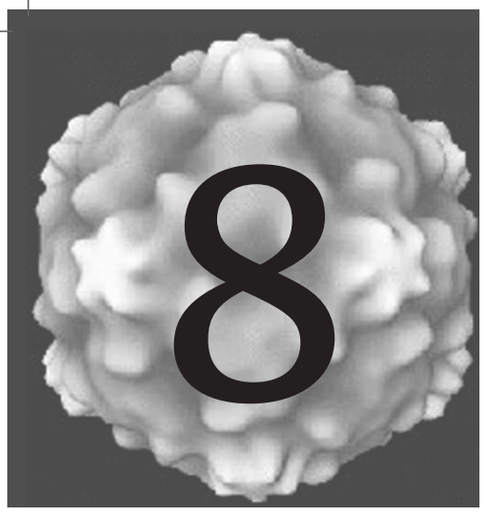


8 Strategies to Protect Against and Combat Viral Infection

CHAPTER

As far as one can tell, viruses and viral disease have coexisted with the bacterial and metazoan hosts in which they replicate since those hosts appeared in the biological universe. While this coexistence is a dynamic process, we humans can envision an "ideal world" where viral disease is controlled, if not eliminated, and its effects minimized. Despite all our knowledge of the biological world, it is clear that there are just two ways to deal with virus-induced disease: prevention and treatment.

Prevention of viral infection can be accomplished by application of public health measures to eliminate the spread of the virus or control its transmission, or it can be accomplished by making sure that there are no susceptible individuals available for the virus to infect. This latter approach can be done by inducing immunity to infection. The specific application of appropriate antiviral drugs also can have a role in preventing virus infection.

Treatment of virus infection can also involve methods to encourage the body's own highly evolved antiviral mechanisms to deploy before virus infection leads to serious damage. Additionally, treatment can be mediated by specific antiviral agents designed to specifically block one or another stage of virus replication in the host.

VACCINATION – INDUCTION OF IMMUNITY TO PREVENT VIRUS INFECTION

Antiviral vaccines

Almost everyone has heard the term *vaccination* and, in fact, has been given a vaccine, whether it be for poliovirus, measles virus, or mumps virus. Just what is a vaccine? How is it prepared and administered? And is it possible to create one for every viral infection of significance?

The term *vaccinate* means to administer, as a single or multiple dose, a nonpathogenic antigen (intact virus or virion subunit) to an animal or human such that the immune system of the individual responds by producing antibodies (humoral immunity) and in some cases, cell-mediated immunity directed against one, several, or all viral antigens. The successfully vaccinated individual retains an immunologic memory of the event. The mechanism of such immunity formation was described in Chapter 7.

Smallpox and the history of vaccination

For more than 2000 years, the scourge of smallpox affected human populations. The virus (member of the Poxviridae family, *Orthopoxvirus* genus) appears to have originated in Asia and made its way into Africa, the Middle East and the Western world by 800 to 1000 AD. The virus was brought to the New World by Spanish and other European explorers and colonizers, and hundreds of thousands of indigenous people in North and South America died as a result. In some cases, such as in the Caribbean, all native populations were wiped out. The process repeated itself with other infectious diseases such as measles both in the New World and in the islands of Oceania in the eighteenth and nineteenth centuries. Some of the processes involved in such spread of novel diseases in populations were briefly described in Part I, especially Chapters 2 and 3. Variola major, the more serious form of smallpox, has a case fatality rate of 30% to 40%. In contrast, variola minor, a less severe form of the disease, kills only about 1% to 5% of those infected.

Differences in disease severity were attributed to slight genetic differences between strains of smallpox virus only in the late nineteenth century. Despite this, perspicacious observers noted that survivors of the disease were immune for life, and those who contracted it late in a local epidemic had a higher chance of survival. This was exploited in the technique called **variolation**, which was introduced into Europe from the Middle East in the early eighteenth century. Lady Mary Wortly Montague, the wife of the British ambassador to Turkey, saw to it that her children underwent variolation, despite prejudices of those who argued that it would not work on Caucasians. Her success was responsible for introduction of the technique into England in 1718. In this (rather heroic) technique, an uninfected person, usually a child, would be exposed to scabs or crusts that formed on the skin of a patient recovering from a natural infection. This method often resulted in inducing disease with mortality rates well below 1% and lifelong immunity. We now know that this method inadvertently exploited the fact that virus in such a healing lesion will tend to be partially inactivated by the patient's own immune response as well as by partial desiccation.

Even though variolation was often successful, the failure rate (number of deaths from the technique) made it a dangerous practice. Still, this was a common preventative method used in many parts of China, the Middle East, and Africa well into the early parts of the twentieth century. In England, Edward Jenner, a country physician working in Gloucestershire, was experimenting with variolation when he learned from his patients—who were milkmaids—that those infected with a disease called cowpox would subsequently be immune to smallpox. Jenner had the insight to exploit this method as a relatively safe way to protect against the scourge of smallpox. As a result, he began experiments to purposely infect his patients with cowpox virus, giving them a mild, asymp-

tomatic disease and subsequent protection against infection with smallpox. Jenner named the method *vaccination* from the Latin word for "cow," *vacca*.

The success of Jenner's technique led to the rapid spread of prophylactic vaccination against infection with smallpox, but the success was largely confined to the developed West until after World War II. Ultimately, the success of vaccination against smallpox culminated in the announcement by the World Health Organization that smallpox has been eradicated from the planet. The last naturally occurring case of smallpox in the world was in October 1977, in a man in Somalia. He died and it was determined that he contracted the virus from an aerosol of desiccated contaminated material that had been improperly disposed of during an earlier epidemic!

The only existing stocks of smallpox virus are at the Centers for Disease Control and Prevention in Atlanta and at the Russian State Research Center of Virology at Kolsovov. By international agreement, these stocks were to be destroyed on June 30, 1999, thus making the virus extinct. However, disagreements over the advisability of this delayed the planned destruction.

This situation changed dramatically on September 11, 2002. After the terrorist attacks on the World Trade Center and the Pentagon, the United States moved into a much different position with regard to the threat posed by potential biological agents that could be used against the population. In fact, the store of smallpox vaccine ready for use was found to be much smaller than needed. As a result, a spate of research has begun on smallpox and preventative measures, including both vaccine production as well as potential therapeutic modalities. The US stores of smallpox virus have not been destroyed and are again being tapped for experimental purposes, using the highest levels of containment.

Despite Jenner's success, little was understood about the dynamics of vaccine production or the reasons for generating avirulent variants of infectious agents, until the germ theory of disease was well established in the latter half of the nineteenth century. Notably, in 1885, Louis Pasteur produced the first effective vaccine for rabies virus, utilizing the technique of culturing the virus in a nonnatural host using laboratory methods of infection. In the case of rabies, Pasteur injected virus isolated from a rabid dog directly into the brain of rabbits, and found that as the virus was maintained in this way, it became **attenuated** in its ability to infect dogs, but more virulent in its ability to cause the disease in rabbits. Considering how dangerous the disease of rabies is and the fact that it can be transferred to humans by needle stick, this method of generating avirulent virus was, indeed, heroic. Current practices take advantage of much more complete understanding of culturing methods as well as better precautions against accidental infection. Still, the generation of a vaccine against a human pathogen can be risky and is a potential hazard to laboratory workers.

