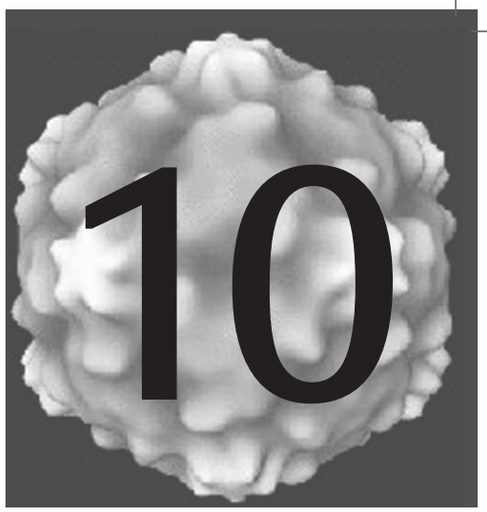


CHAPTER 10

Replicating and Measuring Biological Activity of Viruses

Cell culture techniques

Growing and maintaining cells in the laboratory is an absolute necessity for any molecular biological investigation. Because viruses must replicate within the cell they infect, their study is greatly enhanced by the ability to maintain cultures of the cells in which the viruses grow most conveniently for the study at hand. Ultimately, cell culture involves taking a representative sample of cells from their natural setting, characterizing them to a sufficient degree so that their basic growth properties and any specific functional properties are known, and then keeping them in continuous or semicontinuous culture so that they are in ready supply. Depending on the type of virus being studied, and the specific property of that virus of interest, this task can be routine or daunting.

Maintenance of bacterial cells

The study of bacterial viruses provided the model for the study of all viruses because it was convenient to replicate such viruses in easily grown bacterial cell cultures. Some bacterial cells are exceedingly difficult to grow in culture and have very slow generation times. But standard laboratory culture of the most commonly used prokaryotic cells, such as *Escherichia coli* (*E. coli*), can be grown on simple, defined media consisting only of an energy and carbon source (usually glucose) and inorganic nitrogen, phosphorus, and sulfate sources such as NH_4Cl, $MgSO_4$, and phosphate buffers. Such ability to grow on media containing only sugar and inorganic molecules is called **prototropy** and allows full knowledge of all the ultimate sources of biological reactions. More rapid growth is attained with a broth of yeast or beef extract, possibly supplemented with required inorganic materials.

Bacterial cells can be grown in liquid culture, where densities of 10^8 cells/ml are reached during the exponential (i.e., most rapid) phase of growth. Bacterial cells can also be grown on solid or semi-

solid surfaces, allowing formation of colonies or **clones** where all cells are the descendants of one single cell. The most common material used for this type of growth is agar, poured as a thin slab into glass or plastic Petri dishes.

Such plates are used extensively for *plaque assays* of bacterial viruses. Plaque assays take advantage of the fact that virus replication results in cell lysis and thus a center of virus infection will be devoid of cells. Techniques of plaque assays are described in more detail later in this chapter.

Plant cell cultures

Most plant viruses can be more or less conveniently studied by infection of their intact hosts, which are not difficult to grow and maintain. This method allows basic analysis of many plant virus features. Indeed, early structural study of plant viruses was at a level fully equivalent to studies of bacterial viruses.

Molecular biological studies lagged until recently, however, due to a lack of reliable plant cell culture systems. Plant cell culture techniques have not developed as rapidly as those for animal cells, because plant cell architecture makes the manipulation of cells in culture (which is such a boon to the study of animal and bacterial cells) very difficult and often nearly impossible. These technical problems have resulted in plant cell culture not having a major impact on the development of plant virology. Plant cells without their cell walls can be cultured as protoplasts, and this has provided great impetus to the study of plant molecular biology; but as yet, little virology has been done with such systems.

Culture of animal and human cells

Maintenance of cells in culture

To maintain cells in culture, culture medium approximating blood plasma must be used. This medium contains salts similar to those found in plasma; most amino acids (since animal cells cannot synthesize many of these); vitamins; glucose as an energy source; buffers (usually carbon dioxide/sodium bicarbonate) to prevent lactic acid (resulting from glucose fermentation) from making the medium too acidic; and most importantly, blood serum, which is usually obtained from calves or horses. This serum contains many growth factors (e.g., proteins) that the cell needs for growth. As noted, antibiotics also are included to preclude microbial contamination.

In addition to the uncertainties of exact culture requirements, the same type of cell (e.g., a fibroblast or skin cell) can have strikingly different properties depending on its species of origin, age of the donor animal, state of the cell, and the specific culture history. Thus, each cell culture has its own pedigree and peculiarities.

A general method for obtaining mouse mammary epithelial cells is shown in Fig. 10.1. Similar methods are used to generate cultures of many primary and tumor cells. Cells are usually grown in standard-sized culture dishes with specific areas. Popular sizes range from 25 cm^2 to 150 cm^2, depending on the number of cells needed.

Types of cells

Ultimately all animal cells are derived from living tissue; however, some—such as HeLa cells—have been in culture for so long (about 50 years) that they have lost all resemblance to the tissue from which they were isolated. Such **continuous cell lines** are very useful in that they grow rapidly to provide large amounts of virus for the study of some basic aspects of virus replication. They are not good, however, for studying the relatively subtle effects of virus infection on cell growth and control. Continuous cell lines are also not appropriate for the study of differentiated cell function.

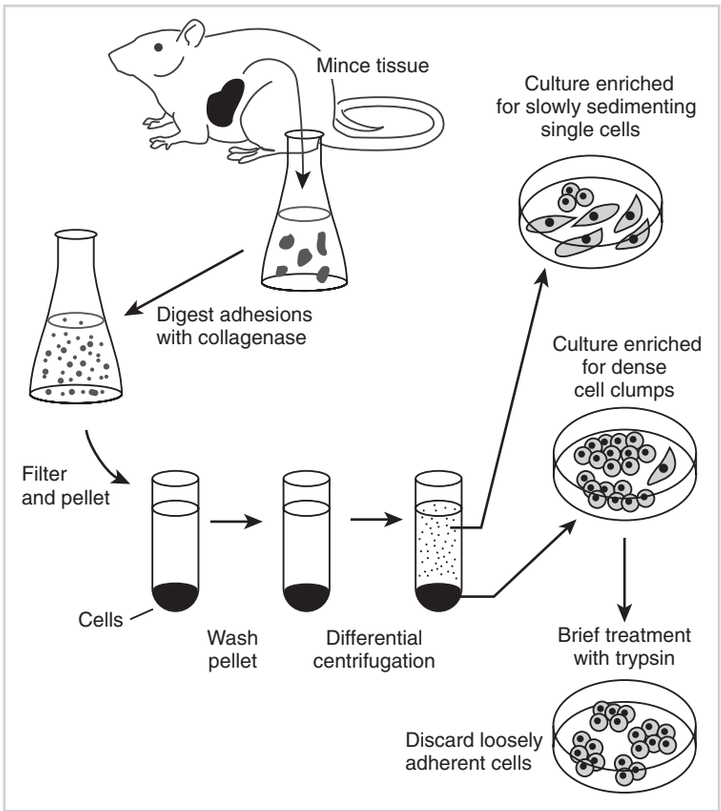


Fig. 10.1 Generating a primary cell culture. Tissue is surgically removed from an anesthetized animal, and then minced and homogenized. Addition of collagenase breaks down extracellular collagen, but the enzyme does not attack intact cells. The cells are purified by filtration through a coarse mesh to remove large fragments, and then concentrated by deposition under a mild centrifugal field in a low-speed centrifuge. The pelleted cells are washed in various buffered media containing serum, and then can be subjected to differential low-speed centrifugation to partially separate cell types based on sedimentation rates (a function of cell size and density). Various fractions are plated onto culture dishes in the presence of a culture medium containing essential amino acids, vitamins, antibiotics, and serum. Cells grow as loose clumps that can be dispersed with mild trypsin treatment, and individual cell types then can be cultured.

