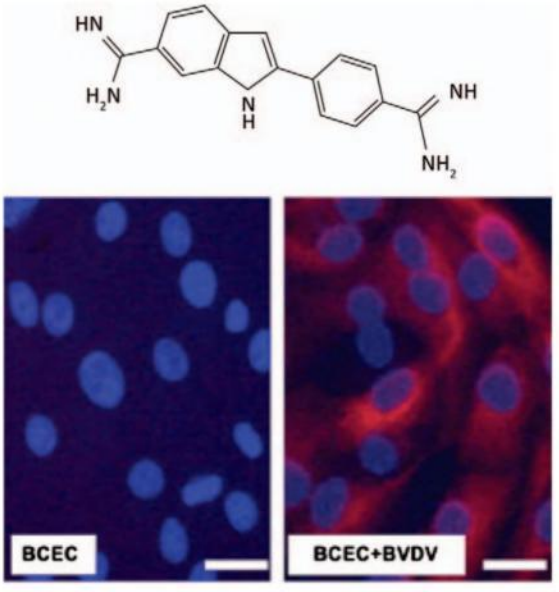

FIGURE 4.3 Chemical structure of DAPI (top) a fluorescent stain used to stain nuclei. Bottom left, bovine caruncular epithelial cell line (BCEC) stained with DAPI and showing blue nuclei. Bottom right, BCEC infected with bovine viral diarrhea virus (BVDV) showing cell nuclei stained with DAPI and fluorescence cy3-conjugated antibody to detect BVDV (red). *Bridger et al. 2007.*

Confocal microscopy offers several advantages over conventional fluorescent microscopy. Confocal microscopy uses "point-like" illumination and detection to reduce out-of-focus glare. The method provides the ability to collect serial *optical* sections through a thick specimen by collecting and measuring the light intensity originating from numerous thin focal areas in a cell. Confocal microscopy can image either fixed or living cells that have been labeled with one or more fluorescent probes. The excitation energies required to reach deep into the cells are very high and specimen damage can result. In contrast *deconvolution methods* employ conventional widefield fluorescence microscopes for image acquisition. Excitation intensities can be kept low, resulting in less damage to the specimen. Deconvolution is often used to image monolayers of living cells. Deconvolution methods have high computational demands as the images are computationally processed.

Electron Microscopy

The development of EM in the 1930s allowed individual viruses to be seen for the first time. EM uses an electron beam in place of light, and the beam is focused by electromagnets, rather than by glass lenses. EM has great resolving power and can magnify objects by up to ~10,000,000 times. Disadvantages of EM are that fixed and processed samples are dead, and biological samples are heavily damaged by the electron beam even as they are being imaged (sample damage results from the interaction of electrons with organic matter). However, the simple structures and crystalline nature of viruses were first revealed by EM and it remains a common tool of the virologist. In a process called negative staining, heavy metals are used to coat the surface of virus particles to make a "cast" or "relief map" (Fig. 4.6). The heavy metal coating is much less

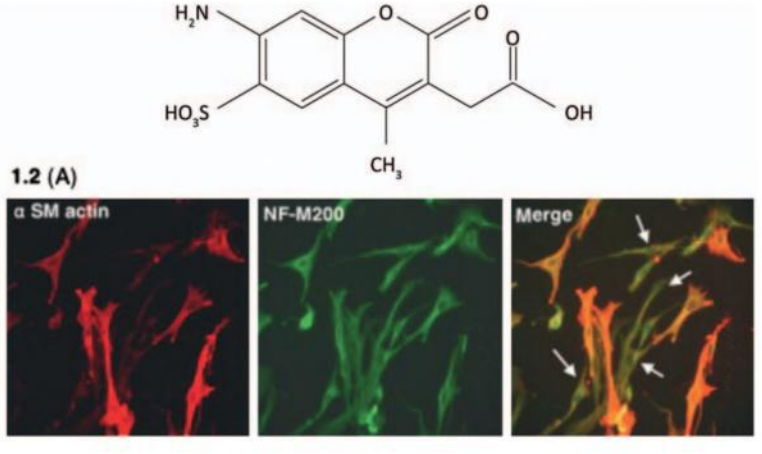

FIGURE 4.4 Chemical structure of Alexa dye (top). Smooth muscle actin (αSM actin, in red Alexa 594) (bottom left) and neurofilament M200 (NF-M200, in green Alexa 488) (bottom middle); merged shows αSM actin-negative cells (white arrows) (bottom right) (magnification 20×). *From Bouchez et al. 2008.*

sensitive to radiation damage than the biological sample, thus the coated surface structures are well preserved. However, information about internal structures is poor.

Thin sectioning is a method that reveals structures inside viruses and cells. Samples are fixed, embedded in resin, and sliced with a diamond knife and a microtome. The sections are carefully placed onto small carbon grids and can be processed in a variety of ways. Samples can be treated with electron dense stains such as uranyl acetate or lead citrate to provide contrast, or samples might be incubated with gold-labeled antibodies. Use of gold-labeled antibodies is a powerful technique that allows the localization of specific molecules within a virus or cell. Micrographs showing the process of virus budding from a cell are produced by thin sectioning (Fig. 4.7 need permissions).

Electron cryomicroscopy (cryo-EM) uses ultralow temperatures (liquid nitrogen or liquid helium) to preserve biological samples. Cryofixation of samples produces a glass-like (vitrified) material rather than crystalline ice. Cryo-EM allows the observation of specimens that have not been chemically stained or fixed, thus showing them in their native aqueous environment. Low temperatures provide for better images with less specimen damage, but cryo-EM micrographs have less contrast than stained images. Cryo-EM images are more complex than negative stains because the entire particle (including the interior) is seen in the image. However, thicker frozen specimens can be cut into thin sections using a cryoultramicrotome. Images generated by cryo-EM are often enhanced by collecting and computationally averaging multiple images. This allows use of lower and less damaging doses of electrons. Tomography is a process whereby a series of images are taken with the specimen tilted at different angles relative to the direction of the electron beam. The images are computationally combined to create 3D images (tomograms) of the sample. Extremely high-resolution (<4 Å) images have been achieved for icosahedral viruses because they are constructed from many repeating subunits that can be computationally averaged. Cryo-EM, while a powerful technique, requires expensive and specialized equipment and highly trained personnel (Fig. 4.8).

COUNTING VIRUSES

Methods of counting or quantitating viruses fall into two discrete categories. Infectivity assays, as the name implies, measure virions that can successfully infect a cell to produce infectious progeny. Inactivated

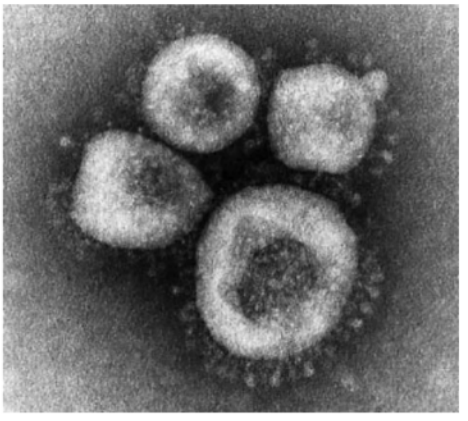

FIGURE 4.6 Negative stain of bovine coronavirus. *Courtesy: HR Payne.*

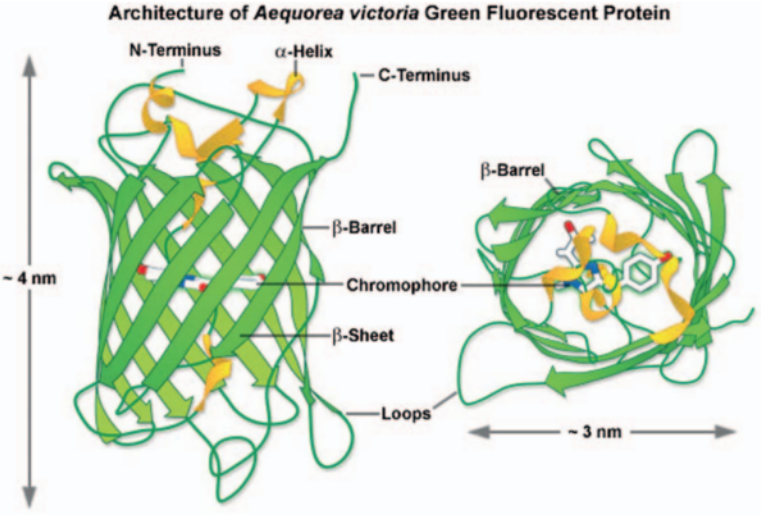

FIGURE 4.5 Ribbon diagram showing the structure of the GFP protein. *From Zeiss Education in Microscopy and Digital Imaging. Contributing Authors Richard N. Day and Michael W. Davidson.*

(noninfectious) virions are not counted. The second category of techniques measures specific virion components, often a specific viral protein, or the viral genome. These techniques are chemical/physical measures of virus quantification and they include serologic assays, polymerase chain reaction (PCR), and hemagglutination assays (HA). Negative staining EM can also be used as a chemical/physical assay to detect and count virus particles. Chemical/physical assays do not distinguish between infectious and inactivated (noninfectious) virions. For example, if one were to take two identical samples and expose one to UV radiation (to damage viral genomes), the amount of viral protein measured would be the same for both samples, even though the irradiated sample contains no infectious virus.

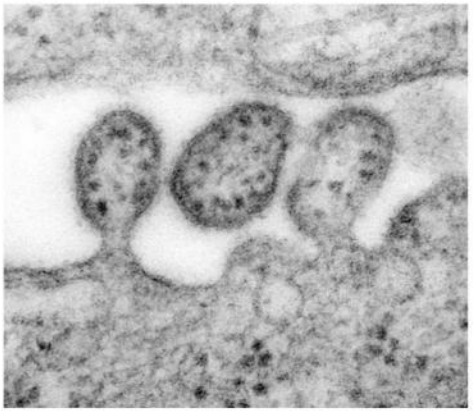

FIGURE 4.7 Highly magnified transmission electron microscopic (TEM) image depicting some ultrastructural details of three Lassa virus virions. The virion in the middle has completely budded from its host cell, while the two other particles are in the process of budding. Content provider: CDC/C.S Goldsmith, D. Auperin. Public Health Image Library, Image 8699.

Infectivity Assays

Infectivity assays require cell cultures, embryonated eggs, or animals (or plants or bacteria). An infectivity assay measures particles capable of replicating in a particular cell type or animal.

Plaque assays are a common type of infectivity assay, used to count discrete "infectious centers." Samples containing virus are serially diluted and aliquots of each dilution are added to a dish of cultured cells (or a plant leaf in the case of a plant virus) (Fig. 4.9). Each dilution is usually tested in triplicate. The process is:

- An individual virion infects a cell.
- The virus replicates, producing progeny virions.
- Second generation virions infect and kill surrounding cells to generate a hole or plaque in the cell monolayer that is visible to the naked eye (or at low magnification). Often the live cells are stained, providing a dark background for the clear plaques (Fig. 4.10).