How a vaccine is produced

The current vaccines available for human use include those shown in Table 8.1. A number of procedures have been developed to produce a vaccine against a particular virus. Whereas Jenner's original vaccine against smallpox began as cowpox virus, the modern vaccine utilizes a virus called *vaccinia*, which is much more closely related to buffalopox virus than to cowpox virus, and is not closely related to smallpox at all! It is not known how vaccinia came to be cultured as a vaccine strain virus or when it became the laboratory entity that it now is. Although vaccinia is an example of the class of vaccines that are live viruses (**live-virus vaccine**) or attenuated viruses, it is quite unlike other attenuated viruses used as vaccines in that it has very little relationship to the virus that it protects against. This type of vaccine is often termed a **Jennerian vaccine**.

Many types of vaccines currently in wide use are produced by inactivation of the virus; these are called **killed-virus vaccines** or inactivated-virus vaccines because the virus in the vaccine cannot initiate infection. With the advent of biotechnology, the ability now exists to produce **recombinant** virus vaccines. Finally, individual viral proteins or groups of proteins either purified from the

Table 8.1 Some human viral vaccines.

Virus	Vaccine type	Route of administration
Polio	Live, attenuated	Oral
Measles	Live, attenuated	Subcutaneous
Mumps	Live, attenuated	Subcutaneous
Rubella	Live, attenuated	Subcutaneous
Rabies	Inactivated	Intramuscular
Influenza	Inactivated	Intramuscular
Yellow fever	Live, attenuated	Subcutaneous
Varicella zoster (chicken pox)	Live, attenuated	Subcutaneous
Rotavirus	Live, attenuated	Oral
Hepatitis A	Inactivated	Intramuscular
Hepatitis B	Subunit (surface antigen)	Intramuscular
Tick-borne encephalitis	Inactivated	Intramuscular
Japanese encephalitis	Inactivated	Subcutaneous
Smallpox (variola)	Live, attenuated (vaccinia)	Subcutaneous

virus itself or expressed from recombinant vectors can be utilized to generate immunity. Such vaccines are called **subunit vaccines**.

Live-virus vaccines

If a live virus is to be administered and is to elicit an immune response, it must be avirulent and cause either a mild disease or no disease at all. Such mutants are produced in an empirical fashion by serial passage of a virulent strain of the virus in cell culture multiple times. Intermediate passages are tested for virulence in appropriate animals, including primates. The process of attenuation introduces a number of point mutations into the viral genome, essentially mutating functions not required for replication but rather for pathogenesis. This technique was used to produce the Sabin strains of oral vaccine directed against the three serotypes of poliovirus.

Serial passage is a blind procedure and the results cannot be predicted. As more information accumulates about the genetic basis of virus–host interactions and virulence, specific mutants can be produced, either as deletions of regions of the genome or as site-specific changes, such that the properties of the putative vaccine can be customized.

One great advantage of live-virus vaccines is that since an actual infection takes place, both humoral and cell-mediated immune responses are stimulated. As a result, immunity develops after one or at most three exposures and usually lasts many years. A disadvantage may be the occasional reversion of virus to virulence. This can take place either by the occurrence of **back mutation** as the vaccine virus is replicating in the individual being immunized, or possibly, by a recombinational event taking place between the genome of a virus in the individual and the vaccine strain. Reversion to virulence by back mutation is a problem with the Sabin type 3 poliovirus vaccine, and virulent virus can be isolated with high frequency from the feces of individuals who have been immunized with the vaccine. While this should not be a problem with a population enjoying good waste-treatment facilities, it could pose a significant problem in mass vaccinations in countries with inadequate public health facilities.

Live-virus vaccines also have other potential problems. A major one is that they must be carefully handled and preserved with refrigeration, which makes their use in the field somewhat diffi-

cult, especially in parts of the world where reliable sources of electrical power are wanting. This problem can be partially alleviated by freeze drying (**lyophylizing**) providing the virus is stable to such treatment, but rehydration will require reliable sources of sterile material among other things.

In addition, there is always the risk of an unknown pathogen being present and undetected in the vaccine stock. As techniques for assay for adventitious contamination become more sensitive and sophisticated, this latter problem becomes less worrisome, but it is important to remember that the earliest preparations of the Sabin polio vaccine were contaminated with SV40 virus, which can replicate in humans. Luckily, this has not led to any sequelae, to date at least.

Killed-virus vaccines

Even though smallpox and rabies vaccines were attenuated viruses, most of the successful vaccines produced in the first part of this century utilized inactivated virus. An inactivated virus for a vaccine is generated from stocks of the virulent strain of the virus grown in cultured cells (or animals). This potentially virulent virus is then made noninfectious (inactivated) by chemical treatment. Originally, formaldehyde (formalin) treatment was used to inactivate virus; the original and highly successful Salk poliovirus vaccine was a formalin-inactivated preparation of the three virus serotypes. Despite its wide use in early vaccines, formalin is difficult to remove and therefore has the danger of residual toxicity. More recently, betapropiolactone is the chemical of choice to inactivate virus because residual amounts of the reagent can be readily hydrolyzed to nontoxic products.

An advantage of the killed-virus vaccines is the absence of the virus's capacity to revert to virulence, since there is no virus replication during immunization. Further, killed-virus vaccines can be stored more cheaply than can live-virus vaccines. These advantages are balanced against the fact that the vaccine must be injected, multiple rounds of immunization are generally required, and vaccination does not result in complete immunity because an active infection does not occur. This latter complication also means that immunity is usually nowhere near as prolonged as it is with a live-virus vaccine.

Recombinant virus vaccines

It is possible to use the process of **genetic recombination** to introduce the genes for proteins inducing protective immunity into the genome of another virus, which itself might be avirulent. For example, the capsid protein gene of hepatitis B virus might be used. The methods and general principles behind the generation of such *recombinant virus* are detailed in Chapter 14. The genes introduced either could replace genes not required for replication of the carrier virus when it is used as a vaccine, or could be added to the viral genome. Such a recombinant virus could then be used to vaccinate an individual, leading to generation of immunity against the proteins in question. Since the carrier virus would be able to replicate, it would (hopefully) be able to generate a full repertoire of immune responses against the immunizing protein or proteins. Further, the carrier could be extensively modified to ensure that it was absolutely avirulent. Possible candidate vectors for such carrier viruses include members of the poxviruses, the herpesviruses, and the adenoviruses, but vaccinia virus has been subjected to the majority of developmental studies to date.