Continuous cell lines generally have the following properties:

1 They have fragmented and reduplicated chromosomes; that is, they are **aneuploid**.

2 They are able to grow in suspension and in relatively low concentrations of serum, and can overgrow each other; they display no response to neighboring cells.

3 They are essentially immortal; if periodically diluted and fed with appropriate nutrients, they will continue replicating.

4 They generally do not display properties of differentiated cells and do not respond to modulators of cell growth or function.

5 If introduced into an animal (even one of the same species from which they were originally isolated), they will not grow and will be eliminated by the animal's immune system.

At the opposite extreme of laboratory cell type are **primary cells**. Primary cells are most conveniently isolated from embryonic (fetal) tissue or from newborn animals or tissue. Cells isolated from older animals tend to be difficult to culture and have a much shorter life in culture before they fall apart (**senesce**) or die.

While the very act of culturing cells leads to rapid changes in the subtle properties of living cells, the earliest stages of culturing primary cells are very nearly identical to those in the tissue from which they derive. Although almost any type of replicating cell can be cultured if the tissue containing it is properly isolated, the more rapidly growing and replicating cells, such as fibroblasts, will outgrow other cells in a mixed tissue source. For that reason, isolation of primary cells from whole embryos generally produces cultures of fibroblasts.

Most primary cells have the following properties:

1 They have normal chromosome numbers and shape.

2 They require high serum concentrations containing numerous growth factors.

3 They cannot divide or even survive for long unless they are maintained in contact with a solid surface.
4 They are subject to **contact inhibition** of growth and of cell movement. Contact inhibition means that when they touch other cells in a culture plate, they stop growing and stop moving. Thus, a given area of culture plate will allow cells to grow to a specific number. During contact inhibition, the cells are healthy and metabolize energy. When they are diluted (passaged) and placed into a new culture dish, they will begin to grow and divide again.
5 They have a finite lifetime measured in divisions. Normally, fibroblasts can divide 20 to 30 times after isolation and then the cells begin to senesce and die. Recent experimental evidence suggests that this finite lifetime is due in part to programmed loss of chromosome end regions (**telomer**) at each cell replication. When enough chromosomal DNA is lost, the cells begin to die.
6 They display all properties of differentiated cells and respond to modulators of cell growth or function.
7 If introduced into an animal of the same species from which the cells were originally isolated, they may survive but will not produce tumors.

This list of properties of primary cells is, of necessity, an idealized one. Some of the properties listed may not apply to a given type of cell isolated from an individual. For example, lymphocytes isolated from the small amount of blood found in the umbilical cord of a newborn will survive but not replicate when maintained in suspension. In contrast, lymphocytes cultured from many adolescents and adults who have had infectious mononucleosis not only will survive but also will divide for relatively long periods of time. Even though these immortalized lymphocytes are ostensibly normal, they maintain copies of the genome of the Epstein-Barr herpesvirus, and the expression of certain viral genes contained therein leads to these unusual properties. This type of transformed lymphocyte is not a tumor cell, but it clearly demonstrates some similar properties.

Loss of contact inhibition of growth and immortalization of primary cells

Immortalized B lymphocytes are but one example of cells available in the laboratory that have properties intermediate between the two extremes of continuous cell lines and primary cells. These cell types have undergone transformation and have at least some of the properties of tumor cells. Transformed cells can be generated by culturing primary cells for long periods. During the time in culture, there is a random accumulation of mutations that alter a critical number of growth control genes encoded by the cell. Cells transformed in their growth properties can be generated by specific mutagenesis, or by the action of the genes of certain tumor viruses following infection.

Cells with the properties of transformed cells also can be isolated from tumors in an animal. Different tumor cells in an animal display one or several of the same transformation levels from normal cells that can be observed with the culture of primary cells. This is an important clue to the nature of the cellular events leading to cancers. It is important to be aware, however, that different tumor cells can display widely different deviations from normal growth properties of the cells from which they derive. Some tumor cells, especially those isolated early in the course of cancer development, display very few differences from normal cells—perhaps only the loss of contact inhibition of growth. Others, especially long after the cancer occurred, have many additional changes.

The process of change from primary cells to continuous line cells and the relationship between these cells and tumors in the animal of origin is shown in schematic form in Fig. 10.2. This process of change is a convincing experimental demonstration that the cellular changes in an organism from normal to cancerous involve multiple steps. The changes multiply as mutations of specific growth control and regulatory genes in the cells alter cell function and the cell's ability to respond to normal signals in the animal, limiting cell growth and function.

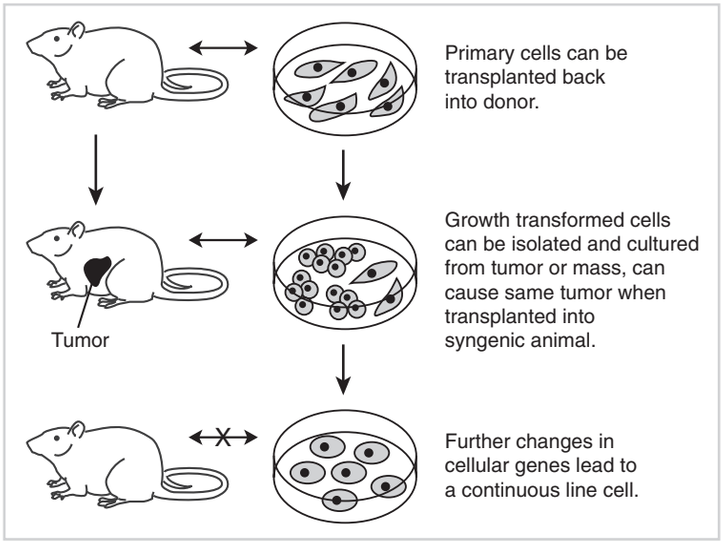


Fig. 10.2 The progression of cells in culture from primary to transformed to continuous lines, and their relationship to tissues in the originating animal.

Depending on the type of tissue from which tumor cells are isolated, tumor cells can display any or most continuous cell line features. But sometimes in a primary cell culture, especially as senescence sets in, a specific period of culture crisis occurs. During this period, most cells die, but growth-transformed cells that have lost the internal limit to their life span survive. These *immortalized* cells become predominant and relatively rapidly overgrow the culture. Such cells eventually can be used to generate continuous lines.

While it is not uncommon to generate an immortalized cell line by lengthy **passage** or other mutagenic processes, it is important to be aware that many tumor cells and some transformed cells that have lost contact inhibition of growth still have a finite lifetime. The genes controlling life span and response to contact inhibition are not identical and can be mutated together or separately.

One of the most fruitful aspects of the study of some viruses is that they can cause transformation of normal primary cells into cancer or tumor cells. Such virus-transformed cells, when reintroduced into animals, can cause tumors. Since this transformation requires a specific interaction between viral and cellular genes or gene products, the study of the process has led to much current understanding of carcinogenesis and the nature of cancer cells.