The purpose of making and testing serial dilutions is to achieve a "countable" number of plaques in the cell monolayer. If too many infectious particles are present, individual cells are likely to be infected with more than one virion or individual plaques will merge to form large areas lacking cells. Plots of the number of plaques versus the dilution factor are linear if a single virion is sufficient to initiate a replication cycle ("single hit" kinetics). Results of plaque assays are reported as plaque forming units (PFU) per volume of measure (usually a mL). The term PFU acknowledges that we cannot know, with certainty, that the visualized plaques were formed by infection with a single virus of interest. Use of the term PFU should also remind us that variables such

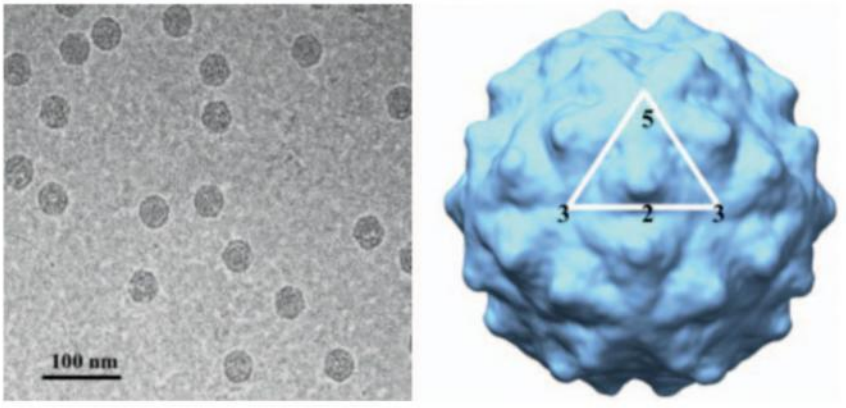

FIGURE 4.8 Cryo-EM structure of Flock house virus, virus like particle (VLP). Left panel: Electron cryomicrograph of frozen hydrated VLPs. Right panel: 3D reconstruction using image processing and viewed down the 2-fold axis of symmetry. The icosahedral asymmetric unit is highlighted by the white triangle, showing the 5-, 2-, and 3-fold axes of symmetry. *From: Bajaj et al. 2016.*

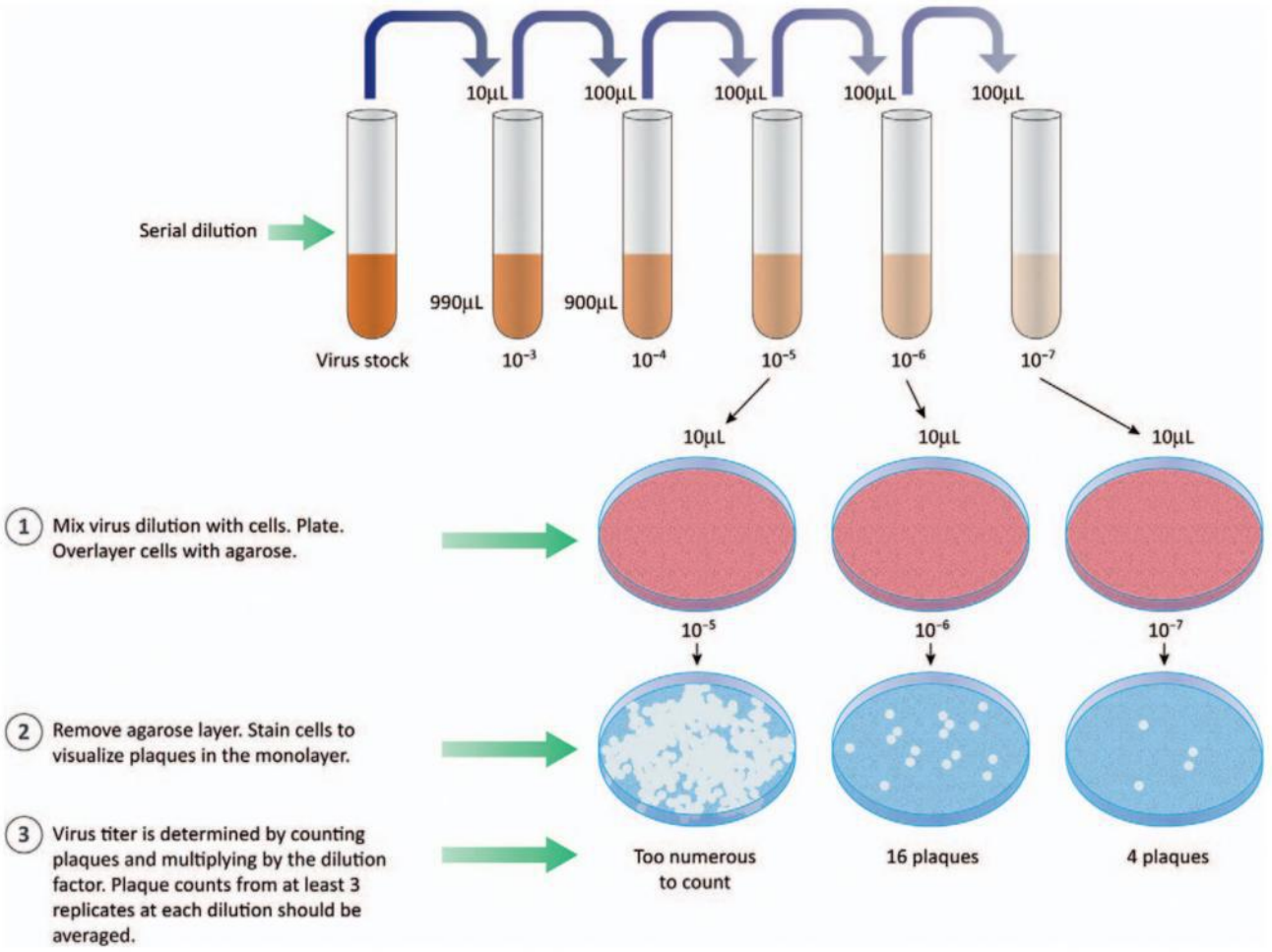

FIGURE 4.9 Plaque assays are used to count infectious particles. Samples are diluted and aliquots of each dilution are added to cultured cells. The cells are covered with an agaroseoverlay. Virus produced from an infected cell can infect nearby cells. If infected cells are killed, a region free of cells (the plaque) develops.

as type of host cell, type of culture media, ion concentration, or pH might affect the apparent concentration of virus. For example, a sample with a titer of 10^3 PFU/mL on mouse cells might have a titer of 10^6 PFU/mL on human cells.

What if the virus of interest does not kill infected cells? Can plaque assays be performed? The answer is yes, if there is a method for counting groups of infected cells. Groups of infected cells are called foci and the assays are focus-forming assays. Results are reported as focus-forming units (FFU). It is common to take advantage of antiviral antibodies to stain groups of infected cells. The antibodies can be detected if they are tagged with a either a fluorescent molecule or with an enzyme that can cleave an uncolored substrate to produce a colored product. There are several variations on the focus-forming assays but all have in common the identification of small, discrete groups of infected cells in a monolayer culture (Figs. 4.11 and 4.12).

Endpoint dilution assays are another type of infectivity assay. The general protocol is as follows:

1. Prepare serial dilutions of the virus stock.
2. Use aliquots of each dilution to infect 3–6 test units. A test unit might be a culture dish, a single well on a multiwell plate, a leaf on a plant, or an animal.
3. Incubate test units to allow virus replication.
4. After a predetermined period of time, check each test unit and determine if virus has replicated. If virus can be detected, the unit is scored positive. If no virus is detected, the unit is scored negative.
5. Count the total number of infected and uninfected units at various dilutions.

When the endpoint dilution assay is done in cell cultures, the titer is reported in Tissue Culture

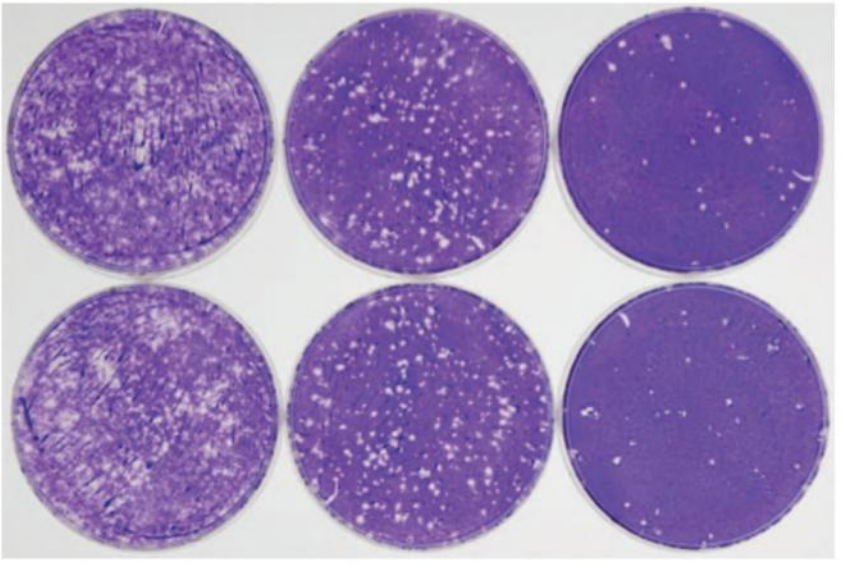

FIGURE 4.10 Stained cell monolayer with plaques. Living cells are stained purple leaving clear areas (plaques) where cells are absent. *From: Cromeans et al. 2008.*

FIGURE 4.11 Red blood cells (RBCs) binding to coronavirus infected cells. Bovine coronavirus infected cells are not killed but do express the viral envelope protein on their surface. The viral glycoprotein binds to RBCs. The RBCs serve to "mark" infected area in this electron micrograph. *Courtesy: HR Payne.*

Infectious Doses 50% ($TCID_{50}$) where one $TCID_{50}$ is the amount of sample that will infect 50% of the test units. For example, if 1 mL of virus sample is added to each of six units, and three of those units are positive at the end of the assay, the $TCID_{50}$ is 1. However, the results of endpoint dilution assays are seldom "perfect" and calculations based on summing all of the data should be used to derive a titer. End-point dilution assays can be performed using animals and results are reported as Infectious Dose 50% (ID_{50}) or Lethal Dose 50% (LD_{50}), if death is the endpoint. For a given virus sample, the $TCID_{50}$ titer is often higher than the ID_{50} titer which is often greater than LD_{50} titer. Note that virus titers are not absolute values, but rather depend on the type of cells or animals used for the assay.

Chemical/Physical Methods of Virus Quantitation

Chemical/physical methods of virus quantitation measure the amount (or relative amount) of a viral protein, genome, or enzyme, in a sample. Types of chemical/physical methods include:

- Direct visualization of virions by EM.
- Hemagglutination (HA) assay.
- Serological assays (based on antigen–antibody interactions, see Box 4.1). Examples of serologic assays include enzyme-linked immunosorbent assays (ELISA), fluorescent-tagged antibody assays, and precipitation assays.
- Genome quantification by PCR.

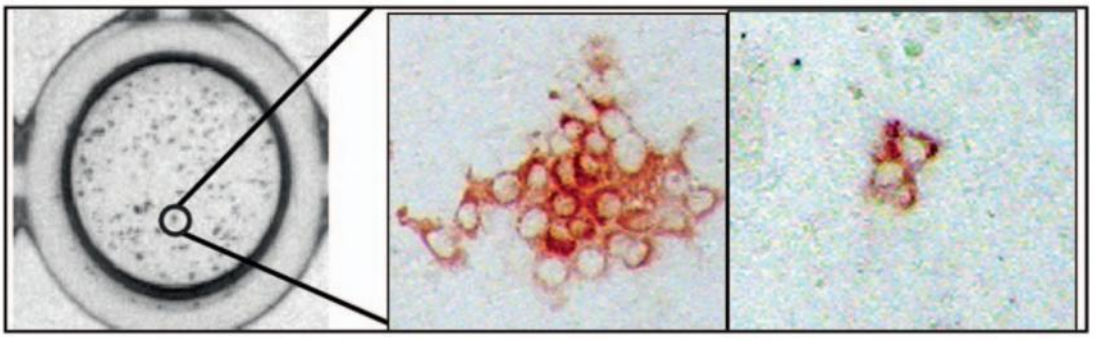

FIGURE 4.12 Focus-forming assay for hepatitis C virus (HCV). Infected cells were stained with an HCV-specific primary antibody followed by a horse radish peroxidase (HRP)-conjugated secondary antibody. *From: Barretto, N. and Uprichard, S. L. (2014).*

BOX 4.1

SEROLOGIC ASSAYS

Serologic assays use the power of antibody–antigen interactions (the ability of an antibody to bind its cognate antigen with high specificity).

A primary antibody is one that binds a specific antigen. If HIV is the antigen, the primary antibodies are those that bind to HIV proteins. For a direct ELISA, the primary antibodies are chemically linked to a fluorescent tag or an enzyme tag.

