Limitations of the Henle–Koch Postulates

*Effect of New Concepts and of Technology**

Robert Koch early recognized that the postulates of causation he had included in 1882 in papers on the etiology of tuberculosis (Koch, 1882a,b; 1938) had their limitations. In a subsequent paper on bacteriological research presented at the International Medical Congress in Berlin (see Koch, 1890) he stated that while the bacteria of anthrax, tuberculosis, tetanus, and many animal diseases fully fulfilled his postulates, many others did not. These latter included the organisms of typhoid fever, diphtheria, leprosy, relapsing fever, and Asiatic cholera. The biggest problem was the failure to reproduce “the disease anew” in an experimental animal. He therefore felt that fulfillment of only the first two postulates was necessary to establish causation. Many other diseases have subsequently failed to fulfill the three postulates, or even two of them. In this chapter, I will discuss the limitations of the postulates from three standpoints: (1) those limitations that directly affected each of the three postulates, as stated in Rivers’s (1937) translation, (2) the effect of newer concepts of pathogenesis and epidemiology on the need to modify the postulates, and (3) the influence of new technological developments that permitted identification of new organisms that could not be grown in pure culture, even if one includes tissue cultures for viruses as the equivalent of standard culture methods for bacteria. A summary of the limitations of the postulates is given in Table 7.1 as derived from previous publications (Evans 1977, 1980).

*A modified version of this article has appeared in the Yale Journal of Biological Medicine (Evans, 1991b)

Table 7.1
Limitations of Koch's^a Postulates of Causation

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1. Not applicable to all pathogenic bacteria
 2. May not be applicable to viruses, fungi, parasites
 3. Do not include the following concepts:
 - A. The asymptomatic carrier state
 - B. The biological spectrum of disease
 - C. Epidemiological elements of causation
 - D. Immunological elements of causation
 - E. Prevention of disease by elimination of putative cause as element of causation
 - F. Multiple causation
 - G. One syndrome has different causes in different settings
 - H. Reactivation of latent agents can cause disease
 - I. Immunological processes as cause of diseases
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^aMore properly termed the Henle–Koch postulates.

Factors Directly Limiting the Henle–Koch Postulates as Originally Stated

“Postulate 1: (a) The parasite occurs in every case of the disease in question, and (b) under circumstances which can account for the pathological changes and clinical course of the disease.” The first part of this postulate suggests that the organism is always *demonstrable* in every case of the disease, a situation that may not always be possible to establish if the organism is a difficult one to culture or if its occurrence preceded the development of the clinical signs, so that an appropriate specimen cannot be obtained. This happens if the clinical manifestations appear at the time when, or shortly after, antibody develops, which combines with the agent making its isolation difficult. This often occurs in viral infections, such as poliomyelitis and infectious mononucleosis.

In the second part of the first postulate, the need to demonstrate that the circumstances under which the organism is isolated “can account for the pathological changes and clinical course of the disease” is satisfied by those organisms in which tissue injury is the direct result of the inflammatory changes induced at the site of multiplication. This is true of many bacterial infections, and is applicable to the tissue damage due to direct lysis of tissues by many viruses. However, there are many exceptions to this concept. The production of disease by bacterial toxins that exert their effect at a site distant from the point of multiplication is an example of this problem. There are three common diseases in which this occurs.

(1) In diphtheria, the organisms multiply locally on epithelial surfaces, where they produce a powerful toxin, which spreads by the blood to the heart and nervous system, resulting in serious involvement in these distant sites. (2) Tetanus bacillus (*Clostridium tetani*) grows in infected wounds, or through a contaminated splinter, or in the umbilical cord of the newborn, where it produces a protein toxin that travels along the axons of peripheral nerves, reaching motor neurons, and eventually diffusing through the central nervous system. There, it binds to a ganglioside receptor, and similar to strychnine, interferes with anterior horn cell activity. (3) The third example is that of scarlet fever in which an erythrogenic strain of streptococcus (*Streptococcus pyogenes*) elaborates a toxin when it is itself infected with a temperate bacteriophage; the toxin then spreads from the local site of bacterial multiplication to the skin via the blood, resulting in the rash that is characteristic of scarlet fever.