THE OUTCOME OF VIRUS INFECTION IN CELLS

Fate of the virus

When a virus infects a cell, its genome enters that cell. Regardless of whether the virus capsid remains at the surface of the host, as in bacteriophage infection, or is internalized, it is modified and then disrupted. If an infecting virus is isolated after attachment and penetration, the virus is no longer stable and cannot initiate a new infection. Thus, following the initial steps of virus—cell interaction, the only way that infectious virus can be isolated is either to block further progress of the infection process, or wait for progeny virus to be formed.

In some types of infections (generally called a **nonproductive infection**), new infectious virus is not produced. This type of infection is also termed an **abortive infection** because it does not proceed to completion of the replication process. Abortive infections can result from the virus infecting a **nonpermissive cell** (i.e., one that, for some reason, does not have the proper machinery for

virus replication). A nonproductive infection could also be the result of infection with a virus that has some defective gene product interfering with replication. A general rule of thumb in differentiating types of infection is the following:

1 productive infection: more virus out than in; and
2 abortive infection: no virus out, virus cannot replicate.

When an abortive infection occurs, the viral genome may be destroyed or it can be internalized. In the latter instance, one or several viral genes might be expressed. This situation could result in the cell's expression of viral antigens at its surface or elsewhere in the cell. Given the proper immune reagents (briefly described in Chapter 12), such antigens can be detected and studied.

From this explanation, it is clear that an abortive infection can have profound effects on the host cell, and perhaps ultimately, on the organism. For example, the continuous presence of noninfectious measles virus in brain tissue can lead to severe complications (see Chapter 4). Also, many DNA viruses that cause cellular transformation do so only under conditions of abortive infection. Understanding the reason why a virus infection is abortive can be very important to understanding and describing the course of virus replication and the effects of virus infection on the host. Some questions important to characterizing abortive infections are the following:

1 Is the virus genome lost?
2 Is part of the genome maintained and expressed?
3 If the genome is maintained, is it physically integrated into the host genome, or is it maintained as a separate "mini-chromosome" or **episome**?

Other types of infection fall between the extremes of productive and abortive. For example, cells can be poor hosts for replication of a specific virus but not strictly nonpermissive for virus replication; often, such a cell is called *semipermissive*. Clearly, there is no real strict point at which a cell is permissive or semipermissive for virus replication; the terms are relative.

Other cell-based impediments to virus replication exist. Viruses can have mutations that are lethal only under certain conditions (conditional lethal mutations) such as high temperature (temperature-sensitive mutations). Dynamic situations can occur in which virus is slowly released over time at low levels. Such a situation can define a persistent, inapparent, or chronic infection. Under some conditions, such an infection in a cell culture or in an animal can lead to episodes of high levels of virus production with obvious cell destruction or disease. These episodic occurrences can alternate with periods in which virus is difficult to detect and the host (or cell culture) appears relatively healthy.

Under certain conditions of infection, many viruses will produce incomplete viral particles, and these particles may be able to infect other cells. Such particles are termed *defective virus particles*, and can be produced by a variety of mechanisms—often involving inefficient steps in virus maturation taking place very late in the infection cycle when the host cell is rapidly deteriorating due to virus-induced changes. The generation of empty capsids of cytomegalovirus as well as enveloped dense bodies made up of tegument proteins shown schematically in Chapter 6 is a good example of such an occurrence.

In addition to the formation of defective particles due to the packaging of empty capsids, viruses can randomly produce partial genomes during their replication. If these partial genomes contain a packaging signal, they can be encapsidated and form a specific class of defective particles. An infection of a cell with one of these particles will be abortive since the genome is not complete.

Interestingly, the simultaneous infection of such a defective particle with an infectious one can lead to interference, which is a result of the smaller fragmentary genome being able to reproduce more copies in a given time than the complete genome. This is purely a mass effect. The shorter molecule can undergo more rounds of initiation and completion of replication per unit of time, but the result is that the yield of infectious particles will be reduced. For this reason, defective virus particles of this type are classified as defective interfering particles. Their presence in a virus stock can be a headache to a researcher trying to get a high yield of virus, but defective particles can be used to

deliver genes to cells under certain instances. The use of viruses to deliver genes is briefly discussed in Chapter 22.

Finally, it should be recalled that herpesviruses (as well as some other viruses) can remain as latent infections in which the viral genome is maintained in the cell or in some cells of the host but no virus is detectable.

Fate of the cell following virus infection

Cell-mediated maintenance of the intra- and intercellular environment

As discussed above, long periods of passage of cells in culture as well as mutations in the genetic information carried by cells can alter their growth properties. Such changes can take place within the animal leading to formation of a tumor, but usually this does not happen. This is because the vertebrate body and the cells comprising it have a number of "check points" that respond to genetic alterations of individual cells. This is a major function of MHCI-mediated antigen presentation. When an abnormal epitope from a genetically damaged protein or a protein that should not be expressed is presented at the surface of the cell a number of programmed responses lead to the death of the cell by the apoptotic pathway. As noted in Chapter 8, apoptosis is a consequence of the action of specific cellular genes that lead to a phased shutdown of cellular functions and cell death. The process has a protective function in the body by inducing the death and elimination of highly differentiated cells no longer needed (such as effector cells of the immune system), aged cells, as well as cells with mutations in genes that normally function to limit cell division. It is important to understand that the apoptotic pathway leads to cell death *without* release of cellular contents to the immune system and resulting inflammation and potential pathology, rather it is a highly regulated process designed only to eliminate those cells that are no longer of value in the tissue in question. The apoptotic pathway should be contrasted with the other major route of cell death, **necrosis**, where the swelling and bursting of the cell targeted for death leads to inflammation in order to stimulate the immune response. The two processes are schematically outlined and contrasted in Fig. 10.3.

Obviously, it is of value for a virus replicating in a cell to ensure that the cell is maintained for a sufficient length of time to ensure an appropriate yield of virus, while at the same time limiting immune responses to the infection. Conversely, it is to the benefit of the cell and the organism comprised of such cells to mount a controlled immune response as rapidly as possible as well as to eliminate infected tissue. It is the tension between these two processes that leads to evolutionary change in both virus and host, and the manifestations of both processes lead to the macroscopic and microscopic changes in virus-infected cells that define cytopathology.

Virus-mediated cytopathology—changes in the physical appearance of cells

Some basic types of virus-induced changes to the host cell (cytopathology) result in changes that are readily observable by eye or with the aid of a low-power microscope. All cytopathology requires some specific interaction between viral gene products and the cell. Even the cell lysis induced by poliovirus or bacteriophage infection, in which the cell "explodes," is the result of very specific modifications to the cell's plasma membrane and lysosomes induced by specific poliovirus gene products. Less dramatic, but still clearly observable changes to the cell include the formation of cytoplasmic inclusion bodies (which is diagnostic for poxvirus infections), generation of nuclear inclusion bodies seen with herpesvirus infections, and alterations in chromosomes.

Cytopathology need not involve cell death. Virus-induced alterations in cell morphology, growth, and life span are all types of cytopathology. Even very subtle changes, such as a virus-induced change in the expression of a protein or appearance of a new macromolecule, are cytopathic changes, as long as they can be observed with some reproducible technique.