A secondary antibody is one that binds to a primary antibody. If the primary antibody is human-anti-HIV (from an infected patient, for example), the secondary antibody recognizes the human antibody. The secondary antibody might be rabbit, mouse, or goat antihuman IgG. The benefit of using a secondary antibody is that a single aliquot of secondary antibody can be used to detect many different human antibodies, regardless of what antigen those antibodies bind.

The readouts from chemical/physical assays provide no information about the amount of *infectious* virus in a sample, but they are often convenient, quick, and quite reproducible. They can often be correlated back to infectivity assays as a quick way to estimate the infectivity of a sample. However, if a virus sample is prepared or stored incorrectly, the protein or genome concentration might remain unchanged despite a significant decrease in the infectivity titer.

Hemagglutination Assay

Some viruses bind to red blood cells (RBCs). Hemagglutinating viruses bind to sialic acid residues on the RBCs. A single virion can bind to several different RBCs, and an RBC can be bound by multiple virions to form a large network, or web, of cell and virus that is easily visualized. The HA is fast and inexpensive and does not require either sophisticated instrumentation or extensive training. It is done by preparing serial dilutions of a virus sample. An aliquot of each dilution is added to RBCs in a microtiter plate well or test tube. One well contains RBCs and saline (negative control) and another contains a known positive reference sample of virus. The samples are gently mixed and allowed to sit at room temperature. In the negative wells the RBCs will slide down to form a tight button at the bottom of the tube. In positive wells the RBCs and virions will bind to each other to form a mesh of cells on the bottom of the tube. The reciprocal of the highest dilution of virus that give a positive HA is the HA titer (Fig. 4.13).

Serologic Techniques

HEMAGGLUTINATION INHIBITION ASSAY

HIA is a serologic assay that be used either to detect antibody to a virus or to identify a suspect virus. The HIA is performed by first mixing virus samples with dilutions of serum. Antibody is allowed time to bind the virus and then RBCs are added to the mix. Viruses that have bound to antibody will be unable to bind to RBCs. Thus in the HIA, the absence of hemagglutination is a positive result. If a hemagglutinating virus is

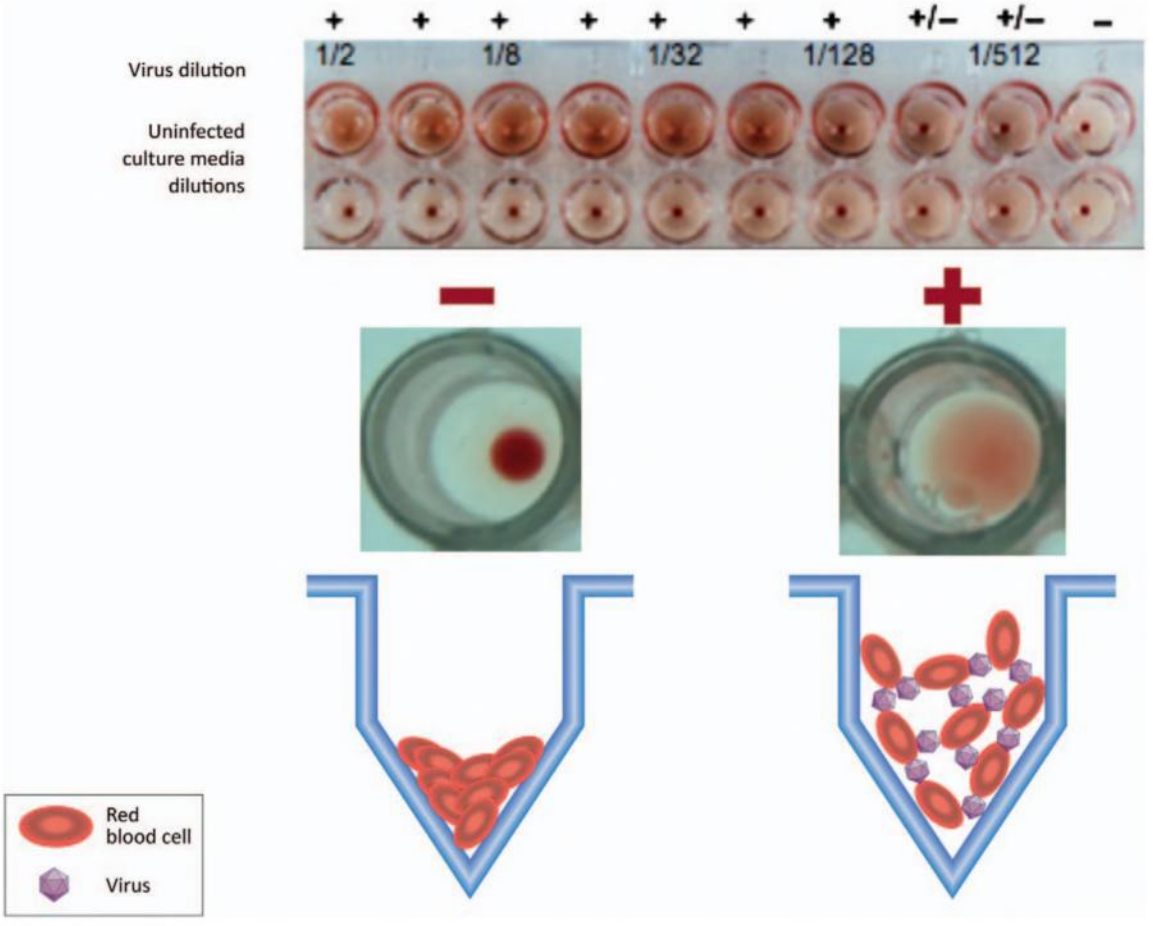

FIGURE 4.13 Some viruses bind to RBCs, causing the cells to form a lattice. Note that positive hemagglutination is the presence of a lacey layer of RBCs. If virus is not present, the RBCs slide to the bottom of the tube to form a tight "button."

the known reagent, the HI assay can be used to detect antibody. If the hemagglutinating virus is unknown, it can be identified by using a panel of known antibodies.

VIRUS NEUTRALIZATION ASSAY

A virus neutralization assay is used in conjunction with an infectivity assay, such as the plaque assay described above. This assay detects antibody that is capable of inhibiting virus replication (or in other words, antibody that can neutralize virus infection). Virus neutralization is a specialized type of immunoassay because it does not detect all antigen–antibody reactions. It only detects antibody that can block virus replication. This is important because related groups of viruses may share common antigens, but only a fraction of these antigens are targets of neutralizing antibody. A virus serotype is usually based on virus neutralization (although this is not always specified). For example, there are three major poliovirus serotypes (neutralization serotypes). In order to protect against poliovirus infection, a successful vaccine must induce neutralizing antibodies to poliovirus Types 1, 2, and 3.

ENZYME-LINKED IMMUNOSORBENT ASSAYS

Interaction of antibodies with their cognate antigens can be visualized if the antibody is "tagged" (Fig. 4.14). The tag can be an enzyme that cleaves a substrate (substrate turns color upon cleavage), a radioactive isotope, or a fluorescent molecule. Serologic assays are commonly used in research and diagnostic laboratories. ELISAs (See Box 4.2) require that either antigen or antibody be adsorbed onto a plate or tube (often plastic). As proteins readily bind to many types of glass or plastic, the adsorption part of the assay is straightforward. The enzyme used for detection is often either horseradish peroxidase (HRP) or alkaline phosphatase (AP). These enzymes relatively stable, cheap, and easy to purify, and they can be chemically linked to an antibody to aid in detection of an immune complex. There are

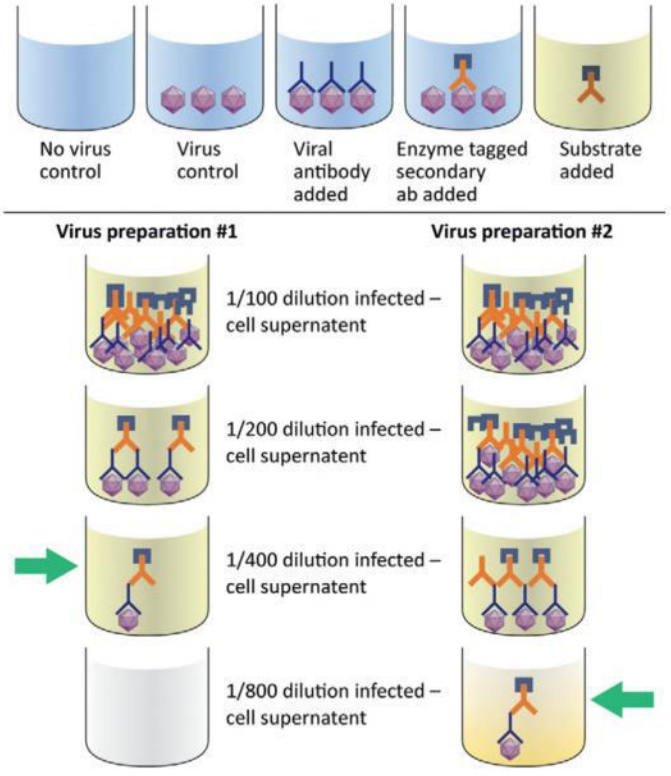

FIGURE 4.14 An ELISA can be used to determine the relative titer of a virus stock. The titer is expressed as the reciprocal of the highest dilution at produces a positive color.

inexpensive substrates that change from colorless to colored when cleaved by these enzymes. ELISAs can be used to detect either antigen (a virus, for example) or antibody (from a potentially infected individual). Antigen detection ELISAs can be used to quantitate the amount of virus in a cell culture supernatant or patient sample. In this case the primary antibody is a purchased and/or standardized reagent. Antibody detection ELISAs demonstrate the presence of antibodies to a specific pathogen (i.e., human immunodeficiency virus, HIV) in patient sera. In this case the antigen is the standardized reagent. The presence of antibody signifies that the patient has either been infected (or vaccinated) with the agent in question.

CELL-BASED FLUORESCENT ANTIBODY ASSAYS

Highly specific antibodies tagged with fluorescent molecules can be used to detect the presence of viral antigens in cells. Assays can be direct (using labeled primary antibody) or indirect (using labeled secondary antibody) (Fig. 4.15). The technique allows one to distinguish infected from uninfected cells, thus can be used to perform focus-forming assays.

WESTERN BLOTS

Western blots are labor intensive and expensive, but provide a method of confirming the identity of a reactive antigen. Western blots are done by electrophoresis of antigen (i.e., purified virus) by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). The proteins in a mixture are separated by size in the gel and are transferred from the gel to a solid (paper-like) substrate often called a membrane. The membrane is incubated with patient sera and the presence of patient antibodies is detected by subsequently incubating the membrane with labeled secondary antibodies. The specificity of the western blot lies in its ability to demonstrate the molecular weights (sizes) of the proteins recognized by patient sera. False positive reactions can be identified when the immunoreactive

BOX 4.2

VARIATIONS OF THE ELISA CAN BE USED FOR ANTIGEN OR ANTIBODY DETECTION

Direct ELISA. Primary antibodies (i.e., anti-influenza virus antibodies) are chemically linked to enzymes such as horseradish peroxidase (HRP) or alkaline phosphatase (AP). These reagents facilitate detection of viral antigens in a sample. If a fluorescent molecule is attached to the primary antibody, the assay is a fluorescence-linked immunosorbent assay.

Indirect ELISA. The primary antibody is untagged, but a tagged secondary antibody is used to detect the presence of primary antibody. For example, if the primary antibody is developed in an immunized rabbit, the secondary antibody is anti-rabbit IgG (perhaps obtained from mice immunized with rabbit IgG). Indirect ELISAs are common because it is cheaper to tag one batch of antirabbit IgG than to tag the specific antibodies directed against hundreds of different viral (or other) proteins.

Sandwich ELISA. The antigen of interest is "sandwiched" between two antibodies. An antibody or a serum sample is used to coat the dish or tube. A sample containing an antigen is added and allowed to bind to the antibody. A second antibody is added to the wells. It will bind to the antigen (if present). In this example the antigen must be able to interact with two antibodies. Requiring that two different antibodies react with the same antigen can improve the specificity of the assay.