Recombinant viruses are currently being tested for use as vaccines. There are two theoretical problems with the use of recombinant virus vaccines. First, it is not clear that the same level of immunity or repertoire of immune responses can be evoked from the expression of a "passenger" protein that has no function in the life cycle of the virus expressing it. Second, once a good carrier virus is produced, its use in a vaccine would provoke immunity against itself. This would preclude use of the same carrier virus for another vaccine at a later time. Thorough testing will resolve the first problem, and if a truly effective vaccine were made against an important pathogen, the second problem could be readily ignored.

Subunit vaccines

Since the desired immune response is most often directed against a critical surface protein of a pathogenic virus, this protein by itself could be used as a vaccine if it were properly presented to the immune system of the vaccine recipient. A subunit vaccine can be prepared by purification of the protein subunit from the viral particle, or by recombinant DNA cloning and expression of the viral protein in a suitable host cell, either bacterial or yeast. Some of the general procedures for utilizing either approach are described in Part III.

Direct administration of a protein will not induce a cell-mediated response the way a live-virus vaccine would. Still, the advantages of a subunit vaccine include the lack of any potential infectivity, either mild in the case of the attenuated strains or severe in the case of the virulent strains or revertants. In addition, subunit vaccines may serve when the virus in question is extremely virulent or when it cannot be grown conveniently in culture.

There are a number of important general problems with the use of subunit vaccines that may not be amenable to easy solution. Still, the speed with which they can be produced makes them very attractive candidates for specific uses. A subunit vaccine currently is available using the hepatitis B virus surface antigen obtained by expression of a cloned gene in yeast cells. This vaccine has been successfully used in Taiwan and its use appears to have reduced the incidence of primary liver cancer in young children there.

Future trends in vaccine development and design

There is a constant effort to improve the efficiency of vaccines in terms of the immunity they provide while at the same time minimizing risks and expenses. It is too early to tell whether there will really be "quantum leaps" in these areas, but several approaches are being actively pursued in hopes of achieving the overall goals.

One approach is to use the technique of transfection, which was briefly described in Chapter 6. In this method, a fragment of DNA containing one or several genes encoding proteins, or portions thereof, whose antigenicity would foster protection is engineered to be expressed via the action of its own or a heterologous promoter. The techniques for generating suitable DNA fragments are generally outlined in Chapter 14. The idea behind DNA vaccines is that if antigen-presenting cells could take up the DNA and express the antigenic proteins, protection could be fostered without the need of inactivated or attenuated virus. Further, methods for the delivery and storage of such a vector might well be cost-effective. While it may seem surprising, considering the inefficiency of the transfection process, DNA-based vaccines have been effective against HSV and several other viruses in animal tests. To date, however, human tests have been rather ambiguous with a major problem being difficulties in getting high antibody titers without **adjuvants**. Adjuvants are compounds added to antigens being prepared for introduction into the host, which increase inflammation leading to heightened infiltration with cells of the immune system. Such inflammation is usually quite painful, however, and their general use is forbidden in humans and discouraged in animals.

Another approach is to express the antigenic protein or antigenic portion of a protein in a form that could be ingested and still generate protective immunity. Obviously, such an antigen would need to be able to survive the digestive system and be assimilated by antigen-presenting cells. While this would appear to be a tall order, the rewards would be immense. Currently, efforts are underway to generate transgenic plants in which antigenic peptides are incorporated into cereal grains, legumes, and even potatoes so that food sources could be made available to provide protection against one or another major human or animal disease. This might be especially important to controlling infectious disease in developing nations.

Problems with vaccine production and use

The great success of a variety of vaccines, including those against smallpox, measles, polio, and rabies, has led to a serious commitment by the World Health Organization and other public health agencies to develop and distribute vaccines for protection against a variety of viral diseases, especially those affecting children. The Expanded Program on Immunization (EPI) of the World Health Organization has targeted six childhood diseases for global immunization, two of which are viral: poliomyelitis and measles.

Two of the major problems that arise to subvert such strategies are genetic instability and heat sensitivity of the vaccines. As mentioned already, in certain cases, such as the type 3 Sabin strain of the oral poliovirus vaccine, revertants that are virulent can occur. Such instabilities can lead to vaccine-associated cases of the disease that is the target of the vaccination. These instabilities may be overcome with the use of recombinant vaccinia virus constructs, where the only gene expressed from the virulent virus is that of the surface antigen used to stimulate the immune response.

A serious problem with administering vaccines in the Third World is the need for refrigeration of some of the preparations. The requirement for a "cold chain" from the site of manufacture to the site of the vaccine's use is critical to efficacy of the immunization. As a result, a good deal of development has gone into two areas, one mechanical and one biological. Portable refrigerators and adequate cold packaging are constantly being redesigned. Accompanying this is the search for vaccine constructs that can withstand ambient temperatures during shipping and delivery. The development of heat-stable and yet highly immunogenic vaccines is a high priority for the World Health Organization and other organizations working to save children from the ravages of these diseases. The campaign for the eradication of poliovirus has made major advances. As of May, 2002, only a few areas in the world still report reservoirs of wild viruses, notably India, Pakistan, and Nigeria.

These obstacles are, at least, surmountable. Others may not be. The most obvious one is that protection against some viruses just may not be controlled by vaccination. This obstacle could be due to some truly significant technical problem with biological specifics of the virus that would preclude it being tractable to scientific study and exploitation, but such technical problems have been addressed and overcome in the past.

The most serious problems are socioeconomic, and these may well persist—all efforts of scientists and medical researchers to the contrary. It is very expensive to move from discovery and characterization of a virus disease to production and use of a truly effective vaccine. This expense will only be borne by for-profit corporations provided they can get a return on their investments. While governments also may be able to cover the costs of vaccine production and application, it is clear that those ultimately supporting such efforts, the taxpayers, must be able to see the need for this expense. This requires education, information, and above all, good will. These items can be either plentiful or in short supply, depending on historical and political background of the disease in question. Clearly, no general solution to such problems can be envisioned. Each disease will need to be dealt with as it occurs. Results inevitably will show both great success and great instances of lost opportunities.

EUKARYOTIC CELL–BASED DEFENSES AGAINST VIRUS REPLICATION

Interferon

The clonal selection of antibody-producing B cells and effector T cells provides an exquisitely sensitive means for the infected host to specifically deal with invading microorganisms and viruses, and to eliminate virus-infected—and thus damaged—cells. However, it does take time for an ef-

fective defense to be mounted. There are more rapid if less specific defenses available. These include inflammation, temperature rise, and interaction with nonspecific phagocytic cells of the immune system.

The ability of cells to produce interferon (IFN) provides another important rapid response. The cells capable of such a response contain a complex set of gene products that can be induced in direct response to virus attack and that render neighboring cells more resistant to virus replication. IFN has a large number of biological effects including the following:

- inhibition of virus replication in IFN-treated cells (target cells);
- inhibition of growth of target cells;
- activation of macrophages, natural killer cells, and cytotoxic T lymphocytes;
- induction of MHCI and MHCII antigens and Fc receptors; and
- induction of fever.