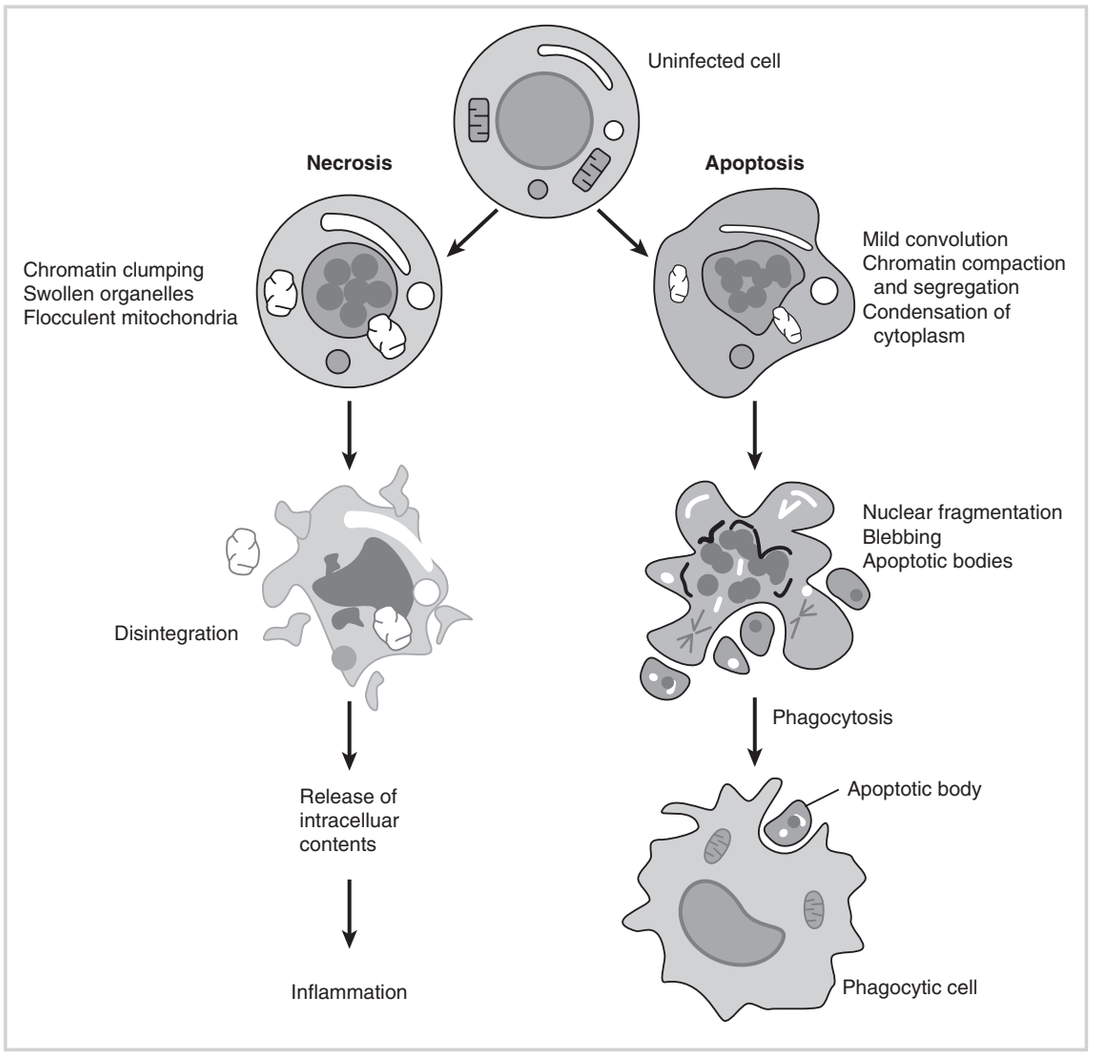


Fig. 10.3 Apoptosis vs necrosis in cell death.

A major type of cytopathology involves changes to the cell surface due to expression there of viral proteins. Among other things, this can lead to the following:

1 Altered antigenicity: the altered cell will stimulate the immune system to generate antibodies to react with viral proteins or previously masked cellular proteins;

2 Hemagglutination or hemadsorption: certain viral proteins will stick to red blood cells and cause these cells to stick together.

3 Cell fusion: changes to the cell membrane can allow formation of large masses of fused cells or syncytia. Specific virus gene products are responsible for this. Such fusion induced by the Sindbis virus (a togavirus) can be used to generate somatic cell hybrids.

Another major type of cytopathology involves changes in cell morphology. An example is demonstrated in Fig. 10.4; an HSV-1 infection is shown disrupting the cytoskeleton of the host cell, thereby changing the cell's shape. In this example, viral infection led to dissociation of the actin fibers, but not degradation of the actin. This very specific biochemical change in the actin subunits results in profound changes to the cell's morphology.

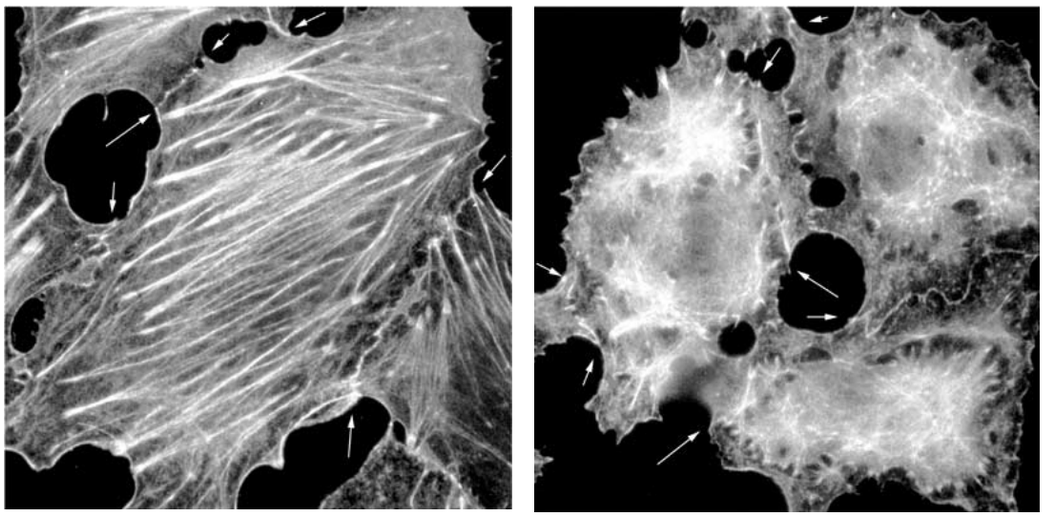


Fig. 10.4 HSV-induced changes in the properties of actin microfilaments of a cultured monkey fibroblast. The cell was stained with a fluorescent dye that reacts with actin fibers so that they can be visualized in ultraviolet light. This technique is similar to immunofluorescence microscopy, which is discussed in Chapter 12. The left panel shows parallel arrangement of the microfibrils in the uninfected cell, while HSV infection (right panel) results in disassociation of the fibrils and diffusion of the actin throughout the cytoplasm. At the same time, the cell loses its spindle-shaped morphology and becomes rounded. The arrows indicate junctions between cells that are also rich in actin fibrils and are not disrupted by HSV infection at this time. (Courtesy of Stephen Rice.)