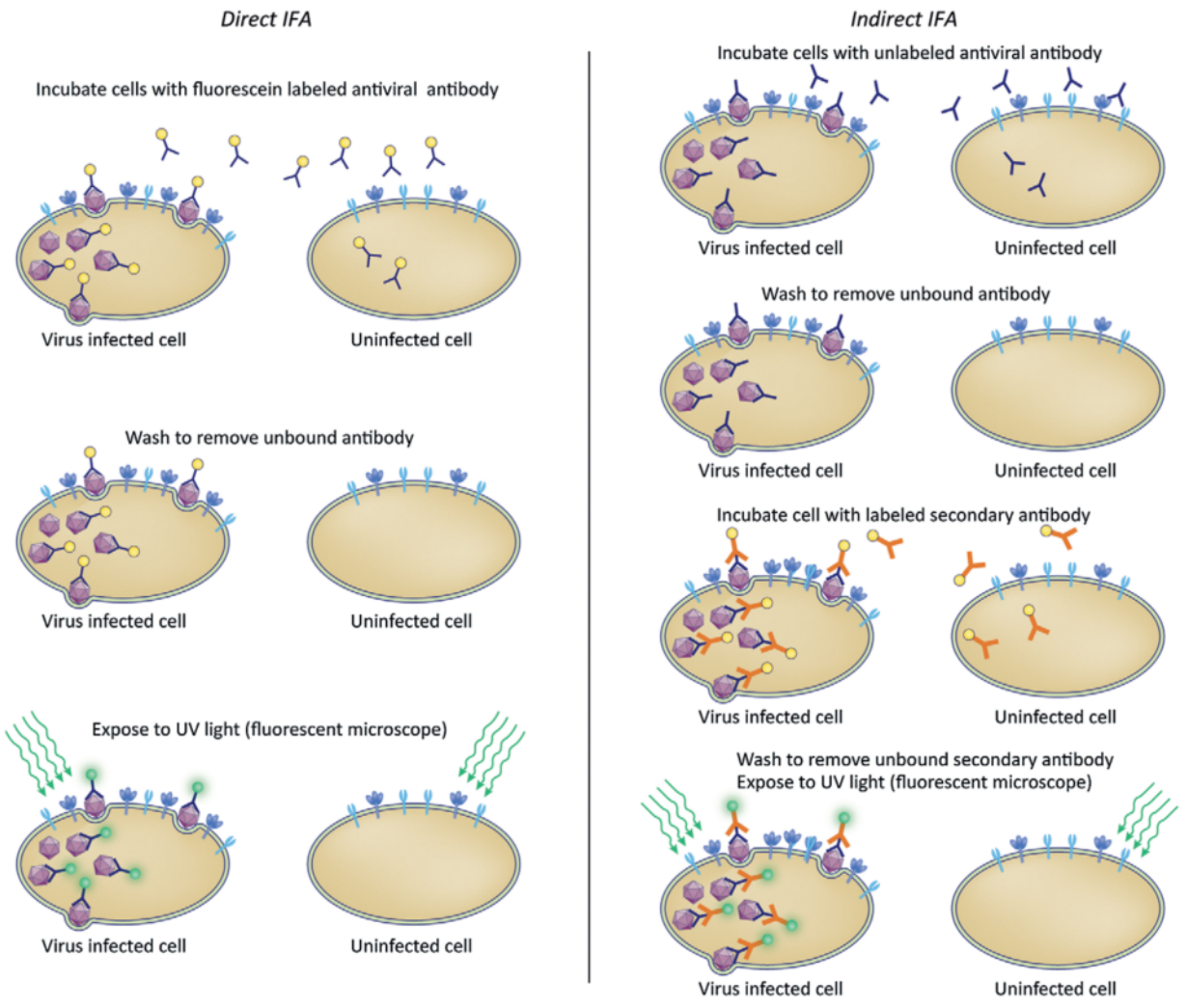

FIGURE 4.15 Cell-based IFAs use tagged antibodies to mark infected cells. Direct immunofluorescence used tagged primary (antiviral) antibodies. Indirect immunofluorescence uses a secondary antibody that is tagged.

protein band does not correspond in size to known viral proteins. For diagnostic purposes, a positive western blot may require that more than one viral protein be bound by patient antibodies.

IMMUNOHISTOCHEMISTRY

The basis of immunohistochemistry is that a tissue section is incubated with enzyme-tagged antibodies. A colorless substrate is added to the sample. If enzyme-tagged antibodies are present, the substrate is cleaved to produce a colored precipitate. This is a powerful technique as it allows one to examine individual virus-infected cells in a tissue section. Patient samples (biopsies) are often preserved in formaldehyde or are stored frozen at ultracold temperatures. If these samples are archived, they can be tested for the presence of viral antigen even after years or decades (Fig. 4.16).

Detecting Viral Nucleic Acids

Polymerase Chain Reaction

PCR can be used to identify and/or quantitate viral genomes in a sample. PCR is a very sensitive method and uses oligonucleotide primers designed to detect suspect viruses. Advantages of PCR include: (1) The PCR product (DNA) can be rapidly sequenced providing genetic information about the virus. (2) Primer sets can be designed to recognize sequences common among groups of related viruses or can be used detect a specific member of a virus group. (3) Multiple primer sets can be used to look for more than one suspect virus in a sample.

PCR assays are very sensitive, but sensitivity can be a disadvantage as well as an advantage. When performing PCR for diagnostic purposes, it is essential that every precaution be taken to avoid contaminating patient samples. This often requires separate

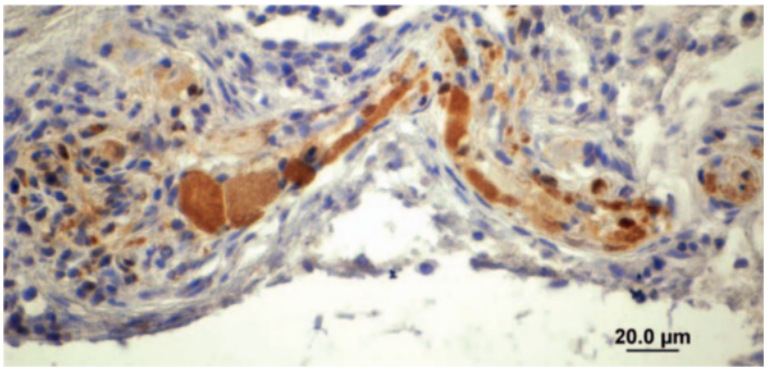

FIGURE 4.16 Immunohistochemistry is a technique used to detect viral antigens in tissue sections. In this example a brain section has been treated with antibodies to a bornavirus. The antibodies are tagged with an enzyme, producing a brown precipitate that indicates bornavirus-infected cells. *Courtesy: I. Tizard.*

equipment and work areas. For example, a "clean" area to process the patient sample, another area to set up the assays and a third area where the PCR products are synthesized and analyzed. It is also important to test all purchased reagents for the presence of contaminating nucleic acids. This requires multiple negative controls. For example, one might apply sterile buffer or water to a nucleic acid purification column to check the column for contamination with viruses that might have been introduced during the manufacturing process.

High Throughput Sequencing

While PCR amplification requires some prior knowledge of a viral sequence, it is now routine to sequence all nucleic acid (DNA and RNA) in a sample, using high throughput, unbiased sequencing techniques. Sequencing technologies can provide a genome's worth of data (billions of nucleotides) in a day. Powerful algorithms are used to analyze the data and compare it to information stored in public databases. Sequences of no interest (human DNA in a patient sample) can be ignored, allowing the investigator to quickly focus on any viral sequences that may be present. As in the case of PCR, the sensitivity of the assay is both a positive and a negative, and the most careful researchers will include multiple negative controls in their assays. Use of unbiased sequencing has resulted in an explosion of new viruses from humans, animals, and environmental samples. The current challenge is to develop an understanding of which viruses might be threats and which are part of our normal viral flora.

BASIC PRINCIPLES OF DIAGNOSTIC VIROLOGY

The purpose of diagnostic virology is to identify the agent most likely responsible for causing disease in a human or animal patient. Virus identification can be used to:

- Determine treatment strategies (although there are only a handful of antiviral medications are available).
- Predict disease course and expected outcome.
- Predict the potential for virus spread.
- Allow identification of, and vaccination of, susceptible individuals.
- Trace the movement of a virus through a community, or world-wide.

For the medical practitioner, methods for identifying the virus in an infected patient ideally should be sensitive, specific, and rapid, as once a patient has recovered (or died) diagnosis has less practical value (although a diagnosis could benefit family and community members). On the other hand, epidemiologic studies may include hundreds or thousands of samples requiring use of low cost, high throughput modalities.

Common targets for diagnostic tests are viral proteins (antigens), viral genomes, and/or antiviral antibodies. Some are designed to detect viral proteins, enzymes, or genomes directly from a patient sample (blood, throat swab) and these are useful in a clinical setting. One type of rapid diagnostic assay design (lateral flow immunoassay) uses the process of diffusion to move a sample across a test chamber. The liquid sample contacts various dried reagents as it flows through the chamber. Among the strengths of the lateral flow immunoassay is the ability provide rapid diagnostic capabilities in a medical/veterinary setting. Tests can be designed to detect either antigen or antibody from a patient sample. An example of an *antigen capture assay* is shown in Fig. 4.17. The test strip contains three key assay reagents (dried on the strip). Closest to the sample addition chamber is an antiviral antibody. In our example the antibody is conjugated to gold nanoparticles. When the liquid from the patient

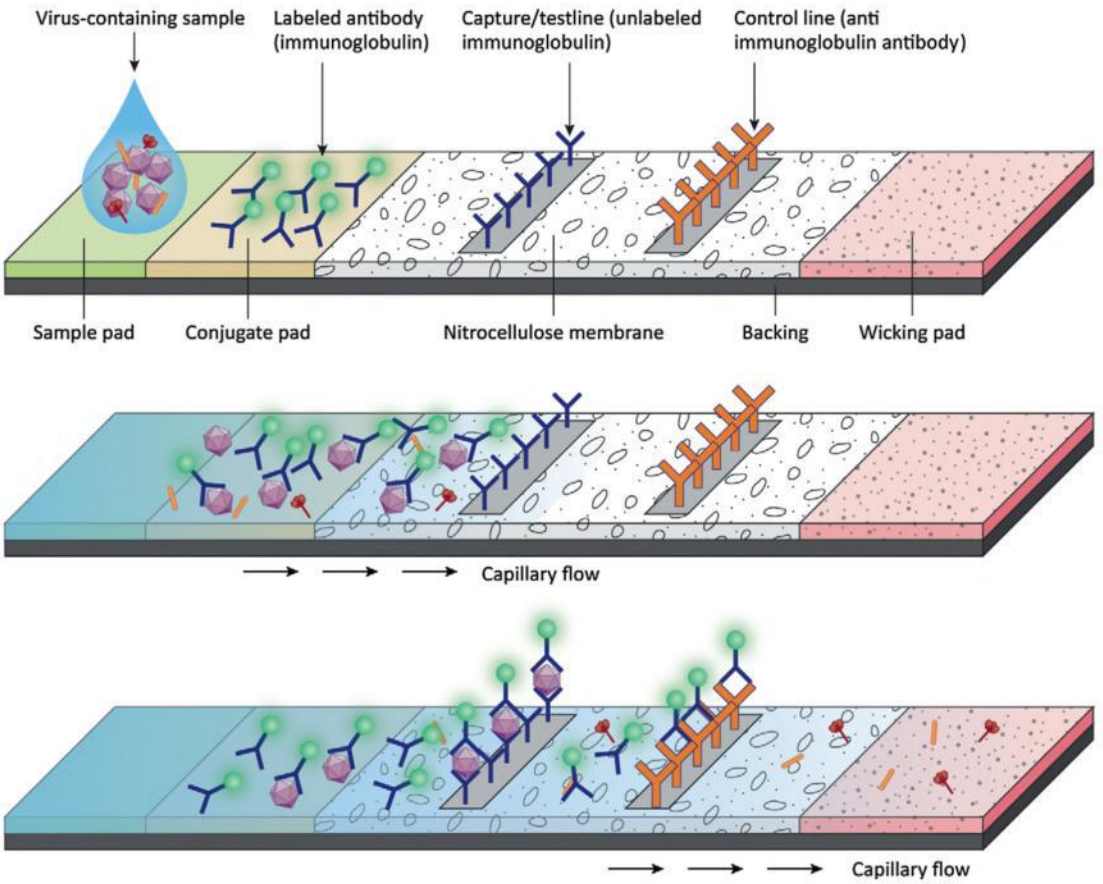

FIGURE 4.17 An antigen capture, lateral flow immunoassay. The test strip contains three key assay reagents. In this example a gold-conjugated antibody binds to virus in the test sample. The liquid moves across the slide by capillary action to the Test (T) strip. The T strip contains membrane bound antiviral antibody. It will bind to capture the virus particles and their associated gold tagged antibodies to generate a visible signal at the T strip. If no virus is present in the sample, the gold-labeled antibody travels past the T strip and binds to anti-immunoglobulin antibodies bound at the C strip. A positive sample must show color reactions at both the T strip and the C strip. A negative sample must show a color reaction at the C strip (to validate that the test worked correctly).