A protein secreted from a cell in order to induce specific responses in other cells having specific receptors for it is generally termed a **cytokine**. IFN is one major group, but there are many others. For example, the proliferation of B cells responding to the presence of an antigen and a helper T cell is the result of specific lymphocyte cytokines (an interleukin) secreted by the helper cell.

It was shown in the late 1950s that culture media isolated from fibroblasts infected with certain viruses contained a substance or substances that would render uninfected cells more resistant to infection with similar viruses (i.e., the infected cells produced a substance that interfered with subsequent infection). Classic protein fractionation methods demonstrated that this substance—IFN—is actually a group of proteins, all very stable to acid pH and all able to function at very high dilutions so that only a few molecules interacting with a target cell render that cell resistant to viral infection.

There are two basic interferons, I and II. Type I IFNs are stable at acid pH and heat. All are distinct and are encoded by separate cellular genes, but all have the same general size and have roughly similar effects. The two major type I IFNs are IFN-α, expressed by leukocytes, and IFN-β, expressed by fibroblasts. There are at least three others in this class. There is only one type II IFN, IFN-γ, expressed by T and (possibly) B lymphocytes. Type I IFNs are most active against virus infections while IFN-γ modulates the immune response. Further, it appears to have some antitumor activity. All IFNs are very species specific; therefore, human IFN is active in human cells, mouse IFN in mouse cells, and so on.

The characterization of IFN followed by cloning and expressing IFN genes resulted in a lot of excitement concerning its potential use as an antiviral and anticancer drug. Its promise is yet to be realized; it is now known that IFN proteins are very toxic to cells and methods for its efficient delivery to regions of the body where it would be therapeutic have yet to be perfected. Thus, although it is clear that the IFN response has a role in natural recovery from virus infection and disease, its complete therapeutic potential is yet to be fully exploited.

Induction of interferon

IFN induction takes place in the infected cell in response to viral products. A major inducer is double-stranded RNA (dsRNA), which is generated in infections by many RNA and DNA viruses. In addition, some viruses (e.g., reoviruses) use dsRNA as their genetic material. A single molecule of dsRNA can induce IFN in a cell under the appropriate conditions.

Because IFN is expressed from cellular genes, only cells that are relatively intact and functioning when dsRNA is present will express it. The requirement for continuing cell function is one reason why viruses that replicate slowly are good IFN inducers. When a virus capable of rapid replication and quick host-cell shutoff initiates an infection under optimal conditions, little IFN is generally induced.

The antiviral state

IFN inducers cause the cell in which they are present to synthesize IFN. This protein is secreted and interacts with neighboring cells to put them in an antiviral state in which **antiviral effector molecules (AVEMs)** are expressed. Cells that have been induced by IFN express new membrane-associated surface proteins, have altered glycosylation patterns, produce enzymes that are activated by dsRNA to degrade mRNA, and inhibit protein synthesis by ribosome modification. These effects are outlined in Fig. 8.1. The antiviral state, thus, primes the cell so that it can trigger a number of responses to virus infection. Just as in the case of IFN induction, the triggering molecule is dsRNA.

To date, expression of more than 300 cellular genes has been demonstrated to be induced or enhanced by IFN—many of these are involved in the establishment of the antiviral state. One—Mx—protein appears solely directed against influenza virus infections, although it also has activity against vesicular stomatitis virus (VSV). Some of these proteins that serve as antiviral effector molecules are listed in Table 8.2. Different mechanisms are involved in the different cellular responses to virus infection. Changes to the cell surface may make it more difficult for viruses to attach and penetrate. When presented with dsRNA, the antiviral cell activates **2′, 5′-oligoA synthetase** and enzymatic activity that is induced by IFN and that produces a bizarre oligonucleotide, 2′, 5′-oligoA. This, in turn, activates a latent mRNA endonuclease (RNAse-L). Finally, this endonuclease rapidly degrades all mRNA (viral and cellular) in the cell. The IFN-primed cell also expresses a **dsRNA-dependent protein kinase (PKR)** that causes modifications resulting in partial inactivation of the translational initiation factor eIF2 in the presence of dsRNA. This makes the cell a poor producer of virus proteins, and thus, an inefficient producer of new infectious virus, since all molecular processes are inhibited.

The action of IFN on cells is not always beneficial. Since IFN also acts as a negative growth regulator (the basis of its activity against tumor cells), its presence can interrupt the function of differentiated cells and tissues. Also, one cellular response to virus infection is the induction of a number of cellular genes that lead to programmed cell death (apoptosis); this process is outlined in more detail in Chapter 10. Such cell death is good for the host, since the reduction of virus replication is well worth the loss of a few cells, but in some cases IFN can block the induction of apoptosis and, thus, actually protect virus-infected cells! Further, IFN causes tissue inflammation and high fevers.

The toxic effects of the IFN response are alleviated by its being carefully balanced and controlled so that it is maintained only as long as needed. The amount of IFN produced by any given infected cell is very small so that only the cells within the immediate vicinity are affected and converted to the antiviral state. If the cells are not infected, they may eventually recover and resume their normal processes.

Measurement of interferon activity

IFN activity is measured in a number of ways because there are so many different types and different effects. An easy and rapid method in virology is the *plaque reduction assay*. This method is quite sensitive; it has been claimed that as few as 10 molecules of IFN can be detected with its careful use. *Plaque assays* are described in detail in Chapter 10, but in essence the process is as follows: Duplicate cell cultures are set up (see Chapter 9), and one culture is treated with IFN for several hours to allow the potential antiviral state to develop. Both are then infected with the same number of infectious units of indicator virus (often VSV since it is so sensitive to IFN). The IFN-treated cells will produce fewer and smaller centers of virus infection (*plaques*) than will the untreated control. Serial dilutions of the original sample can be made until the effect is no longer seen, and a measure such as *median effective dose* **(ED_{50})** can be calculated. The ED_{50} is that dilution in which the number of plaques is reduced by 50% or plaques are 50% smaller than untreated ones. This reduction can be related to units of IFN activity and to the number of IFN molecules present.