Virus-mediated cytopathology—changes in the biochemical properties of cells

Virus infection leads to specific changes in biochemical processes of the cell. Some viruses, such as HSV and poliovirus, specifically inhibit cellular protein synthesis. The mechanism for such inhibition is complex and differs for different viruses. Viral infection also can lead to specific inhibition of cellular mRNA synthesis. Gross inhibition of cellular macromolecular metabolism will lead to cell death. However, there are complex and multifaceted effects of virus infection on cell function resulting from subtle changes in cellular functions that do not result in cell death.

A striking example of the ability of certain DNA tumor viruses to prevent cell death long enough to allow efficient virus replication is found in viral inhibition of apoptosis. Another very important consequence of infection is, as discussed earlier, changes in the growth properties and life span of virus-infected cells. The growth rate, total number, and life span of differentiated cells are tightly controlled through the auspices of specialized **tumor suppressor genes**, so named because they block the formation of tumors. The interactions between viral genes and tumor suppressor genes are generally well understood in the replication of papovaviruses and adenoviruses, and are described in Chapter 17. For the purposes of this discussion, it suffices to note that DNA-tumor viruses inhibit the tumor suppressor genes as a method to "activate" the cell for their own replication. The induction of apoptosis would interfere with the cell's ability to support virus replication. The mechanism of transformation varies between different tumor viruses, but in many cases specific virus-induced inhibition of apoptosis as well as inactivation of cellular genes actively inhibiting cell division are both important factors.

Another major effect of virus infection is interaction between the infected cell and the host's immune system. As briefly outlined in Chapter 8 and more specifically in chapters describing specific viruses (Part IV), many viruses contain genes that function to specifically inhibit the production of interferon in the infected cell. Further, certain viruses, such as HSV, can specifically inhibit major

histocompatibility complex class I (MHC-I)—mediated antigen presentation at the early stages of infection. Although eventually the cell will express viral antigens as infection proceeds, this early inhibition of antigen processing can provide the virus with a vital head start in its infection.

Virus infection of cells can lead to a number of specific cellular responses that involve the expression of new cellular genes, or the increase in expression of some cellular genes. The interferon response described in Chapter 8 is a good example of this. Several techniques of modern molecular biology allow very precise identification of cellular genes induced by virus infection.

One method is termed **differential display analysis** and requires the use of groups of oligonucleotide primers, retrovirus reverse transcriptase, and the **polymerase chain reaction (PCR)** to generate and amplify complementary DNA copies of cellular transcripts. By comparison of the amplification patterns of products isolated from uninfected and infected cells, increases in levels of specific cellular genes can be determined.

Other methods used involve microchip technology in which numerous (up to 64,000) oligonucleotide probes specific for various cellular genes are bound to a very small **microarray** and hybridized with PCR-amplified complementary DNA (**cDNA**) samples made from mRNA isolated from uninfected and infected cells and labeled with different-colored fluorescent dye. Comparison of the patterns of light emission when the microchip is scanned with a laser beam leads to identification of novel transcripts. The general methodology for microchip analysis and PCR is discussed in the two following chapters.

MEASUREMENT OF THE BIOLOGICAL ACTIVITY OF VIRUSES

Quantitative measure of infectious centers

Plaque assays

Cytopathic effects on the host cell by the great majority of viruses cause observable damage or changes to the cells in which they are replicating. Even if cells are not killed or lysed, the alteration of cells in a local area due to a localized virus infection can readily be observed as a plaque or **focus of infection**. With proper dilutions and conditions, such a localized infection can be the result of infection with a single biologically active virus. A virus particle able to initiate a productive infection is termed a **plaque-forming unit (PFU)**.

The process of plaque formation is easy to envision. The first infected cell releases many viruses. If the viruses (big compared to even the most complex molecules in the growth medium) are kept from wide diffusion, they will remain in the vicinity of the original infected cells and will infect neighboring cells. This process is repeated a number of times. As long as the virus-cell interaction is kept localized (often by making the cell culture medium into which the virus is released quite viscous), the area of cytopathology resulting from a single infection of a single PFU will remain localized and can be readily observed and counted a few days after infection. Some examples of plaques on cultured cells are shown in Fig. 10.5.

Cell cultures are not the only way to obtain infectious centers. Infection of plant virus on a leaf of a susceptible plant, along with some type of mechanical abrasion to initiate the infection (perhaps rubbing the leaf with Carborundum powder), results in formation of visible centers of infection. Examples are also shown in Fig. 10.5.

The **chorioallantoic membrane** of developing chick or duck embryos can be used (and indeed, must be used) for assays of certain viruses. In this assay, fertilized eggs are incubated for 2 weeks, then carefully opened to expose the membrane (the embryo is below this within the egg). Virus suspension is then placed on the membrane, the egg resealed, and virus pocks or plaques allowed to develop.

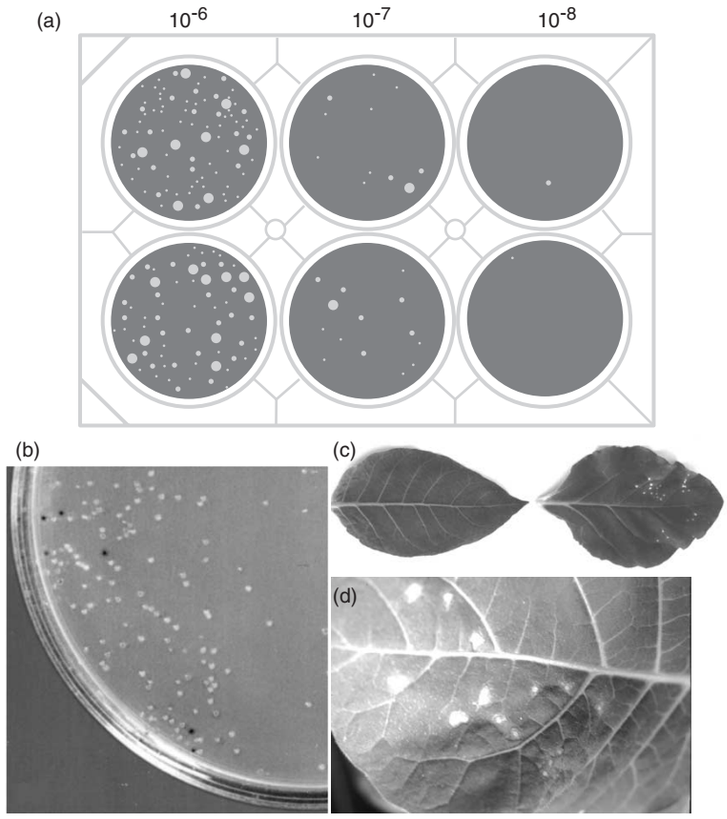


Fig. 10.5 Visualization of virus plaques. Under appropriate conditions, virus infection can be localized to the vicinity of the originally infected cells. If a limited number of infectious units of virus (PFUs) are incubated in a culture dish or on tissue in which virus can cause a cytopathic effect, virus plaques can be visualized. *a.* A continuous line of monkey cells (Vero cells) was grown in the six-well culture dish. When the cells reached confluence, they were infected in duplicate with a series of 10-fold dilutions of an HSV-1 stock. After 48 hours, the cells were partially dehydrated (fixed) with ethanol and stained. Areas of cell death show as white plaques, each representing a single infectious event with the input virus solution. *b.* A portion of the surface of a Petri dish containing agar with bacterial nutrient medium. A "lawn" of *E. coli* was grown on the plate's surface, and this layer of cells was infected with a solution containing a genetically "engineered" version of bacteriophage λ that can be used to clone inserted genes. (See Chapter 14 for some general details.) Bacteriophages that contain an inserted gene form clear plaques due to inactivation of an indicator gene (β-galactosidase) and viruses without the insert form dark-colored plaques. *c.* Assay of tobacco mosaic virus (TMV), showing a leaf of a resistant (left) and a susceptible (right) plant that have been infected with small amounts of virus. *d.* A higher magnification of plaque development. (Photographs in *c* and *d* courtesy of J. Langland.)