sample is applied, it flows, by capillary action, across the slide and will encounter the labeled antibody. The labeled antibody is picked up in the flowing liquid and will bind to any viral antigen present in the sample. The liquid continues to move across the slide (by capillary action) until it hits the Test (T) strip. In our example the T strip contains antibody to the virus in question. If the labeled antibody is bound to virus, it will be stopped (captured) at the test strip. If the labeled antibody is not bound to virus, it will move past the T strip and reach the control (C) strip. Anti-immunoglobulin antibodies are bound at the C strip and will capture any labeled immunoglobulin in the liquid. Thus if the sample is positive for virus, a colored line should appear at the T strip and the C strip (because there is excess labeled antibody). If the sample is negative for virus, the T strip will remain colorless but the C strip will be positive.

Often there is not enough virus present in the infected host to allow for *direct detection* in a patient sample. In that case the sample may be sent to a diagnostic laboratory for inoculation into cultured cells (or fertile eggs) to generate higher concentrations of virus. The infected cultures are closely observed for visible changes, such as cell killing (cytopathic effects), changes in cell morphology, or formation of syncytia (fused cells). Any of these changes in the infected cell cultures (as compared to uninfected controls) provides an indication that an agent is replicating, and that further testing is warranted to identify the agent. Growing viruses is labor intensive, takes days to weeks, and presents biosafety issues. Patient samples are often inoculated into several different types of cells in the hopes that at least one type will be susceptible. Infected cultures can provide abundant material for detection of viral antigens by ELISA, IFA, immunohistochemistry, or western blots. They can also provide material for PCR or direct sequencing, or EM can be used to look for the presence of virus particles in samples. (EM is not usually helpful for direct examination of patient

BOX 4.3

COMMON BIOCHEMICAL AND MOLECULAR TECHNIQUES FOR INVESTIGATIVE VIROLOGY (1)

In addition to the techniques described above, the virologist has a wide array of molecular and biochemical tools that can be used to study virus replication and/or pathogenesis. The scope of this text does not allow a complete discussion of these techniques but short descriptions of some common techniques are provided.

Electrophoresis. Charged molecules can be separated in an electric field. Often the electric field is applied to a semisolid gel (agarose or polyacrylamide) so the components in a sample can be separated by charge and/or size. Addition of SDS to a protein sample coats the proteins with a uniform negative charge so they are separated based on size alone. Nucleic acids have a uniform net negative charge, thus are easily separated by size using agarose or polyacrylamide gels. Gels are treated with specific stains to detect the presence of proteins or nucleic acids. Materials in the semisolid gel can be transferred to a solid support, or membrane, for additional manipulations, such as incubation with antibodies (western blot).

Chromatography is the collective term for a group of techniques used to separate molecules in a mixture. A liquid mixture is applied to material packed in a column (column chromatography), or layered onto plate (thin layer chromatography). Separation is based on the relative ability of the components in the mixture to move through or across the support materials. Chromatography can be applied to any mixture of molecules but the research virologist most often uses column chromatography to separate viral proteins.

Size exclusion chromatography employs beads with pores of defined sizes. Larger molecules are excluded from the beads, thus move quickly through the column. Smaller molecules enter the pores in the beads and this slows down their passage through the column. The result is that larger molecules are eluted from the column before smaller molecules. Thus molecules (i.e. different viral proteins) are separated by size.

Ion exchange chromatography uses charged materials in a column to separate macromolecules based on their charge. If the material in the column is negatively charged, molecules with a positive charge will be retained in the column. By changing buffer conditions (pH and ion concentrations), macromolecules can be separated on the basis of charge.

The basis of *affinity chromatography* is the very specific interaction between two molecules (for example, the interaction of an antibody to its cognate antigen). If an antibody is attached to a solid support, it can be used to capture antigen from a dilute solution. After washing away unbound material, the antigen can be released from the column by changing salt and or pH of the buffer. Proteins A and G are bacterial proteins that bind to immunoglobulins. They are used to affinity purify antibodies and antibody/antigen complexes.

samples as the amount of sample on one microscope grid is very small and it can be tedious to survey more than a handful cells at the highest magnifications. (However, some enteric viruses, for example, rotaviruses, are present in high enough concentrations in stool samples and can be visualized by EM.)

Recent or past exposure to viruses (or immunization) can be determined by detecting antiviral antibodies in patient samples. This type of assay requires a panel of viral antigens against which antisera can be tested. It is possible to detect not only antiviral antibody, but also to determine the class of antibodies in the sample. Detection of IgM indicates recent or acute infection, as these are the first antibodies produced in response to infection. Detection of antiviral IgG indicates that the patient is, or has been, infected with a particular virus; however, IgG may also be present due to prior immunization. Measuring IgG levels can be helpful for diagnostic purposes if *two* patient samples are available: one collected early in the disease process (acute serum sample) and one collected after recovery (convalescent serum sample). If the IgG titer in the convalescent serum is higher (>fourfold higher) than in the acute sample, a diagnosis can be made.

Immunoglobulin titers can be measured using ELISA or IFA. Immunoglobulin can also be measured by its ability to precipitate a particulate antigen (precipitation assays), to block hemagglutination (HIA) or to inhibit virus infectivity (virus neutralization assay).

A few biochemical and molecular techniques commonly used by virologists are described in Boxes 4.3 and 4.4.

In this chapter we have learned:

- A general method for obtaining and culturing cells. Primary cultures contain cells obtained from an animal and they have a limited lifespan. Tumors are

BOX 4.4

COMMON BIOCHEMICAL AND MOLECULAR TECHNIQUES FOR INVESTIGATIVE VIROLOGY (2)

Flow Cytometry. Cells are suspended in a stream of fluid and flow past an electronic detection apparatus. The number of cells passing the detector is counted. Thousands of cells per second can be counted. Often cells are labeled with fluorescent dyes (tagged antibodies, for example) and are not only counted, but are separated into different collection tubes based on labeling patterns. The process is often used to get pure populations of cells from a mixture. Immunologists take advantage of the fact that different types of proteins are found on the surfaces of different types of lymphocytes. Thus labeled antibodies can be used to quantitate and separate different lymphocyte subsets from a blood sample. Not only can the presence of a molecule on the cell surface be detected, but the relative amounts can be determined as well. Flow cytometry is also used for viral diagnostics. Cells infected with an unknown agent can be incubated with panels of antibodies.

Reverse Genetics. Virologists study viral genes to determine their functions. Historically this was accomplished by finding and purifying mutant viruses to determine how they differed from their "wild-type" parents. With the development of gene cloning technologies, virologists are able to clone entire viral genomes, manipulate them in the laboratory (introduce specific mutations) and examine the effects on virus replication or disease. Introducing specific mutation is the "reverse" of traditional genetic method of identifying a mutant phenotype and then determining the genetic cause. In order to do reverse genetics, the virologist usually starts with a cloned viral genome that can be introduced into cells to produce infectious virus.

Designer Cells and Designer Animals. In addition to mutating viral genes, virologists manipulate the hosts as well. It is relatively easy to add, delete, or modify the genetic makeup of cultured cells. Animal genomes can be modified as well. Systems to create designer mice are reasonably efficient and hundreds of types of genetically modified mice are commercially available. What is gained by modifying the host? At every step in the virus life cycle, viral proteins interact with cellular proteins and a single viral protein may interact with dozens of cellular proteins. To analyze the effects of just one type of interaction, it may be preferable to modify the host genome. It is also possible to genetically alter a resistant host to render it susceptible to a virus. For example, the receptor for a human virus could be added to mouse cells to generate a tractable animal model. It can be challenging for a student of virology, new to reading scientific literature, to determine the types of virus or host mutations that have been used to obtain data for a specific study. But this is critical to understanding and evaluating experimental results.

a source of immortalized or transformed cells that can divide continuously.
- A basic method for virus purification using centrifugation.
- Methods to visualize viruses including negative staining EM, thin sectioning. EM, cryo-EM, and confocal microscopy.
- Methods to count infectious virus particles (plaque assays and endpoint dilution).
- The basic features of common techniques used to detect viruses and antibodies.

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CHAPTER

5

Virus Transmission and Epidemiology

OUTLINE

After studying this chapter, you should be able to:

- List some factors that impact virus transmission.
- Define or explain the terms virulence, pathogenicity, prevalence, incidence, incubation period, latent period, and infectious period.
- Explain how serosurveys provide information needed to determine the pathogenicity of a virus.
- List some of the factors that impact the spread of a virus.
- List some of the factors that impact the outcome of a viral infection.
- List modes of virus transmission.

Infectious disease epidemiology (which includes the epidemiology of viruses) is the study of the complex relationships among hosts and infectious agents. Epidemiologists are interested in virus spread or transmission, with or without disease. Viral epidemiologists try to predict the potential for development of epidemics, and a very important part of their job is to define the kinds of interventions that could contain a virus outbreak. Veterinarians are often concerned with threats to food animals (how a disease of food animals might be spread, or be introduced into a disease-free area). In order to model virus transmission, epidemiologists must try to account for a variety of factors involving both host and virus. Factors that can impact virus transmission and spread include:

- Prevalence of the agent within the population.
- Mode or method of transmission of the agent.
- Duration of the infection and the window of transmissibility.
- Numbers of susceptible and nonsusceptible individuals in the population.
- Population density.
- Patterns of travel or associations (for example, schoolchildren and their families form interconnected networks).
- Living conditions.
- Climate and/or season.

Predicting the course of an outbreak is particularly challenging if a new or novel virus is involved, as we often do not have adequate information about modes of virus transmission, duration of infection, window of transmissibility, or stability of the virus in the environment. This can be further complicated by additional factors that may impact the outcome of infection. For example, differences in age, gender, nutrition, and genetic susceptibility of the host are important factors in the outcome of infection. Thus some infected individuals may have an asymptomatic infection while others develop severe, life-threatening disease.

Two key terms used by epidemiologists are "incidence" and "prevalence." These terms have very specific meanings and are not interchangeable.

- *Incidence rate* (also called the attack rate for acute infectious diseases) is a count of the number of

Viruses. DOI: http://dx.doi.org/10.1016/B978-0-12-803109-4.00005-2

new infections during a specific time period. A specific population and a time frame are defined, and the number of new cases is counted in order to arrive at the numerator of the ratio. The denominator of the ratio includes both the size of population and time frame, and is often expressed as person-years.

- *Prevalence* refers to the total number of cases present or counted. In the case of a persistent infection such as human immunodeficiency virus (HIV), the numerator includes patients infected for many years or decades. Prevalence is expressed as a ratio such as "cases per million." Note that there is no time parameter in this ratio.