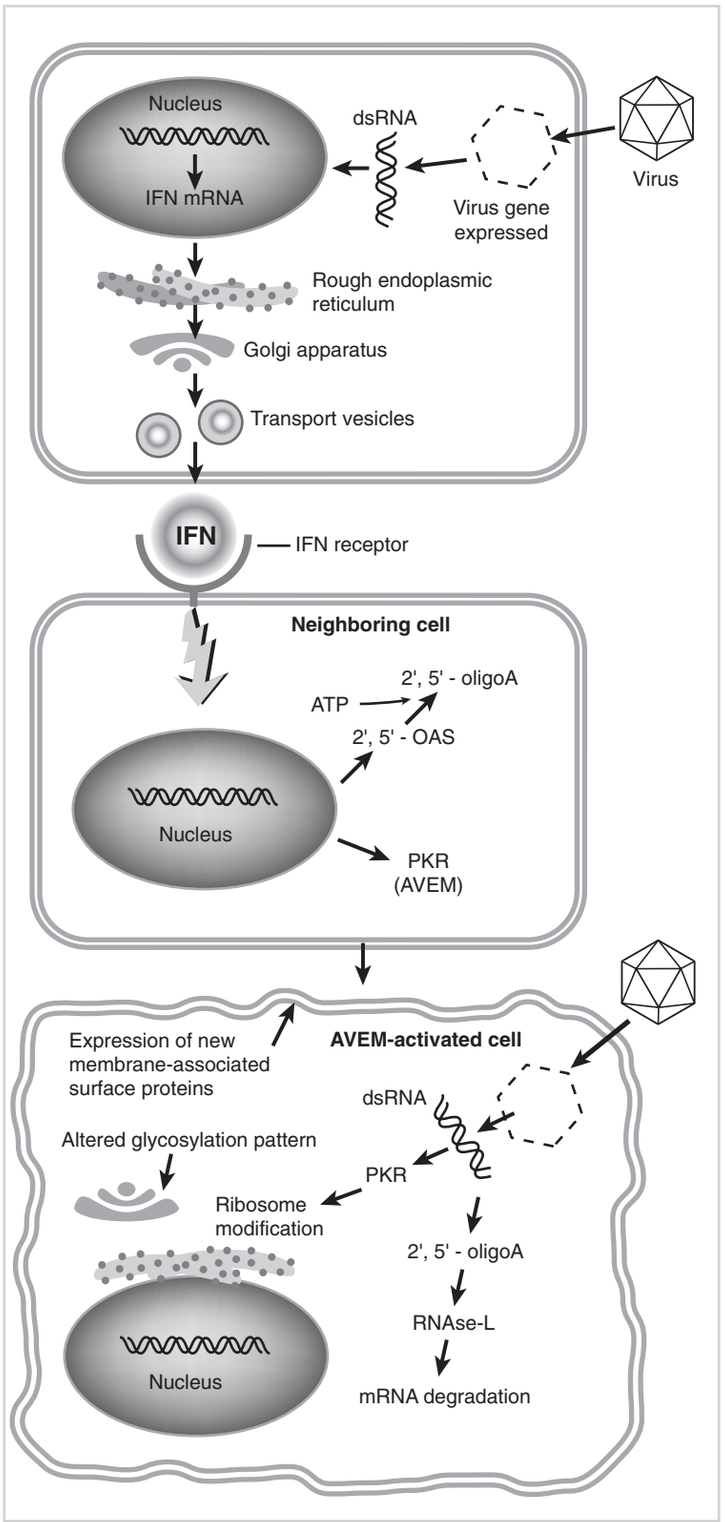


Fig. 8.1 The cascade of events leading to expression of interferon (IFN) and induction of the antiviral state in neighboring cells. The interferon producer (dsRNA) produced during virus infection leads to an infected cell secreting small numbers of the IFN proteins, which are extremely stable glycoproteins. These interact with neighboring cells to induce the antiviral state in which a number of antiviral effector molecules (AVEMs) are expressed and can be triggered by the presence of dsRNA to alter the cell to markedly reduce the yield of infectious virus. (PKR, dsRNA-dependent protein kinase; 2′, 5′-OAS, 2′, 5′-oligoA synthetase; dsRNA, double-stranded RNA.oligoA.)

Table 8.2 Some antiviral proteins induced or activated by interferon.

Protein	Function
2′,5′-oligoadenylate synthetase	Activates latent RNAse-L
dsRNA-dependent protein kinase (PKR)	Phosphorylates eIF2
RNAse-L	mRNA degradation
IFN-1, -2	Transcriptional regulation
MHC-I	Antigen presentation
Mx	Specific blockage of influenza (and vesicular stomatitis virus) entry

ANTIVIRAL DRUGS

All drugs effective against pathogenic microorganisms must target some feature of the pathogen's replication in the host that can be efficiently inhibited without unduly harming the host. Some drugs are effective against the earliest stages of infection and can be given to an individual before he or she is exposed or for a short time after exposure. Such prophylactic use cannot be effective in large populations except under very specific circumstances (e.g., military personnel prior to entering a biological hazard zone).

Despite the value of some prophylactic drugs, the most desirable drugs are ones that can effectively interrupt the disease at any stage. The dramatic effectiveness of penicillin in treating numerous bacterial infections after World War II has proved a model for such drugs, but the earliest specific antibacterial drugs were made up of complex organic molecules containing mercury that Ehrlich utilized to combat syphilis at the end of the last century. He termed these "magic bullets" and developed them to reduce the toxicity of mercury, whose use as an antisyphilitic agent was known to be effective since the Renaissance in Europe. Perhaps not surprisingly, Ehrlich's success was marred by the anger of some moralists who argued that the disease was a punishment for sin! While science progresses, society does so more slowly, and in the past few years similar arguments have been made against developing treatments of AIDS.

The problem of therapeutic drug toxicity is a continuing one. Many effective inhibitors of metabolic processes, even if more or less specific for the pathogen, will have undesirable side effects in the person being dosed. The general ratio of benefit of a drug to its undesirable side effects is termed the **therapeutic index**. Determination of a drug's therapeutic index requires extensive animal testing and extensive documentation, and is a major factor in the expense involved in developing effective pharmaceuticals for any purpose.

Targeting antiviral drugs to specific features of the virus replication cycle

Given the fact that viruses are obligate intracellular parasites, it is easy to understand why a chemotherapeutic approach to halting or slowing a viral infection is difficult to achieve. Unlike bacterial cells, which are free-living, viruses utilize the host cell environment for much of their life cycle. Therefore, chemical agents that inhibit both virus and host functions are not a good choice for therapy.

The preferred strategy has been to identify the viral functions that differ significantly from or are not found within the host and are therefore unique. For each virus of clinical interest, a good deal of effort has been expended on understanding the virus's life cycle and attempting to develop drugs that can specifically block critical steps in this cycle. Table 8.3 lists targeted stages in the virus life

Table 8.3 Some targets for antiviral drugs.

Step in virus life cycle targeted	Molecular target of inhibitor	Example
Virus attachment and entry	Surface protein-receptor interaction	Receptor analogues, fusion protein amantadine
DNA virus genome replication	Viral DNA polymerase	Acyclovir
RNA virus genome replication	Viral RNA replicase	(Theoretical)
Retrovirus – reverse transcription	Reverse transcriptase	AZT, ddC, ddI
Retrovirus – integration	Integrase	(Theoretical)
Viral transcriptional regulation	HIV *tat*	(Theoretical)
Viral mRNA posttranscriptional processing (splicing)	HIV *rev*	(Theoretical)
Virion assembly	Viral protease	Protease inhibitors (ritonavir, Saquinovir)
Virion assembly	Capsid protein–protein interactions, budding	Rimantadine, protease inhibitors

cycle along with examples of existing or proposed agents that could block the cycle with some measure of specificity. With each of these, the problem of resistant mutants always arises, leading to limitation of the drug's usefulness.