Another unusual situation is that in cholera where, despite the enormous outpouring of fluid from the intestinal mucosa which leads to severe dehydration and sometimes death, *no* pathological changes in the involved intestine have been seen pathologically on intestinal biopsy (Gangarosa *et al.*, 1960). The outpouring of fluid is due to a complicated series of chemical events induced by an exotoxin and by a neuraminidase (or sialidase) produced by the organism.

Other indirect mechanisms of tissue injury not explicable simply by the mere presence of the organism involve immunological events. One example is the formation of immune complexes of antigen and antibody of a critical size, such as occurs in the glomerulonephritis that follows infections by certain strains of streptococcus, in the nephrotic syndrome that occurs in severe malarial infections, and in the secondary stage of syphilis. Similarly, the occurrence of rheumatic fever in the wake of group A hemolytic streptococcal infections involves a poorly understood immunological mechanism, perhaps related to the production of an antibody due to an antigen shared by the organism and by heart muscle. In viral infections, a wide variety of immunological responses may lead to the clinical manifestations of the disease. These have been classified as anaphylactic, cytotoxic, immune complex, and cell-mediated, and are discussed in Chapters 3 and 4. They are simply mentioned here to indicate that the establishment of the circumstances under which an organism can account for the pathological changes and clinical course of a disease is sometimes much more difficult than suggested by the Henle–Koch postulates. While the relationship between the organism and the disease should make general biological sense, knowledge of the exact pathogenic events leading to tissue damage often comes long after the organism has been fully accepted as the cause of the disease.

“Postulate 2: It occurs in no other disease as a fortuitous and nonpathogenic parasite.” This implies that the organism should always display pathogenicity and not exist as “an accidental tourist” in the presence of some other disease. Several limitations to this concept arose almost immediately. The first was the

discovery that organisms could persist long after recovery from the clinical disease. Thus, an organism, say organism A, could still be present and isolable when the individual developed a subsequent disease due to organism B. A second circumstance was when organism A resulted in a completely asymptomatic, subclinical infection and at the same time the patient was infected with organism B, which was the real culprit in producing the clinical manifestations. The third circumstance is when organism A exists in a completely latent state until organism B infects the individual producing an illness, but also reactivating organism A from latency to active, asymptomatic multiplication. Many bacterial forms, especially intracellular organisms, such as the tubercle bacillus, brucella, parasitic infections like malaria, and many viruses, especially of the herpes family, are examples of these three possible circumstances.

Historically, the discovery of the asymptomatic and carrier state occurred shortly after Koch's 1890 lecture in Berlin. In 1893, Koch, himself, demonstrated the existence of a convalescent carrier state for the cholera bacillus, as well as the presence of a healthy carrier state, although he did not place much emphasis at that time on the latter in the transmission of the infection (Koch, 1893). At the same time, Park and Beebe in the United States clearly established both the importance of the carrier state in diphtheria and the importance of the healthy carrier in its transmission within a family (Park and Beebe, 1894). In a third disease, typhoid fever, the role of the healthy carrier in the spread of infection was recognized by Reed *et al.* (1900) in studies of the disease in military camps during the Spanish–American war in 1898 and later by Koch (1903). The importance of the long-term carrier state of the typhoid bacillus was later illustrated by the famous case of Mary Mallon, known as “Typhoid Mary,” whose chronic carrier state and occupation as a household cook resulted in a series of outbreaks in New York City over almost two decades, as described by Soper (1907, 1939). The carrier state has been reviewed by Ledingham and Arkwright (1912).

Had Koch known in 1882 of the asymptomatic and carrier states and of the persistence of the organism long after recovery, it is not clear how they would have affected his phrasing of the second postulate. His concern was in establishing the *pathogenicity* of the tubercle bacillus, not its *nonpathogenicity*, and to stress that its isolation from cases was a constant feature, not the accidental growth of some nonpathogenic, contaminating organism that had nothing to do with the disease in question. This same issue has plagued microbiologists ever since, both in differentiating the pathogenicity of two or more organisms isolated from involved tissues of an ill person, as well as the presence in experimental animals or tissue cultures of indigenous organisms derived from the animal or tissue itself, or from airborne, commensal organisms contaminating the culture media. Today, we would insist that the presence of such extraneous organisms be fully excluded by careful studies of uninoculated animals or media and/or confirmation of the results in several laboratories.