For an acute viral infection, such as measles, the incidence rate, or attack rate may be similar to the prevalence of the virus, given that virus is present and transmissible for a relatively short period of time. In contrast, when considering persistent or chronic infections, such as those caused by the HIV or hepatitis C virus (HCV), incidence and prevalence may actually be quite different. In the case of HIV, education about safer sex practices can decrease incidence (new infections), while the availability of antiviral drugs results in significantly longer survival times, thus increasing prevalence. Interestingly, it has been shown for HIV that drug treatment decreases incidence while at the same time increasing prevalence, because treatment not only prolongs life but decreases transmission by drastically reducing viral loads in infected patients (Fig. 5.1).

METHODS TO COUNT VIRAL INFECTIONS AND DISEASE

Accurate determination of incidence rates or prevalence of a virus depends on the ability to count infections. The values obtained depend on the methods used for counting. Changes in the case definition of a disease, or the diagnostic tools available to detect a viral infection can lead to sudden apparent changes in incidence or prevalence.

Passive surveillance is done when healthcare workers report cases of disease. Many viral diseases are designated as "reportable" and in theory, each and every case should be reported. The weak link in passive surveillance is the filing of the initial report, as in practice only a small fraction of cases of common, reportable viral diseases are actually reported (reporting frequency may be as low as 10%–15%). Even so, passive surveillance is useful for monitoring infectious disease trends.

Active case detection through investigation is another way to collect information on disease outbreaks. Active case detection seeks to classify an illness and determine the causative organism, to assess the extent of an outbreak and its economic and health impact, to stop the outbreak and to inform the public.

Serological surveys are an important tool for the viral epidemiologist. They are useful because many viral infections stimulate the production of antibodies (for example, immunoglobulin G) and detectable antiviral antibody titers are often maintained lifelong.

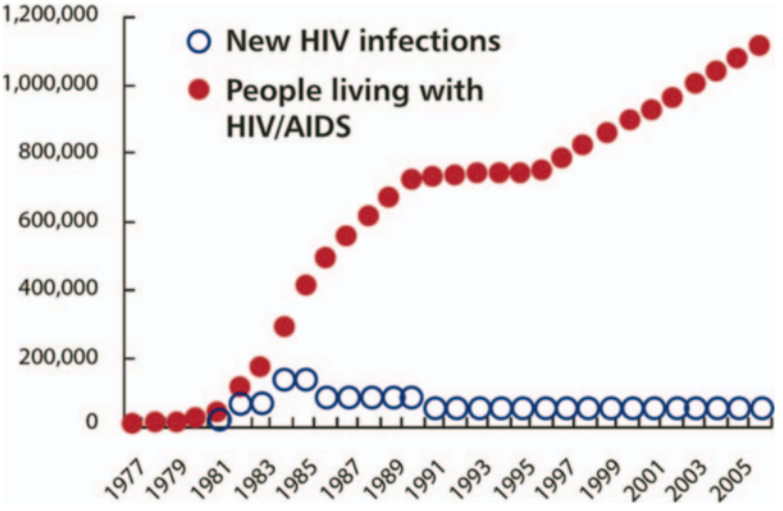

FIGURE 5.1 Incidence versus prevalence of HIV infections. The number of new HIV infections per year in the United States has remained steady but most infected individuals live years to decades with treatment, hence the yearly increase in prevalence. Source: *Campsmith, et al., 2008. CROI 2009. JAMA 300 (5), 520–529.*

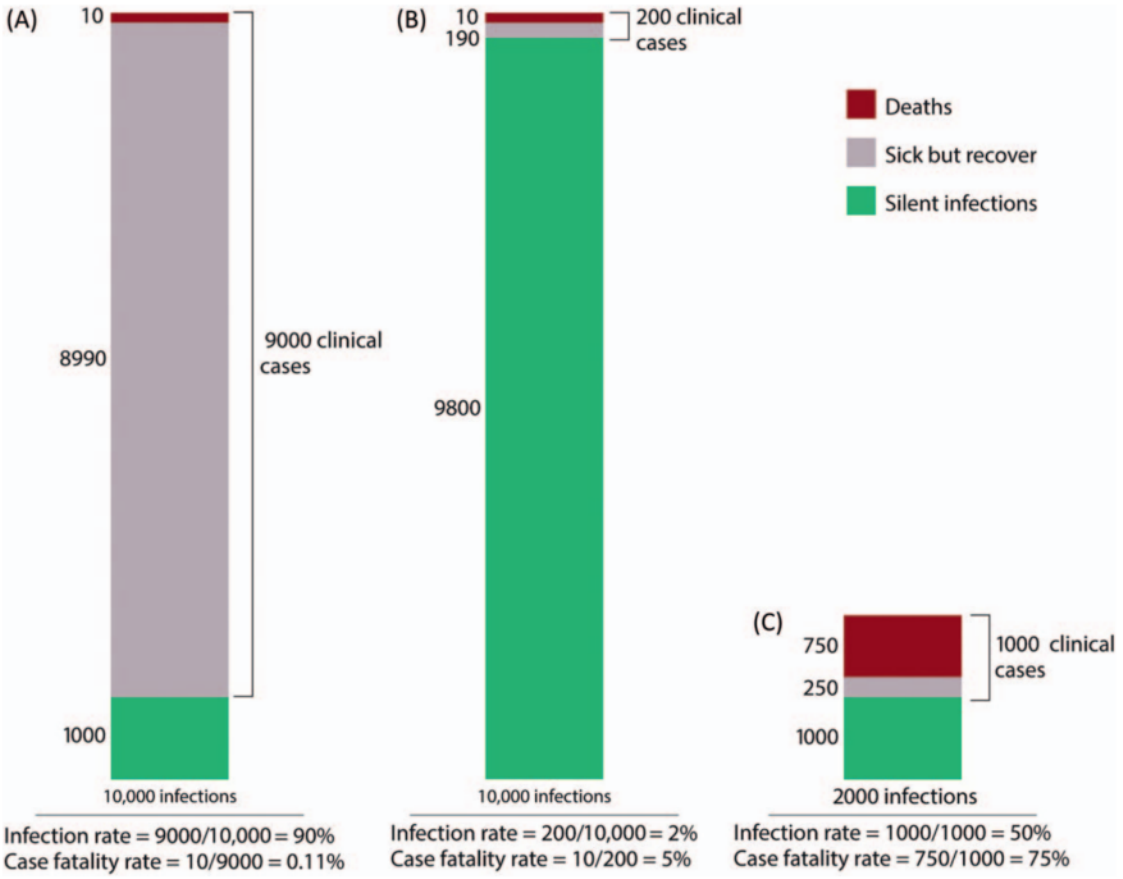

FIGURE 5.2 Infection ratio versus fatality ratio. The infection ratio is the number of clinical cases per case of infection. The case fatality ratio is the number of deaths per clinical cases. Three different scenarios are presented. The virus in panel A has high pathogenicity but low virulence. The virus in panel B has low pathogenicity. The virus in panel C has high virulence.

An important feature of serosurveys is that they provide a history of both clinically apparent and clinically inapparent infections. A question that can only be answered by serosurvey is whether or not those severely impacted by an infection represent the total number infections, or if severe disease is a rare outcome on a background of a many inapparent infections (Fig. 5.2).

INFECTION VERSUS DISEASE

Another important concept in infectious disease epidemiology is the difference between infection and disease. For example, one reason for the successful eradication of the smallpox virus was that over 95% of infections resulted in observable, distinct, disease. The virus was not able to hide or spread silently. In contrast, poliovirus has proven more difficult to eradicate. One reason is that there is less than one case of observable disease per 100 infections.

Serosurveys are needed to determine the case infection ratio, the number of *clinical* cases per 100 infections (the number of clinical cases/the number of infections). To evaluate and express the severity of clinical disease, another measure is used. This is the case fatality ratio or the number of deaths per 100 clinical cases. The case infection ratio of a virus might be 0.001 (1 in 10,000 infected individuals develops disease) but the case fatality rate for the same virus might be 1 (every clinical case results in death).

As regards serosurveys, it is also important to note that a serologic assay may not be able to differentiate between natural infection and immunization. Recently there has been increased interest in developing vaccines designed to allow for discrimination between infection and vaccination (DIVA vaccines).

Serosurveys can assist in determining the pathogenicity of a virus. *Pathogenicity* is a measure of the proportion of infections resulting in overt disease. Measles virus is highly pathogenic as over 95% of infected individuals will experience a disease episode. A related, but distinct term is virulence. Virulence is a measure of the *severity* of the disease and is often measured as the number of deaths per number of infections. A virus can have low pathogenicity (few infections lead to a disease episode) but high virulence (when disease does occur it is often fatal) or the opposite might be true (most infections lead to disease, but disease is almost always

prior infection and/or vaccination reduces the number of susceptible individuals in the population. The effective or net reproductive number (R) is the actual average number of secondary cases and it equals the product of R_0 and the proportion of susceptible individuals in the population. R is smaller than R_0 because not all individuals in real populations are susceptible.

What is the value of calculating R? When R is greater than 1, we expect the incidence of infection to increase, but when R is less than 1, the infection burns itself out. When R equals 1, the number of infections is constant. Thus infection control programs can be modeled to determine the most effective methods available (for example, vaccination, quarantine, use of antiviral drugs) to control an outbreak. Of course we must also consider the human factor, the ability, and willingness of a population to adhere to recommended control methods and guidelines.

In summary, understanding the epidemiology of an outbreak requires knowledge of the particular virus, the host, the place (geography), and the time (season). Host factors include: age, nutrition, immunity, personal conduct, occupation, and interaction networks. Geography includes important factors such as temperature and humidity but also includes factors such as types of dwellings, water sources, and sanitary infrastructure. Many viral outbreaks are seasonal and the factors that influence seasonality include both host factors and geography.

In this chapter we have learned that:

- A variety of environmental, biological, and societal/behavioral factors impact virus transmission.
- Viruses (even closely related viruses) differ in their virulence, pathogenicity, and transmissibility, and these attributes are not linked. A virus may be highly transmissible but has low pathogenicity. A virus may have high pathogenicity but low virulence. Recall that pathogenicity is a measure of how many clinical cases are seen among infected individuals. Virulence is a measure of the severity of disease.
- Serosurveys are important for gathering information about the total number of infections within a group. Including those instances where infection was inapparent.
- A variety of factors impact the outcome of an infection. These include the age, sex, immune status, nutritional status, and overall health of the individual or animal.
- Modes of virus transmission include respiratory, fecal-oral, direct contact, insect vectors, vertical.

CHAPTER

6

Immunity and Resistance to Viruses

OUTLINE

After studying this chapter, you should be able to answer the following questions:

- What are the major differences between innate and specific immune responses?
- What kinds of unique molecules (proteins, nucleic acids, carbohydrates) are associated with pathogens?
- How do cells detect the presence of viral pathogens?
- What are the first responses to viral infection?
- How are interferons (IFNs) induced and what do they do?
- Describe at least one IFN-induced antiviral pathway.
- What is intrinsic viral immunity? Provide an example.

Immunity is the defense of the body against microbial invaders such as bacteria, viruses, parasitic protozoa, parasitic worms, and cancer cells. Immunity includes physical barriers to infection such as mucus, skin, and normal flora. Innate immunity involves cells and signaling pathways that are triggered by large groups of infectious agents. Specific immune responses use cells and antibodies that recognize *very specific* pathogens. There is a memory component to specific immunity that allows an animal to respond faster and more robustly the second (or third, fourth, . . .) time an infectious agent is encountered. In this chapter we review some basic information about innate and specific immunity (Fig. 6.1), with a focus on viral infections. If you are not familiar with the basic principles of immune responses, it would be helpful to read about the immune system in a general microbiology text. The following terms should be very familiar: antigen, antibody, lymphocyte, macrophage, T-cell, and B-cell. A brief description of the major cellular player in the immune response is provided in Fig. 6.2.