Acyclovir and the herpesviruses

The development of acycloguanosine (acG) for use in herpesvirus infections marked a great advance in the chemotherapy of viral infections. This compound, prescribed under the name acyclovir, is the first of the nucleoside analogues that are chain-terminating inhibitors. When the triphosphorylated form of acycloguanosine is incorporated into a growing DNA chain in place of guanosine, no further elongation can take place because of the missing 3′ OH. The structure is shown in Fig. 8.2.

The specificity of acyclovir for herpesvirus-infected cells results from two events. First, after the nucleoside is transported into the cell, it must be triphosphorylated to be utilized as a substrate for DNA replication. The first step in this process, the conversion of acG to the monophosphate (acGMP), requires the presence of the herpesvirus-encoded thymidine kinase (TK). Following this, a cellular enzyme is able to add the next two phosphates, producing the triphosphate acGTP. This acGTP inhibits the synthesis of viral genomes by acting as a substrate for herpesvirus DNA polymerase. When this happens, the DNA chain is terminated — no additional bases can be added because of the missing 3′-OH group. The drug will inhibit the viral enzyme about 10 times more efficiently than it will the cellular DNA polymerases. In addition, when acGTP is utilized by the herpesvirus polymerase as a substrate, the resulting viral DNA synthesis is halted.

As a result of the requirement for herpesvirus TK and the inhibition and chain termination of herpesvirus DNA synthesis, acyclovir is highly specific for herpes-infected cells and is nontoxic to uninfected cells. Acyclovir has been used successfully as both topical and internal applications with both HSV type 1 and HSV type 2.

Chemical modification of aG's structure has resulted in ganciclovir [9-(1,3-dihydroxy-2-propoxy)methylguanine] (Fig. 8.2). This drug has the same properties as acG, except that it is specific for cells infected with cytomegaloviruses. Unfortunately, this drug has a severe toxicity when given intravenously and must be used with caution.

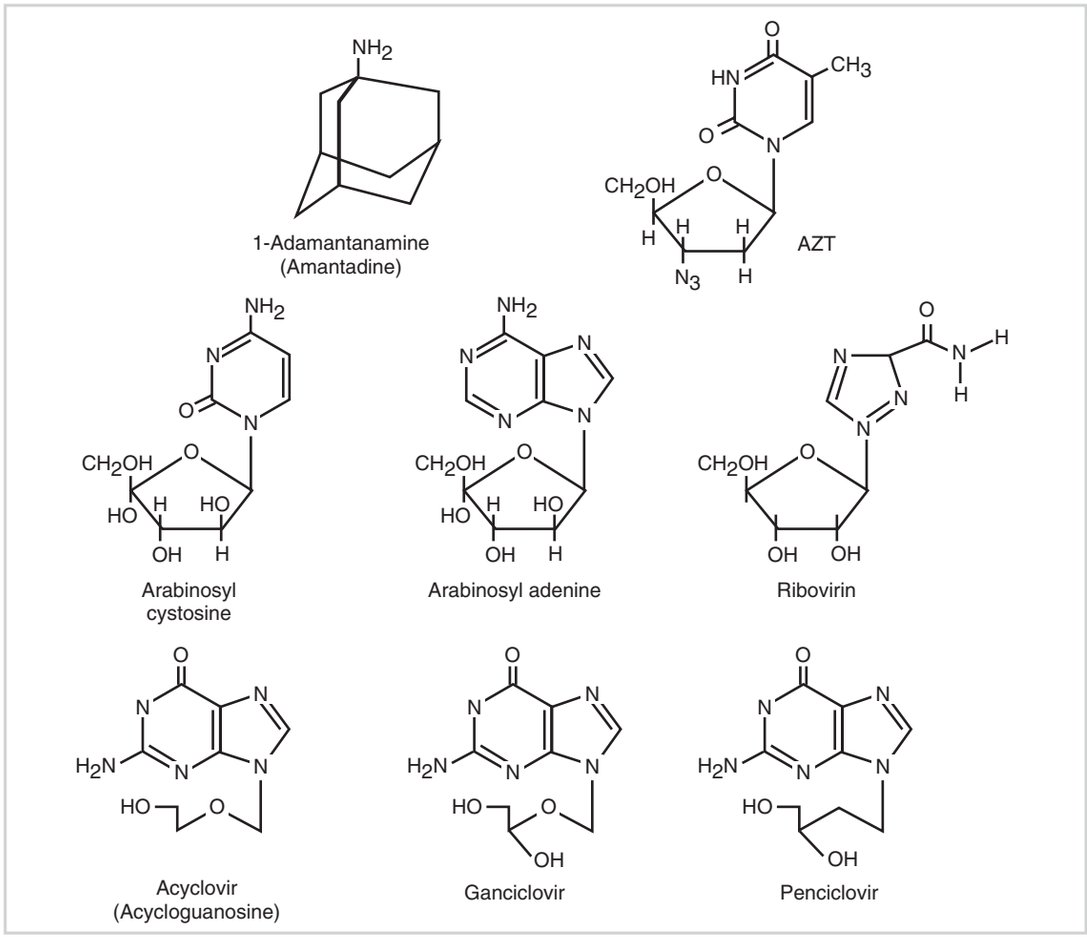


Fig. 8.2 The structure of some currently effective antiviral drugs.

Amantadine and influenza A viruses

Type A influenza viruses enter their host cells by means of the receptor-mediated endocytotic pathway. In this process, the viral hemagglutinin molecules in the membrane of the particles undergo a conformational change when the pH of the endocytotic vesicle is lowered to around 5 after fusion of the vesicle with an acidic endosome. At this lower pH, the viral membrane undergoes fusion with the vesicle membrane and viral nucleocapsids enter the cell cytoplasm (see Chapter 6).

Two compounds that have been developed interfere with the ability of the cell to change pH within influenza virus-modified vesicles—amantadine and rimantadine. Amantadine (1-aminoadamantane hydrochloride), whose structure is shown in Fig. 8.2, is a basic primary amine, and can prevent the acidification that is essential for completion of viral entry.

The drug also works during virus assembly and maturation. At this time, newly synthesized hemagglutinin must be transported to the plasma membrane prior to particle budding. During this transport it is important that the exocytotic vesicle not become acidified, or the hemagglutinin will assume its fusion conformation and be unavailable for correct assembly. The small viral protein M2 serves as an ion channel protein in the vesicle membrane that blocks this acidification. Amantadine inhibits the action of M2 and thus serves to block correct maturation of type A flu virus particles.

Amantadine must be administered as early as possible after the initial infection in order to have any efficacy in reducing disease symptoms. Prophylactic administration of the drug during epidemics is not considered to be a practical approach in the Western World because of the high dosages required and problems with side effects; it has been used with some success in isolated flu outbreaks in Russia, however.

The related drug rimantidine appears to have fewer side effects and is now the preferred drug. Viral mutants resistant to both these drugs are readily observed; all have alterations in the M2 protein.