Generation of transformed cell foci

The same titration principles can be used to measure other biological effects of virus infection. Under certain conditions, some DNA viruses can transform cells so that normal growth control is altered. As outlined earlier in this chapter, transformed cells have a different morphology and tend to overgrow normal cells to form clumps of proliferating cells. Each infectious event, even if it is abortive and does not produce new virus, will result in the formation of transformed cell clumps, called a *focus of transformation.* An example of a focus of transformed cells is shown in Fig. 10.6. The changes in cell morphology (a type of cytopathic effect of transformation) is clearly evident. The number of focus-forming units can be counted just as with PFUs, but here one is counting the spread of transformed cells, not the spread of virus.

Use of virus titers to quantitatively control infection conditions

There are two important definitions relating to infectious virus particles or PFUs. The **particle to PFU ratio** measures just that: the proportion of total number of virus particles to infectious particles of virus. To obtain the ratio, one must count the total virus particles and do an assay for biologically functional ones.

Some types of viruses (bacteriophages, and under very special circumstances, poliovirus, for example) have particle to PFU ratios approaching 1. Careful preparations of viruses such as adenovirus and HSV can have ratios of less than 10, but the best ratios for influenza A virus are on the order of 10^3. This high particle to PFU ratio is unusual but is inherent in the way that flu virus

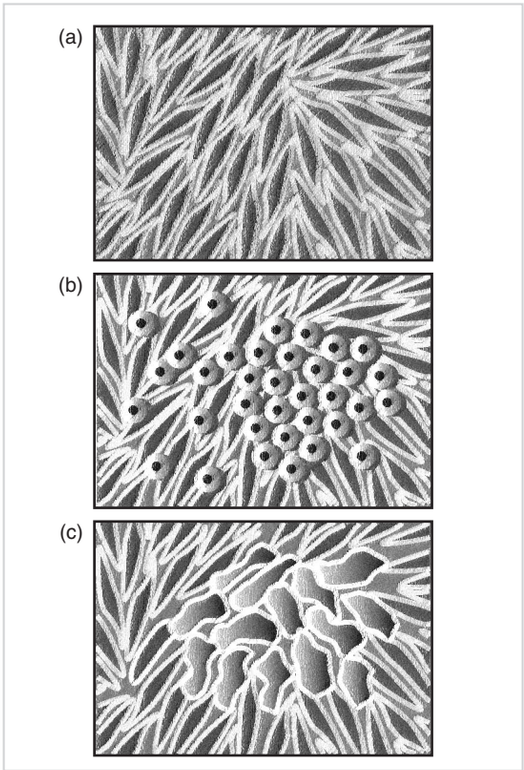


Fig. 10.6 Some representative morphologies of rat fibroblast cells (F-111) infected with different transforming viruses. The top panel shows normal cells with their characteristic parallel orientation. A focus of transformed cells generated by infection with Rous sarcoma virus (an oncornavirus) is shown in the middle panel. Note the rounded morphology and density of these cells. The bottom panel shows the subtle difference in morphology when normal F-111 cells are infected with SV40 virus, for which they are nonpermissive. (Based on portions of a photograph in Benjamin, J., and Vogt, P.K. Cell transformation in viruses, in Fields, B.N., and Knipe, D.M., eds. *Fundamental Virology*, 2nd edn. New York: Raven Press, 1991: chapter 13.)

virions are formed. Particle to PFU ratios can vary depending on the specifics of the particular infection and virus, and each virus type has a characteristic optimum value that tells something about the efficiency of encapsidation and release of infectious virus from infected cells.

A second quantitative measure of conditions of virus infection is the **multiplicity of infection (MOI)**, which is simply the average number of PFUs per cell utilized in the original infection. An MOI of 1 means 1 PFU per cell, so if 10^6 cells were infected at an MOI of 1, one would need to add 10^6 PFUs of virus. It is important to note that an MOI can vary from zero to a very high number, depending on the concentration of virus in the original stock, the type of experimental problem being studied, and so on. MOI measures an average value; statistical analysis that demonstrates the number of PFUs interacting with one individual cell can vary over a wide range when a culture is infected at MOIs greater than 0.1 or so.

Examples of plaque assays

In the plaque assays shown in Fig. 10.5a, the cell culture dishes contained 10^6 cells. Thus, the MOI used to generate the average of 40 plaques seen in the 10^7 dilution shown was calculated as follows: PFU/cell = $40/10^6 = 4 \times 10^{-5}$. One should be able to see that where the plaques could be readily counted, the MOI must be quite low, and any cell initiating a focus of infection or plaque must have been infected with only 1 PFU. This is a simple demonstration of the fact that with normal (wild-type [*wt*]) animal viruses, only one viral genome delivered to the right place in the cell is sufficient to carry out the whole infection. Indeed, a very high MOI may actually inhibit the replication process because particle to PFU ratios may increase rapidly with a high MOI. One way this can happen is by the generation of defective interfering particles as outlined earlier.

To do a plaque (or focus) assay, serial dilutions of a virus stock are made and aliquots of each dilution are added to a culture dish. The plaques are allowed to develop and then are counted. Simple arithmetic yields the original number of PFUs in the solution.

Fig. 10.5a shows an example of HSV plaques developed on Vero cells. Serial 10-fold dilutions were added to separate plates in duplicate. After adsorption, the cells were rinsed and covered with a special overlay medium that inhibits virus spread beyond neighboring cells. Following incubation for 48 hours at 34°C, the cells were rinsed, fixed, and stained. The clear areas are plaques. The average number of 40 plaques in the 10^7 dilution means that about that number of PFUs was added to each plate at that dilution of virus.

Another example is shown in Fig. 10.7. Here a 100 ml stock of HSV was diluted and infectious units measured by plaque assay as shown in Table 10.1. One can readily calculate that the original stock was about 6×10^7 PFU/ml or 6×10^9 total units of infectious virus (PFU). The following formula is useful to make the calculation:

$$V_f = V_o / D$$

V_f is the final concentration of PFU (units/ml), V_o is the original concentration, and D is the dilution factor.

Also note in this example that the number of plaques counted in two plates infected with the same amount of diluted stock varies quite a bit. Some of this variation is due to experimental error,

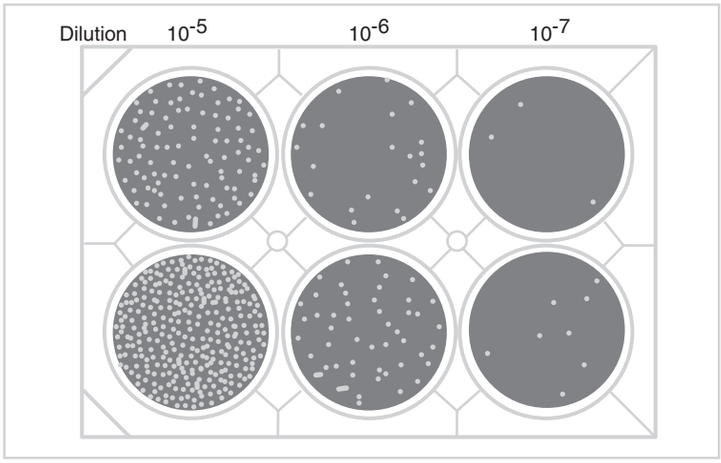


Fig. 10.7 Serial 10-fold dilutions of HSV to determine the titer of virus in a stock solution. The details of the infection are as described in the legend to Fig. 10.5a, and the calculation of the titer is shown in Table 10.1.