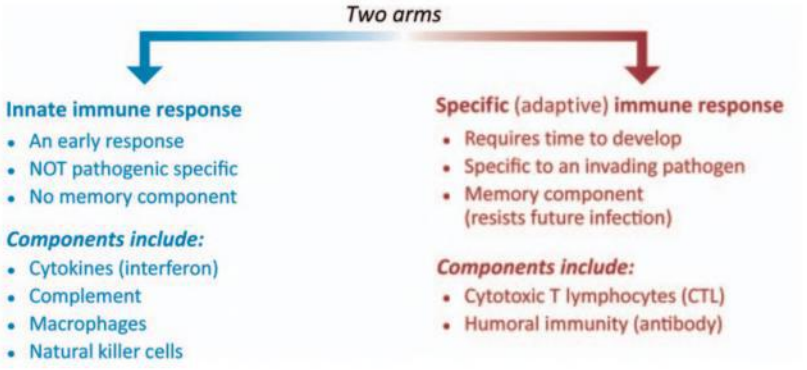

FIGURE 6.1 Overview of immune responses. A general comparison of innate and specific immune responses to pathogens.

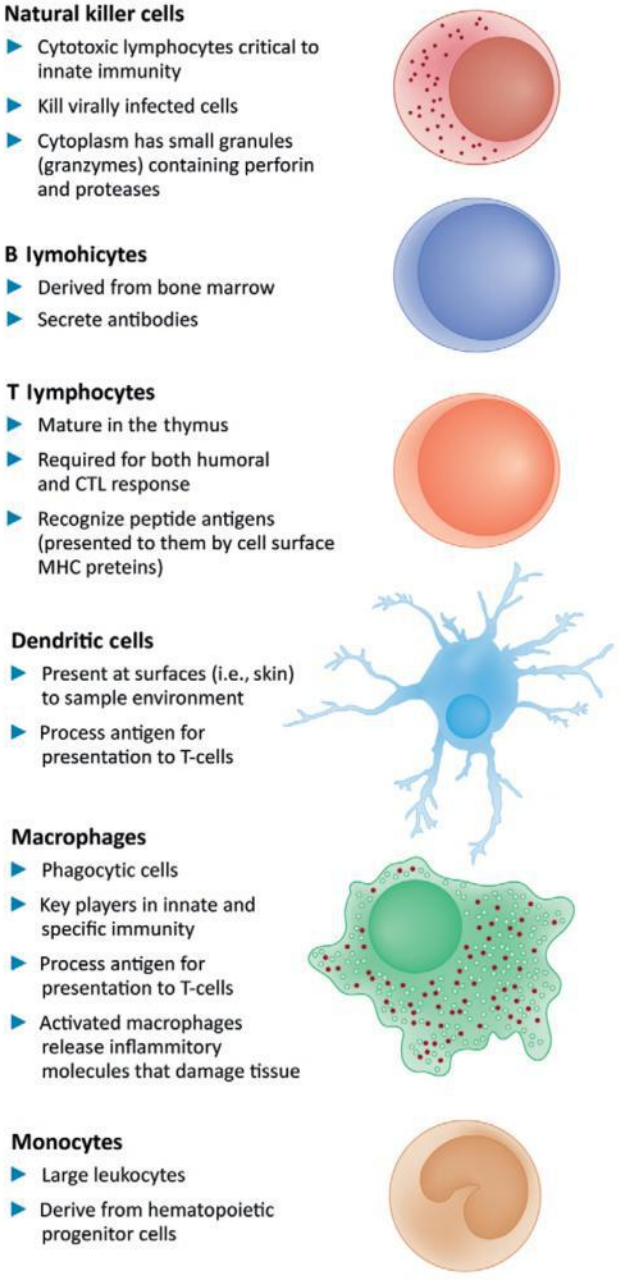

FIGURE 6.2 Cells of the immune system. Types of cells and their key roles in immunity to viruses are summarized.

INNATE IMMUNITY

The innate immune system is complex, involving multiple activators, signaling pathways, and responses. The signaling pathways connect activators to responses and these pathways intersect at many points. If signaling pathways are a roadmap, this chapter will introduce some highways and major intersections but will not consider the many smaller roads that may lead to the same destination. When reviewing this material try to keep the "big picture" in mind by considering the following questions:

- If the immune system recognizes molecules unique to pathogens, why do not mutations arise that allow the pathogens to escape? (Sometime they do!)
- If pathogens change in response to the body's defenses, what is the response of the host? (Develop new defenses!)
- Can host and infectious agent reach a balance? (Often, but not always.)

The foundation of innate immunity is the ability to detect and respond to foreign (pathogen-associated) macromolecules (Fig. 6.3). Thus when a virus breaches physical barriers and infects a cell, hard-wired, innate responses to infection are immediately triggered. The virus is recognized as an invader because the body recognizes and responds to specific groups of molecules, collectively called pathogen-associated molecular patterns (PAMPs). This phenomenon is called pattern-recognition and is accomplished by groups of proteins called pattern-recognition receptors (PRRs). In the case of viruses, several receptors recognize viral nucleic acids. When PRRs bind to a target they initiate signaling cascades that modulate cellular gene expression prompting the cell to synthesize many inflammatory and defensive molecules central to antiviral defenses. Three groups of PRRs are important for innate immunity to viruses:

- Toll-like receptors (TLRs),
- RIG (retinoic acid-inducible gene)-1-like receptors (RLRs),

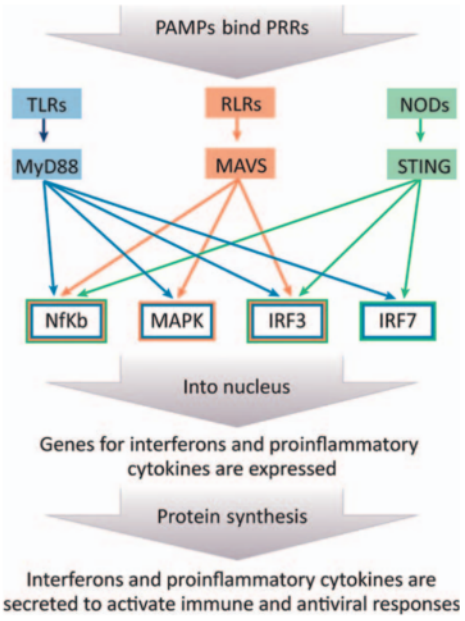

FIGURE 6.3 Signaling cascades are activated by PAMPs. PAMPs interact with classes of receptors (NODs, TLRs, RLRs) that in turn interact with major signaling molecules (MYD88, MAV, STING) that then activate specific transcription factors enabling genes encoding interferon and proinflammatory cytokines to be activated.

- NOD (nucleotide-binding oligomerization domain)-like receptors (NLRs).

Toll-Like Receptors

The word "toll" in German, means amazing or weird, and was used by geneticists to describe abnormal-looking fruit flies. The toll molecule was eventually found to be responsible for antifungal activities in *Drosophila*, thus when proteins with similar sequence, structure, and function were found in mammals, they were called TLRs). TLRs are transmembrane proteins. Some TLRs are found in the plasma membrane where they sense extracellular PAMPs. Intracellular TLRs are important in virus recognition as they detect intracellular viral nucleic acids. When triggered, TLRs stimulate the production of antiviral cytokines, particularly IFNs.

Most TLRs signal through a protein called MyD88. MyD88 in turn activates transcription factors such as nuclear factor kappa-β (NF-kβ), MAP kinase (MAPK), and IFN regulatory factor (IRF) 3 and IRF7. Activated transcription factors move from the cytosol into the nucleus where they bind DNA to turn on genes that code for IFNs and proinflammatory cytokines. Binding of PAMPs to TLRs not only triggers the innate immune response, but is also required to activate the adaptive immune system.

Retinoic Acid-Inducible Gene-Like Receptors

Two proteins, RIG-1 and melanoma differentiation-associated protein 5 (MDA5), are members of the RLR family. RLRs are found in the cytosol where they bind to certain types of viral nucleic acids. RLRs are helicases. RIG-1 senses RNAs with a 5′-triphosphate and base pairing at the 5′ end. Recall that cellular mRNAs have 5′-methyl guanosine caps, thus do not display a triphosphate at the 5′-end. However some RNA viruses lack "caps" and many have complementary sequences at their 5′- and 3′-ends. MDA5 recognizes the long dsRNA molecules that are formed during replication of the genomes of plus-strand RNA viruses.

When RIG-1 and MDA5 encounter their ligands, they are recruited to the mitochondrial signaling protein, MAVS, initiating a multistep signaling pathway leading to activation of the transcription factors MAPK, NF-kβ, and IRF3 with the subsequent induction of antiviral cytokines. Recall that these transcription factors are also key players in TLR signaling.

NLRs Are Receptors With NOD-Like Domains

The NOD domain is a nucleotide-binding oligomerization domain, thus NLRs bind to nucleic acids and form oligomers. NLRs are PRRs that detect pathogens in the cytosol. These DNA sensors include IFI16 (p204), the helicase DDX41, and cyclic GMP-AMP synthase. The endoplasmic reticulum resident protein, stimulator of IFN genes (STING), is the converging point of these DNA sensors. STING activates IRF3, IRF7, and NFkβ.

Inflammasomes

After PRRs interact with specific specific PAMPs, they act as scaffold proteins for assembly of multiprotein complexes called inflammasomes. Assembly of inflammasomes leads to the activation of caspaces (a type of protease) that cleave and activate proinflammatory cytokines such as IL-1β and IL-18.

Proinflammatory Molecules

Proinflammatory molecules and IFNs are released from cells as a result of PRR activation. These include cytokines such as interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α), and IL-6. Other proinflammatory molecules include nitric oxide (NO) (produced by the enzymes nitric oxide synthase 2 and cyclooxygenase-2) and proinflammatory lipids such as prostaglandins and leukotrienes. These molecules increase local blood flow, attract defensive cells (for example, neutrophils) and increase blood vessel permeability so that

IFN-Stimulated Genes and the Antiviral State

There are over 300 ISGs and they code for proteins with a variety of activities. Some ISGs are regulators of IFN signaling. Others have specific or general antiviral effects. Cells responding to IFN binding are said to be in an "antiviral state" and they are refractory to infection by many viruses (not just the specific virus that that originally triggered the IFN response). Some types of viruses are more sensitive to the effects of IFNs than others. The following sections describe the activities of a few ISGs.

Protein Kinase RNA-Activated Pathway

The enzyme protein kinase RNA-activated (PKR) is produced in response to Type I IFNs. PKR remains in an inactive or latent state until, as its name suggests, it is activated (auto-phosphorylated) upon binding to viral dsRNA. Once active, PKR phosphorylates the eukaryotic translation initiation factor EIF2A. Phosphorylation of EIF2A inhibits mRNA translation, thereby preventing host and viral protein synthesis. Active PKR also induces cellular apoptosis, to prevent further viral spread.

2′-5′ Oligo A Synthase Pathway

Two ISGs are key to this pathway: ribonuclease L (RNaseL) and 2′-5′ oligo A synthase (OAS). RNaseL (for latent) is inactive when synthesized, but when activated destroys RNA within the cell, leading to autophagy and apoptosis. (Both of these processes are robust antiviral responses.) In order for RNAseL to become active, it must bind to polymers of 2′-5′-linked oligoadenylate (2′-5′ oligo A) and these are synthesized by OAS. However OAS is also produced in an inactive form and only becomes active upon binding to dsRNA. The requirement for a multistep pathway helps insure that RNaseL is not activated in the absence of viral infection. To summarize the pathway: (1) RNaseL and OAS are ISGs. (2) If a cell expressing these proteins is infected by an RNA virus, long dsRNA accumulates in the cytoplasm. (3) OAS is activated and synthesizes 2′-5′ oligo A. (4) RNaseL binds to 2′-5′oligo A and is activated. (5) Active RNaseL cleaves viral and cellular RNA.

Other Interferon Stimulated Genes

Tetherin is an ISG that inhibits a variety of enveloped viruses, including human immunodeficiency virus (HIV), Ebola virus, Lassa virus, vesicular stomatitis virus, and Nipah virus. As the name implies, tetherin inhibits release of enveloped viruses by tethering them to the cell surface. Tetherin is a small, glycosylated membrane protein (181 aa). It is found associated cholesterol-rich microdomains in the PM, preferential budding sites of several enveloped viruses. In polarized cells, tetherin is found specifically at the apical surface. The antiviral role of tetherin appears to be to physically crosslink virions to the PM. A number of viruses, including HIV and Ebola virus, have evolved mechanisms to counteract tetherin.