Chemotherapeutic approaches for HIV

When it was discovered that the viral agent that causes AIDS is, in fact, a retrovirus, the immediately obvious goal was the development of a drug that could specifically inhibit the unique viral replicative enzyme of the retroviruses: reverse transcriptase. A drug that had been developed as an antitumor agent was found to inhibit this enzyme: 3′-azido-2′ 3′-dideoxythymidine, commonly called azidothymidine or AZT (see Fig. 8.2). Like acG, this drug, when transported into the cell and phosphorylated, can be utilized by the HIV polymerase to produce a chain termination because of the missing 3′ OH. Although the drug exhibits a good specificity for HIV reverse transcriptase compared with cellular DNA polymerases in vitro, severe toxic effects are still seen when the drug is administered to patients. Most importantly, because of the high mutability of HIV replication (see Chapter 20), the development of AZT-resistant mutants occurs rapidly.

Other nucleoside analogues have been produced for therapeutic use. Notable are dideoxycytidine (ddC) and dideoxyinosine (ddI). Since development of resistance to these two drugs does not occur in the mutation of the virus to AZT resistance, the drugs are commonly used in combination.

The most recent advance in the chemotherapeutic treatment of HIV infection has been production of the class of drugs known as *protease inhibitors*. Retroviruses, as well as many other viral families, require proteolytic processing of initial translation products so that the active viral proteins can be made. For HIV (like all retroviruses), this is carried out by a viral-encoded protease. The drugs known commercially as Saquinovir or ritonavir act by inhibiting HIV protease. As a result, the posttranslational processing of viral products as well as the final proteolytic steps required during viral assembly are blocked (see Chapter 20).

Multiple drug therapies to reduce or eliminate mutation to drug resistance

The most promising therapy against HIV now being used involves the use of multiple drugs. The original protocol required the simultaneous administration of AZT, another nucleoside analogue such as ddC, and a protease inhibitor. Initial results with this cocktail were quite impressive. Clinical observations of AIDS patients showed reversal of symptoms and rebound of $CD4^+$ cellular levels. Viral loads decrease and circulating virus all but disappears. With the wide application of these therapies in the United States, most cities reported a decrease in deaths from AIDS by the end of 1997. Currently, the therapy is called Highly Active Antiretroviral Therapy (**HAART**) and entails the use of four inhibitors. For instance, one treatment uses a protease inhibitor (lopinavir) along with three reverse transcriptase inhibitors (3TC, tenofovir, and efavirenz). Ongoing trials are even using as many as a five-inhibitor combination. In all of these cases rapid reduction in viral load is the objective.

This exciting picture must be tempered by words of caution. First, the therapy itself is quite complicated and expensive. It certainly cannot be readily applied to developing nations and to individuals at risk in this and other developed countries who do not have the financial or emotional resources required for the treatment, which requires a lot of self-discipline. Patients on the three-drug cocktail must take on the order of 20 pills or more each day. If dosages are skipped or missed,

there is the great danger of developing resistant mutants that would effectively destroy progress made by the patient. This fear was recently underlined by the finding that even after long periods of treatment, HIV genomes still exist in critical lymphocytes and can be recovered as infectious virus if drug is removed. At this point, it is assumed that the therapy must be followed for the rest of the patient's life. There are no data yet on the long-term effects of this therapy. Thus, a major question yet looms: What will be the ultimate effect on the patient?

Other approaches

The goal of developing methods for specifically targeting virus replication is so important that other methods are being actively pursued. One approach is *precise targeting*. The toxicity of many antiviral drugs is exacerbated by the fact that the drug must be presented to the whole body, thus affecting tissue that is free of virus. Localized HSV reactivation can be effectively treated with iodouridinedeoxyriboside (IUdR) by local application to the lips or genital area, even though this drug is relatively toxic when taken internally. Presently, research is directed toward the development of protocols that combine methods for ensuring the delivery of small amounts of even highly toxic drugs only to virus-infected tissue.

A second promising approach is the generation of short oligonucleotide polymers that have sequences complementary to specific portions of viral mRNA molecules. Such **antisense oligonucleotides** can be designed to specifically inhibit the translation of an important viral gene product with little or no attendant toxicity. Some antisense drugs are already being clinically tested.

BACTERIAL ANTIVIRAL SYSTEMS – RESTRICTION ENDONUCLEASES

Bacterial cells do not have the ability to produce antibodies or IFN as do animal cells. However, they have evolved mechanisms through which viral infections can be aborted, or at least limited. **Bacterial restriction** is the most common type of antiviral defense. The discovery of bacterial restriction systems not only led to a basic understanding of bacterial–viral interactions but also provided one of the most critical set of tools used in modern molecular biology and biotechnology: **restriction endonucleases**.

Bacterial cells can "mark" their own DNA for identification by the covalent addition of methyl groups to critical bases within the nucleic acid. For example, adenosine residues can be enzymatically converted to 5-methyl adenosine by transfer of a methyl group from *S*-adenosylmethionine, catalyzed by bacterial enzymes called *DNA methylases*. These modifications are made at specific sites within the DNA. These sites are specific sequences of 4, 5, 6, 7, or 8 nucleotides; such sequences often display a dyad symmetry (GAATTC, for example) for *Eco*RI, one of the first restriction endonucleases characterized. Note that the sequence reads the same on both DNA strands; that is, it is a **palindromic sequence**.

Any DNA entering cell cytoplasm that does not have the host bacteria's specific modifications at these sites will be cleaved with a **restriction enzyme** that can recognize the unmodified sequence. Thus, the system functions to restrict the growth of a virus whose genome has found its way into the cell. In effect, the host cell can recognize its own DNA as well as foreign viral DNA and destroy the invader before viral gene expression begins.

There are some cells in which a viral genome will be able to avoid the restriction enzymes for one of a number of reasons (perhaps the concentration of enzyme is too low to act quickly enough). These cells will produce progeny virus particles whose DNA is modified (methylated) in the same pattern as the host's DNA since host enzymes will work on this DNA as it is replicated. As a result, these progeny particles will be able to grow productively on cells of this particular restriction

modification type. Thus, a balance is achieved in a population of cells between lytic replication of the virus with subsequent destruction of the host and complete inhibition of virus growth.

Later sections will explain that the exquisite specificity of restriction endonucleases (of which more than 500 are now known) makes them extremely valuable tools for manipulating DNA molecules. They can be used to cut genomes into specifically sized pieces, and are vital to the isolation and direct manipulation of individual DNA pieces containing genes of particular interest. The Nobel Prize was awarded in the early 1980s to W. Arber, H. Smith, and D. Nathans for their characterization of restriction endonucleases, and it is fair to say that this single discovery is probably the most directly seminal in the development of modern molecular genetics and recombinational DNA technology.