Table 10.1 An example of a set of dilutions for a plaque assay.

Operation	Dilution of stock	Plaques per dish
0.01 ml of stock diluted into 10 ml of buffer	10^3	Too many to count
1 ml of above diluted into 10 ml of buffer	10^4	Too many to count
1 ml of above diluted into 10 ml of buffer	10^5	500–1000 (estimated)
1 ml of above diluted into 10 ml of buffer	10^6	$(20+100)/2=60$
1 ml of above diluted into 10ml of buffer	10^7	$(3+8)/2-5$
1 ml of above diluted into 10 ml of buffer	10^8	0
1 ml of above diluted into 10 ml of buffer	10^9	0

but there also is an inherent statistical variation because one cannot be sure that the same amount of virus particles will be in a small volume at a given time. This type of variation is inherent when working with samples that contain a small number of particles.

Statistical analysis of infection

The statistics of chance mean that at low and moderate MOI values, the actual PFU number infecting any one cell will vary widely. For example, at an MOI of 2, a significant number of cells will *see* no *virus*, and a larger number for MOI will *get* 1 PFU. Some cells will get 3 PFUs, and some others (a smaller number) will get 4, 5, or more PFUs. The proportion (or probability) of any given cell being infected with any specific number of PFU can be calculated using a statistical method originally developed for analyzing gambling results. This is the **Poisson analysis**, which describes the distribution of positive results in a low number of trials as

$$P_i = (m^i e^{-m})/i!$$

P_i is the probability that a cell will be infected with exactly i number of virus and m is the MOI (average number of PFUs added per cell). Using this equation, one can always calculate the probability of a cell being uninfected, and thus, the number of uninfected cells (if you know the number of cells in the sample). Since $m^0 = 1$ and $0! \equiv 1$:

$$P_0 = e^{-m}$$

For the MOI of 2 mentioned previously, the proportion (probability) of cells getting i number of PFUs is:

$$P_0 = e^{-2} = 0.135$$
$$P_1 = 2e^{-2} = 0.27$$
$$P_2 = 2^2 e^{-2}/2 = 0.27$$
$$P_3 = 2^3 e^{-2}/6 = 0.18$$
$$P_{4\text{ or more}} = 1.0 - (P_{0-3}) = 0.145$$

One gets the last number ($P_{4\text{ or more}}$) from the fact that the total probability of a cell being infected with no PFUs or any number of PFUs must be 1.0.

This fact can be used in another way. For example, what MOI is needed to ensure that at least 99% of cells in a culture are infected?

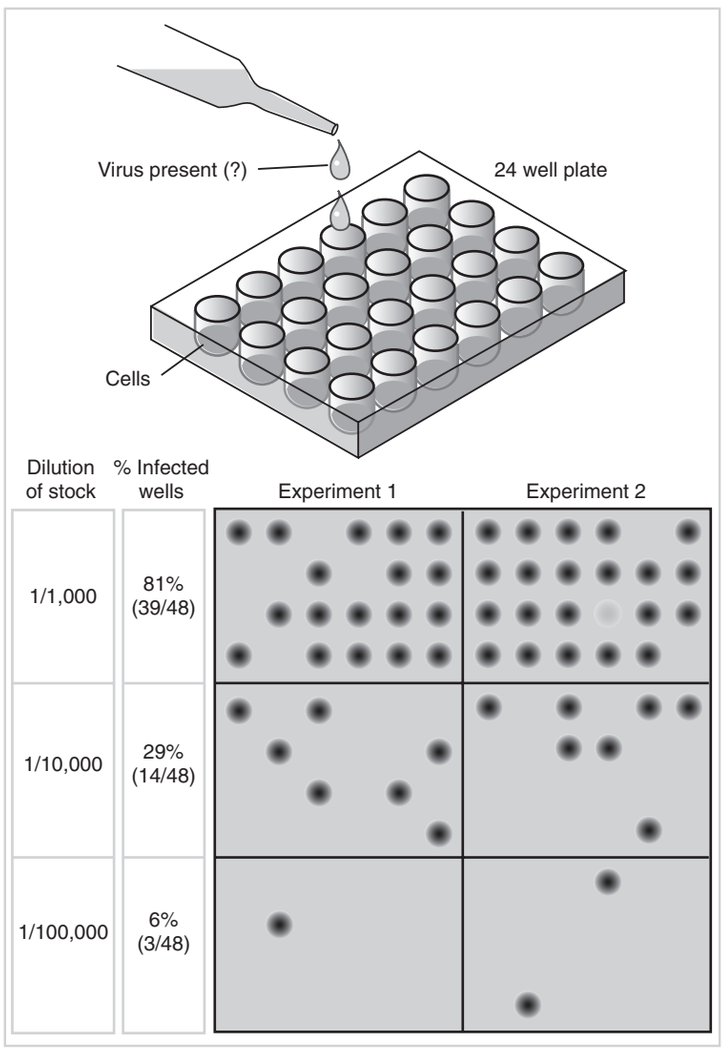


Fig. 10.8 Quantal (endpoint dilution) assay of HSV in tissue culture wells. Replicate cultures of rabbit skin fibroblasts were grown to a density of about 5×10^4 cells per well of a 24-well tissue culture plate. Aliquots of the indicated stock virus dilutions were pipetted into the cultures and the plate was incubated for 48 hours and then developed with a stain that indicates black for virus-infected cells. Any well that received at least 1 PFU of virus stained black (two separate experiments are shown). The percentage of positive (infected) wells is shown at each dilution.

$$P_0 = 1 - 0.99 = 0.01 = e^{-m}$$

Thus,

$$\ln(0.01) = -m \text{ or } 2.3 \log(0.01) = -m$$

So m (MOI) must be at least 4.6 PFU/cell.

Dilution endpoint methods

In the hemagglutination inhibition (HI) and hemagglutination (HA) assays (described in Chapters 7 and 9, respectively), there comes a point in the dilution of any virus stock below which a desired property cannot be observed. If a virus stock is diluted far enough, and then a small measured sample (an **aliquot**) is taken, chances are that there will be no infectious virus present. The virus has not been destroyed, just diluted so much that its concentration is well below, say, 1 PFU/ml so that in any 1 ml, there is no virus.

Because virus stocks can be diluted so much that any given aliquot will usually have no PFUs, one can measure infection by dilution instead of by titration. This type of endpoint dilution method is often called a **quantal assay** because it is a statistical analysis, not a quantitative one. In this type of assay, a given number of subjects (animals, cell culture wells, etc) must be infected with increasing dilutions of virus and then scored for illness, death, or cytopathicity.