“Postulate 3: (a) After being fully isolated from the body and repeatedly grown in pure culture, (b) it can induce the disease anew.” The growth in pure, or even impure culture is not possible for many bacteria. Let me give two examples. The leprosy bacillus, *Mycobacterium leprae*, was discovered by Hansen in 1874 (Hansen, 1875), and known as a disease for some 2000 years, but has yet to be cultivated on artificial media or in tissue culture. It does multiply readily in the armadillo, but without reproducing the disease. Thus, it fails to meet both parts of the third postulate. *Chlamydia trachomatis*, the cause of trachoma, which was described in the Ebers papyrus papers in 1500 B.C. and causes a wide spectrum of other clinical syndromes involving the eye, lung, and genital tract, cannot be cultivated on artificial media. However, it can be grown in the yolk sac of eggs and, much more easily, in certain types of tissue culture, such as McCoy cells, but even here requires centrifugation onto the medium to ensure growth (excepting the LGV strain). This organism, too, does not reproduce disease in an experimental animal. The limitations of the Henle–Koch postulates to viruses have been discussed in Chapters 3 and 4 but a couple of examples are of interest to reemphasize here. Hepatitis B virus cannot be grown in any type of tissue culture and fails to reproduce clinical hepatitis in experimental animals, although it multiplies in marmosets and chimpanzees. Thus, it fails to meet the two parts of the third postulate. Yet, an excellent vaccine has been prepared using virus circulating in infected humans as the source of antigen, and more recently, by DNA recombinant technology, in which the infectious nucleic acid is inserted into a yeast carrier. Hepatitis A virus has recently been grown in specialized tissue cultures after almost 40 years of failure to do so. From this successful propagation of the virus, efforts to produce both live and killed vaccines are under vigorous development, and at the moment a killed, attenuated vaccine has shown the most promise in vaccine trials. Rapid advances are occurring in identifying forms of viral hepatitis that are neither HAV nor HBV and were formerly designated in the past simply as “non-A, non-B” hepatitis, or NANB. One of these, which was transmitted by parenteral routes, and most closely resembled HBV in its epidemiology, has now been designated as type C (HCV). It produces severe and chronic forms of hepatitis that lead to cirrhosis, is the most common form of posttransfusion hepatitis in the developed world, where HBV has been controlled by screening blood donors, and is probably the major cause of hepatocellular carcinoma (HCC) not due to HBV. In Japan, for example, an increasing percentage of HCC is associated with HCV rather than HBV. The virus has been cloned, serological tests developed, and viral identification made by a nested polymerase chain reaction test in blood and tissues. A second type of non-A, non-B hepatitis has been identified that is transmitted by the fecal–oral route and is similar to hepatitis A (HAV), except that it produces high attack rates among adults and a high case–fatality rate in pregnant women. It has now been redesignated type E (for enteric) hepatitis (HEV). Its capacity to result in water-

borne outbreaks has been deduced from earlier, large waterborne outbreaks, such as the one in India in the mid-1950s, by the lack of serological evidence of HAV infection. Diagnosis can now be made by immunoelectron microscopy on viral preparations from the stool. Neither HCV nor HEV has been grown in tissue culture, but both produce an asymptomatic infection of nonhuman primates, such as cynomolgous macaques, tamarins, and marmosets. Both viruses have been cloned so that rapid progress in diagnosis and vaccine developments may be expected. However, at the present time neither fulfill the Henle–Koch postulates.

In summary, many bacteria and viruses fail to fulfill the classical postulates of causation proposed by Henle and Koch, yet the agents are widely accepted as the cause of the diseases with which they are associated. Such incomplete evidence has not hampered successful efforts directed at their prevention and control. More modern criteria of causal proof should be used to establish causal inferences (Evans, 1976, 1988, 1991a).