QUESTIONS FOR CHAPTER 8

1 Describe how bacterial restriction enzymes can cleave bacteriophage DNA as a part of a host defense mechanism.

2 Interferons (IFNs)-α and -β are expressed in response to a virus infection and are released from the cell in which they are produced. IFNs induce an antiviral state in other neighboring cells.

a Which cellular process is inactivated when IFN-treated cells are infected with a virus?

b One arm of the IFN-induced antiviral state is the synthesis of 2′, 5′-oligoA in response to viral infection. In one sentence or a simple diagram, what is the effect of this on the cell?

c Another arm of the IFN-induced antiviral state is activation of the protein kinase in response to viral infection. In one sentence or a simple diagram, what is the effect of this on the cell?

d All cells contain the genes for IFNs. IFN synthesis is stimulated by virus infection. Would you expect a cell that has been *treated with IFN* to synthesize IFN in response to a viral infection? Explain your answer.

3 The IFN response is one of the two major defense systems of animals in reaction to virus infection. The table below lists several activities that are associated with this response. Indicate which, if any of them, might be readily observed in cells before or after IFN treatment, with or without virus infection.

	Uninfected		Virus Infected	
Activity	Normal cells	IFN-treated cells	Normal cells	IFN-treated cells
mRNA for IFN found in the cells				
mRNA for 2′,5′-oligo A synthase found in the cells				
2′,5′-oligo A found in the cells				
Inactive protein kinase found in the cells				
Receptor for IFN found on the surface of the cells				

PART II Problems

1 The table below shows the properties of the genomes of three different viruses. The data were obtained as follows: Nuclease sensitivity was measured by the ability of deoxyribonuclease (DNase) or ribonuclease (RNase) to destroy the genome (a "+" means sensitivity). The ability of the genome to act as mRNA was tested by incubating it in a cell-free system. If amino acids were incorporated into protein, the data are shown as a "+." Finally, the virus particles were tested for the presence of a virion polymerase. If an enzyme was present, the data show whether it could polymerize deoxynucleotide triphosphates (dNTPs) or nucleoside triphosphates (NTPs).

	Genome properties				
	Nuclease sensitivity?		Can genome be an	Virion polymerase?	
Virus	DNase	RNase	mRNA?	with dNTPs	with NTPs
#1	–	+	+	–	–
#2	–	+	–	–	+
#3	–	+	+	+	–

For each virus, indicate the strategy of the genome, using the Baltimore classification. What is the nature of the product of the virion polymerase when present?

2 Interferons are synthesized by cells in response to many different viral infections. The common result of the interferon-induced antiviral state is the cessation of protein synthesis. Predict the effect of the following treatments of the indicated cell on *protein synthesis in that cell*. (Assume, for the purpose of this question, that the virus does not inhibit cellular protein synthesis as a result of the infection.)

	Viral infection of cCell	Insertion of dsRNA into cell
Normal cell		
Interferon-treated cell		

3 You wish to produce a subunit vaccine for a *positive-sense RNA virus* that will stimulate the production of neutralizing antibodies in the person receiving it. Indicate which of the following viral proteins would be a logical candidate for such a subunit vaccine and state a brief justification.

a Viral capsomer protein
b Viral protein that is bound to the RNA genome inside of the virion
c Viral RNA polymerase

4 Each year in late winter a behavioral "disorder" engulfs the people of New Orleans, Louisiana, reaching a climax on the day before Ash Wednesday. Together with virologists at Louisiana State University, you have isolated a virus from the affected people that you suspect is responsible for this condition. You have named the new isolate Mardi Gras virus (MGV). You have found a convenient host cell in which to grow MGV in the laboratory. The following table lists some of the properties of MGV you have discovered.

Experiment	Initial Data for Mardi Gras Virus Observation
Physical nature of the virion	Electron microscopy (EM) reveals 100 nm particles; shape indicates presence of envelope with visible surface projections; ether destroys particle integrity.
Chemical nature of viral genome	Digested with RNase; degraded by alkali; resistant to DNase.
Informational nature of viral genome	Genome cannot be translated in cell-free protein synthetic system.
Enzymatic analysis of virion	With NTP precursors: catalyzes RNA synthesis; with dNTP precursors: no reaction.
Biological analysis of virus	HeLa cells (human): attachment and penetration (observed by EM) and progeny virus produced; AGMK cells (simian): attachment and penetration (observed by EM) but *no* progeny virus produced.

a What would you predict to be the effect of treatment with ether or other lipid solvents on the infectivity of MGV?
b To which Baltimore class would you assign MGV? Give two reasons for this classification, based on the data in the table.
c Based on the data in the table, would you say that MGV is a human or a simian virus? Justify your answer briefly with reference to the data.

5 If a virus has a negative-sense RNA genome, what enzymatic activity (if any) will be found as part of the virion structure *and* what will be the first step in expression of the viral genome?

6 Influenza viruses gain entry into their host cells by attachment to *N*-acetylneuraminic acid residues on the cell surface, followed by receptor-mediated endocytosis. Predict what effect the treatments shown in the table below will have on (a) the attachment of an influenza virus to a susceptible host cell, *and* (b) the subsequent uncoating of influenza virus in the same cell. Use a "+" to indicate that the event will take place or a "—"

to indicate that it will not take place. Note: In each case it is assumed that the events would be occurring in the same cell that has undergone the treatment.

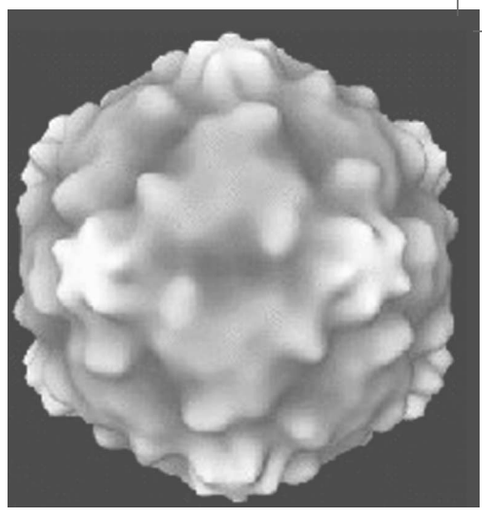
Treatment	Attachment?	Uncoating?
Treatment of the host cell with neuraminidase		
Treatment of the host cell with NH_4Cl, which prevents lowering of the lysosomal pH		
Treatment of the host cell with actinomycin D, which prevents synthesis of messenger mRNA		

7 Cells produce mRNA by transcription of their DNA genomes. In contrast, single-stranded RNA genome viruses have three different strategies with respect to viral mRNA production. Briefly describe the production of mRNA for each of the following viruses.

a Poliovirus
b Vesicular stomatitis virus
c Rous sarcoma virus

8 Infection of a human with influenza virus can trigger both host defense systems: the interferon response and the immune response. In the table below, indicate with a "Yes" or a "No" regarding which of the events is characteristic of which defense system (either, both, or neither).

Event	The interferon response	The immune response
Both host and viral mRNA are degraded in the cell after infection.		
A fragment of viral protein in complex with class I MHC is displayed on the surface of the infected cell.		
The virally infected cell dies.		
Capped mRNA is no longer translated in the infected cell.		



Additional Reading for Part II

Note: see Resource Center for relevant websites.

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