The *IFN-inducible transmembrane protein (IFITM) family of proteins* inhibits a long list of enveloped viruses including influenza A and B viruses, dengue virus, hepatitis C virus (HCV), and Ebola virus among others. IFITMs contain two membrane-associated domains separated by a conserved intracellular loop. They are found on cytoplasmic and endosomal membranes and block viral replication by preventing fusion of the viral envelope to the host membrane. Imaging studies suggest that IFITMs trap infecting virions leading to their destruction by lysosomes and autolysosomes.

How do IFITMs work to inhibit fusion of enveloped viruses? They may alter the physical properties of the membrane of the host cell. IFITMs seem to associate with proteins that reside in lipid rafts, a location favored by some enveloped viruses. The importance of IFITM-mediated virus restriction has been demonstrated in mice, where lack of IFITM3 increases susceptibility to influenza virus infection. A role for IFITM3 in human susceptibility to influenza virus infection has been suggested, based on evidence linking a minor human allele of IFITM3 with more severe disease.

Viperin (virus inhibitory protein, endoplasmic reticulum-associated, IFN-inducible, also known as RSAD2) is an ISG induced by Type I, Type II, and Type III IFNs. Viperin has broad antiviral activity against both RNA and DNA viruses including some herpesviruses, flaviviruses, retroviruses, orthomyxoviruses, paramyxoviruses, togaviruses, and rhabdoviruses. Viparin is expressed by a variety of animals, including mammals, fish, and reptiles. Viparin inhibits the release of some viruses but may have other means of inhibiting virus replication. At least one virus, human cytomegalovirus (CMV or human herpes virus 5 (HHV-5)) induces viparin expression to enhance viral infectivity.

Mx proteins are IFN inducible, large GTPases that belong to the *dynamin superfamily* of high molecular weight GTPases. (Dynamin in involved in membrane budding and is required for scission of nascent vesicles from parent membranes.) Mx proteins are expressed by most vertebrates and they commonly localize to the cytoplasm. An exception is mice, whose Mx proteins are nuclear and cytoplasmic. Mx proteins assemble to form large, highly ordered oligomers that associate with intracellular membranes. Humans encode two Mx proteins, MxA and MxB,

BOX 6.1

AUTOPHAGY

Autophagy is a complex and highly regulated process whereby cellular components (for example, protein aggregates or damaged organelles) or infectious agents (viruses) are engulfed by double-membrane vesicles in the cytosol. These vesicles, called autophagosomes, then fuse with lysosomes leading to degradation of their contents and providing building blocks for the cell. Not surprisingly, a general trigger for autophagy is nutrient deprivation.

The selective removal of intracellular infectious agents by autophagy is called xenophagy and the process has been shown to restrict virus titers. Autophagy also provides peptides for presentation to the adaptive immune system. In addition autophagy controls inflammasomes triggered by innate immunity signaling cascades, possibly preventing excessive immune activation. There are more than 30 autophagy-related gene products that control and drive these diverse processes.

Viral pathogens manipulate autophagy as an immune escape mechanism. Some viruses have been shown to interfere with the formation of autophagosomes while others inhibit their fusion with late endosomes or lysosomes. There are also examples of viruses coopting autophagosomal membranes for use as platforms for genome replication and to facilitate their release from infected cells. For example, picornaviruses and flaviviruses seem to rely on autophagosomal membranes for optimum replication and release from cells.

infection, autophagy can act as a surveillance mechanism that delivers viral antigens to endosomal/lysosomal compartments enriched in immune sensing molecules. Activated immune sensors can signal to activate autophagy. To evade this antiviral activity, many viruses actively block the autophagy pathway. Alternatively, some viruses subvert autophagy for their own benefit. Manipulated autophagy has been proposed to facilitate nearly every stage of the viral lifecycle in direct and indirect ways.

Natural Killer Cell Responses to Viral Infection

Natural killer (NK) cells are large granular, cytotoxic lymphocytes that, as part of the innate immune system, play a key role in host defense against viral infections. NK cells provide rapid responses to virally-infected cells, acting at around 3 days postinfection. NK cells may recognize viral antigens directly and may kill some virus-infected cells. The importance of NK cells in controlling virus infection is underscored by the ability of some viruses to alter NK function.

SPECIFIC IMMUNITY

Specific immunity refers to pathogen-specific immune responses that get better with time and experience (practice makes perfect). Two major types of specific immunity are antibody responses (humoral immunity) and cell-mediated immunity (CMI). These processes involve the activities of T and B lymphocytes. As summarized in Fig. 6.5, the general steps in activating specific immune responses are:

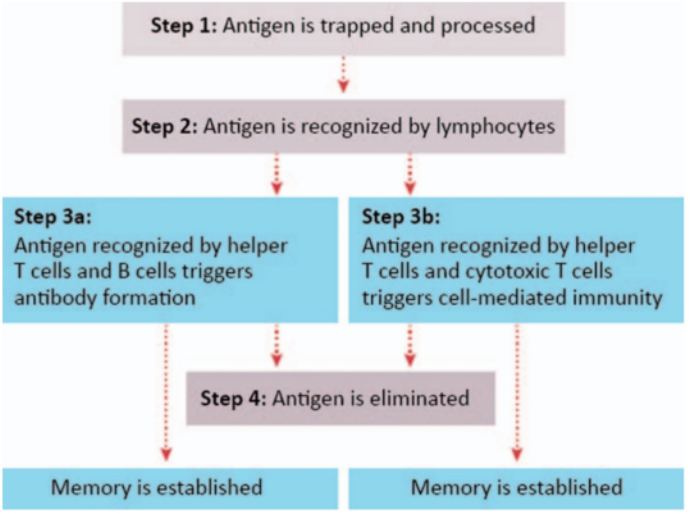

FIGURE 6.5 Overall steps in development of specific immunity.

- Antigen is trapped and processed.
- Antigen is recognized by T and B lymphocytes.
- Antigen is eliminated.
- Memory is established due to the presence of long-lived T and B lymphocytes.

Antibody Responses

Antibodies are produced by B lymphocytes in response to foreign antigens. Antibodies are found in the blood, lymph, and mucosal secretions. Their role is


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CHAPTER

7

Viral Vaccines

OUTLINE

After reading this chapter, you should be able to answer the following questions:

- What is the purpose of vaccination?
- When considering vaccine development, what are "correlates of protection" and why is it helpful to determine them?
- What are the advantages of a killed/inactivated vaccine? What are the disadvantages?
- What are the advantages of a replication competent viral vaccine? What are the disadvantages?

The purpose of a vaccine is to provide protection against a pathogen (in our case a viral pathogen). Early attempts at "vaccine" development were based on the observation that persons surviving a disease, for example, smallpox, were protected against further occurrences of that particular disease. Today we know that immune protection against a specific viral infection is provided by "specialized" (also called "adaptive") responses that inhibit virus replication or kill infected cells. The goal of vaccination is to stimulate the body to develop a specialized, protective, immune response in the absence of disease. Immune responses target one or more viral proteins and there are a variety of ways that viral proteins can be delivered as vaccines. These include administration of:

- Weakened (so-called attenuated) viruses that replicate in the host without causing disease.
- Inactivated (killed) virions.
- Purified protein products.
- Nucleic acids (genes) to direct synthesis of desired proteins.

CLASSICAL VERSUS ENGINEERED VACCINES

Traditional or classical methods to produce a viral vaccine required growing large quantities of virus. Attenuated viruses were usually obtained by repeated passage in animals or cultured cells; unfortunately the results were impossible to predict and some viruses were never adequately attenuated even after prolonged passage (Fig. 7.1).

Today recombinant DNA technologies, along with our understanding of the molecular details of virus replication and pathogenesis, provide an array of opportunities to achieve vaccine goals. Recombinant DNA technologies can be used to make targeted mutations in a virus or to clone and express specific viral proteins. As shown in Fig. 7.2, examples recombinant DNA strategies for vaccine development include:

- Virulent viruses can be weakened by deletion or modifications of *specific* genes.
- Specific genes from a virulent virus (often genes for capsid or envelope proteins) can be transferred to a

Cytotoxic T-lymphocytes recognize and kill virally infected cells thereby limiting the amount of virus produced. Recall that the T-cell response depends on recognition of peptides bound to MHC proteins and presented on the cell's surface. In the case a viral infection the cell will process (digest by proteolysis) some viral proteins as soon as they are synthesized, signaling to surveying T-cells that the cell is infected.

CORRELATES OF PROTECTION

An important consideration for vaccine development is to determine the type(s) of response that is (are) protective. Protection may require neutralizing antibodies, virus-specific cytotoxic T-cells, or both. Protection may require recognition of one or more viral proteins, or recognition of a specific epitope on a target protein. An obvious way to determine if a vaccine is protective is to compare the response of vaccinated versus nonvaccinated animals to a viral challenge. (We anticipate that the unvaccinated animals will get sick but the vaccinated animals will remain healthy.) This direct approach is the "gold standard" but uses a lot of animals and can be quite costly. Thus during the vaccine development process, it is often helpful to determine the specific type and magnitude of response that confers protection. Or in other words, what responses (both type of response and magnitude of response) *correlate* with the desired outcome: protection. For example, a specific titer of neutralizing antibody may serve to protect against infection by a particular viral pathogen. Various combinations of antigens, doses, delivery strategies, and adjuvants can then be administered, followed by measurement of neutralizing antibody titers. Only those preparations that induce adequate levels of neutralizing antibody need be tested further. Another advantage of understanding correlates of protection is that vaccine recipients can be tested to determine their status (protected or not protected). For example, in the case of rabies vaccines, protection is well correlated with high titers of antibody to the G protein. Among veterinarians or other "at risk" persons, antibody titers can be measured to provide information about the need for a booster dose (Box 7.1).

In the case of natural infections (for example, Ebola virus) we may examine survivors to determine the types of specific responses that correlate with survival. We can then design vaccines that mimic the natural, protective response (Box 7.2). But what happens when natural infection is not protective? When our immune system is unable to clear or control the virus after a natural infection? The best (worst?) example of this is infection with the human immunodeficiency virus (HIV). An untreated HIV infection is almost universally fatal, despite the fact that patients do mount an immune response. The inability for to control infection is due, in part, to the fact that HIV attacks the very immune cells we need to sustain a protective response. HIV also mutates rapidly and its surface proteins are designed to evade host responses. In short, there is little/no natural *protective* response, thus there are no well-defined correlates of protection to serve as the standard for vaccine design. (Worldwide, a very few persons have been identified who seem to be naturally resistant to HIV. They are an important resource in the fight against HIV!)

Vaccines to protect against influenza virus infection have been used for many decades, but we still face

BOX 7.1

RABIES VACCINE SUCCESS IN 1885

Louis Pasteur was a talented and innovative microbiologist. His work (among others) was key to understanding that diseases are caused by infectious agents that can be identified and grown in the laboratory. Pasteur initially developed and tested animal vaccines for the bacterial diseases anthrax (*Bacillus anthracis*) and fowl cholera (*Pasteurella multocida*). His studies of rabies were more challenging as he could not grow agent on an agar plate. Instead Pasteur used monkeys and rabbits to grow and attenuate the virus. He successfully used dried spinal cord material from infected rabbits to protect against rabies in dogs. He even determined that the vaccine could be administered to dogs *after* they were exposed to the virus. In 1885 Pasteur was asked to treat Joseph Meister, a 9 years old who had been bitten multiple times by a rabid dog. No one doubted that without treatment the boy would die from rabies. Pasteur treated Joseph Meister with injections of rabbit spinal cord material and the boy survived. This was such a celebrated and important event that people from around the world made contributions support Pasteur's work. Thus the Pasteur institute, dedicated to the study of biology, microorganisms, diseases, and vaccines, opened in 1888. To this day the institute continues to support cutting edge biomedical research.

Index

Note: Page numbers followed by "*f*," "*t*," and "*b*" refer to figures, tables, and boxes, respectively.

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