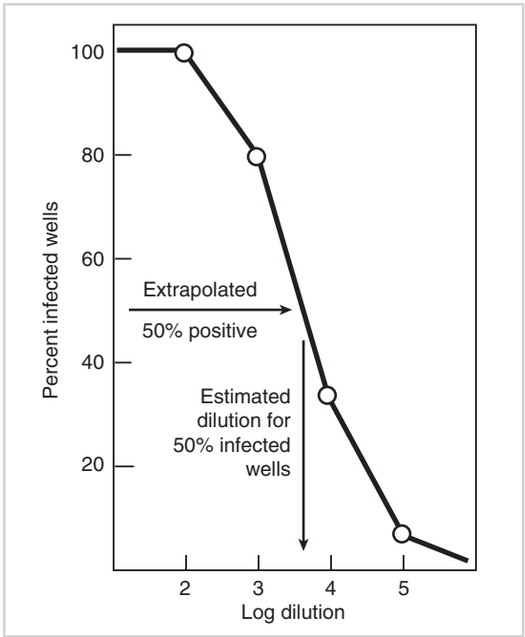


Fig. 10.9 Graphic analysis of the data from Fig. 10.8. The percentage of infected wells as a function of dilution is shown on a semilogarithmic plot. The dilution at which 50% of the wells would be infected (the $TCID_{50}$) can be estimated by graphic interpolation.

In a quantal assay, localizing plaques is not necessary. By plotting log dilution versus percentage of infected subjects, one can estimate a virus dilution that results in half the aliquots in that dilution containing virus and half not. In an assay of a disease in animals, this endpoint is called **ID_{50} (median infectious dose)**, or **LD_{50} (median lethal dose)**. For measurement of gross cytopathology in tissue culture wells, it might be called **$TCID_{50}$ (median tissue culture infectious dose)**. The ED_{50} assay described to measure interferon activity in Chapter 8 is another example of a quantal assay.

The relation between dilution endpoint and infectious units of virus

Quantal endpoints are simply a measure of dilution of infectious virus, but they relate to the average number of PFUs in the aliquot. An example of a quantal assay is shown in Fig. 10.8. An HSV stock was diluted as shown and equal aliquots were added to individual wells of 48-well culture plates. Evidence of virus infection (CPE) is shown by the black wells. For the titration, one can construct a table such as Table 10.2, and from the tabulated data, one can make the graph shown in Fig. 10.9. In the graph, one can estimate that a dilution at which 50% of the wells would be infected is

Table 10.2 An example of a quantal assay for virus infectivity.

Sample dilution	Log dilution	No. of infected wells	Total no. of wells	% infected
None	0	100	100	100
1/1000	3	39	48	81
1/10,000	4	14	48	29
1/100,000	5	3	48	6
1/1,000,000	6	1	100	0

about 4×10^3; therefore, the $TCID_{50}$ was 4×10^3 in the original sample. More accurate measures of the ID_{50} of a virus stock can be obtained by using statistical methods such as the method of Reed and Munch, which is described in a variety of basic statistical texts.

Although ID_{50} is a measure of dilution, an ID_{50} *unit* is directly related to PFU; 1 ID_{50} unit measures a dilution required to ensure that 50% of the aliquots in that dilution have infectious virus in them. This will only occur if there are 0.7 PFU (average) per aliquot, or 7 PFUs in 10 ml in the above example.

This finding follows from certain rough arithmetical considerations: If a certain X number of PFUs per milliliter in the original concentration was diluted by a factor D so that each animal or tissue culture well has a 50% probability of being infected with a PFU, then the final concentration of virus defines a type of multiplicity of infection (call it m) where the probability of a positive infection is 50%. This value (m) should have the dimensions of units of infectivity in a standard volume (here 1 ml). Then

$$P_0 = 0.5 = e^{-m} = 0.7 \text{ PFU/ml}$$

QUESTIONS FOR CHAPTER 10

1 You have diluted a 1 ml sample of virus stock by taking 100 µl from the stock solution and adding to it 0.9 ml of buffer. You then take 10 µl of this dilution and dilute it into 1 ml. You then infect two plates that contain 10^5 cells each with 100 µl. One plate had 25 plaques while the other had 29 plaques. What was the titer in the original stock?

2 One milliliter of bacterial culture at 5×10^8 cells/ml is infected with 10^9 phages. After sufficient time for more than 99% adsorption, phage antiserum is added to inactivate all unadsorbed phage. Cells from this culture are mixed with indicator cells in soft agar and plaques are allowed to form. If 200 cells from the culture are put in a Petri dish, how many plaques would you expect to find?

3 You have a series of culture dishes that contain "lawns" of HeLa cells (human cells). You plan to infect these cells with poliovirus type 1 under a variety of conditions. You will measure the ability of the virus to form plaques on these cells. In the table below, predict which of the conditions will result in plaque formation by poliovirus type 1 on HeLa cells. Indicate your answer with a "Yes" or a "No" in the table.

Experiment	Virus added	Treatment of cells	Plaques?
Negative control	None	–	No
Positive control	Poliovirus type 1	–	Yes
A	Poliovirus type 1	Cells treated with interferon	
B	Poliovirus type 1	Antibody against rhinovirus added	
C	Poliovirus type 1	Antibody against poliovirus type 2 added	
D	Poliovirus type 1	Antibody against poliovirus type 1 added	

Continued

4 You have performed a plaque assay on a stock of bacteriophage T4. Your results show an average of 400 plaques when you assay 0.1 ml of a dilution prepared by mixing 1 part of the original virus solution with 999,999 parts of buffer.

a What is the titer of the original stock of bacteriophage?

b What volume of this stock would you have to use to infect a 10 ml culture of *E. coli*, containing 4×10^6 cells/ml, such that the multiplicity of infection will be 10?

5 A stock of poliovirus is measured by plaque assay on a "lawn" of HeLa cells. When 0.1 ml of a 10^5 dilution of this stock is plated, an average of 200 plaques are observed.

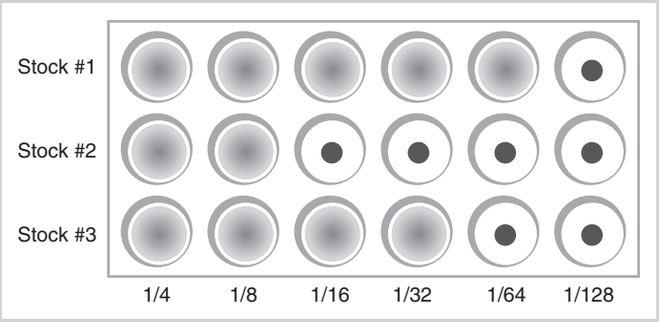
a What is the titer of this stock?

b If 0.1 ml of this stock is used to infect 10.0 ml of HeLa cells containing 10^5 cells/ml, what is the multiplicity of infection (MOI) in this case?

6 Using the Poisson distribution, calculate the proportion (probability) of cells infected with the indicated number of plaque-forming units (PFUs), given the multiplicity of infection (MOI) shown in the table.

	Proportion (probability) of cells infected with		
MOI	0 PFU	1 PFU	2 PFUs
0.01			
0.1			
1			
10			

7 You have three stocks of influenza virus that you have assayed by hemagglutination. The microtiter plate is shown below:



a Which of the virus stocks has the highest hemagglutinin (HA) titer? Which has the lowest HA titer?

b What would you report as the endpoint HA unit for stock 2?

8 Virus particles are very carefully isolated from an infected cell stock. You use this material to infect a culture of 10^6 cells with an MOI of 7 PFUs/cell. What is the maximum percentage of cells which *could* be productively infected?

9 You apply a virus stock solution containing 3×10^6 virus particles to 3×10^5 cells. What is the MOI for this infection?