Role of New Developments in Technology on Causation

Introduction

Our knowledge of the causation of infectious diseases depends on two major aspects: the conceptual and the technical. The former is concerned with our knowledge of the natural history of disease and its pathogenesis; the latter is concerned with the laboratory techniques available at the time to identify the organism, visualize it, grow it in the laboratory, reproduce the disease in an experimental animal, and explain how the organism causes the disease. The famous Henle–Koch postulates of causation (Table 2.5) (Henle, 1938; Koch, 1882a; 1938), which for over 100 years have guided investigators in establishing the causal relationship of an organism to a disease, were limited by conceptual issues, which have been reviewed (see Evans, 1976) and which have been summarized in Table 7.1.

This section will review the technical developments that preceded Koch and that permitted him to evolve his evidence of causation, particularly for tuberculosis. I will then present subsequent technical advances up to the present time that have permitted the identification of new organisms, each of which involved establishing the possible causal relationship to the disease from which it was isolated. In some cases, the discovery of the organism preceded recognition of the disease state with which it was associated. In other cases, the organism discovered by the new technique could not be cultivated in the laboratory or could not be reproduced in an experimental animal, thus not fulfilling the Henle–Koch postulates for causation.

Bacteriology

The major discoveries and examples of the organisms found are given in Table 7.2.

A few points should be emphasized at the start of this discussion. As has been stated so well by Bulloch (1938) in his great and comprehensive book, *The History of Bacteriology*, most advances have evolved in a series of small steps based on the work of several successive investigators, and the application of the method was not always made by its discoverer or even by those who later perfected the technique.

The discussion that follows was derived largely from the works of Bulloch (1938) and Foster (1970) for bacteriology and Fenner and Gibbs (1988) and Hughes (1977) for virology and in which the original references are cited. Bul-

Table 7.2
Technical Developments and the Discovery of Microbial Causes of Disease

Development	Examples
Light microscope	<i>Mycobacterium leprae</i>
Laboratory animals	
Guinea pigs	<i>M. tuberculosis</i> , Legionnaire's disease
Ferrets	Influenza
Adult mice	Herpes simplex, yellow fever
Suckling mice	Coxsackie, newer arboviruses
Chimpanzees	Hepatitis B, kuru, Creutzfeldt–Jakob
Armadillos	Leprosy
Bacterial culture (agar culture)	Most bacteria, especially Legionnaire's, campylobacteria, <i>Yersinia</i>
Embryonated eggs	Herpes, smallpox, some influenza viruses
Tissue culture	
Monkey kidney	Enteroviruses, Lassa
Adult human	Polio, adeno, RSV
Embryonic human	
Lung WI-38	CMV, rhinoviruses, corona virus
Cord lymphocytes	EBV, HIV, HTLV-I, HHV-6
Brain	Papova (JC strain)
Electron microscope	Hepatitis A, rotavirus, EVB
Fluorescent antibody	<i>M. pneumonia</i> , etiology of infectious mononucleosis

loch, in particular, has an excellent reference section and a brief sketch of all of the important microbiologists.

The Light Microscope

This instrument evolved at the end of the 16th or the beginning of the 17th century, and was based on much older knowledge of the art of making convex and concave lenses. The Dutch microscopist Antonie van Leeuwenhoek was said to have been the first to apply the light microscope to the identification of living protozoa and to bacteria (both in 1675) (Bulloch, 1938). This remarkable man ground his own lenses and made some 400 single biconvex microscopes (really more like magnifying glasses) that were capable of enlarging objects some 300 times. Besides his activities as a lens grinder and microscopist, he was also a draper, haberdasher, wine gauger for the town of Delft, and a qualified surveyor. His discoveries with his microscopes included descriptions of bacteria found in his own teeth and various morphological forms of bacteria such as coccil and spiral. His description of a motile animalcule that he found in his own feces was, in all probability, *Giardia lamblia*, and if so, it was the first parasitic protozoan to be observed in man (Dobell, 1932).

Bacterial Stains

Proper morphological description of organisms under the light microscope required some method of staining them to identify their characteristics better. The earliest efforts were apparently those of Hermann Hoffman in 1869 who employed carmine and fuchsin stains; the former was also used by Weigert in 1871 (Bulloch, 1938). However, the staining of bacteria as an art is said to have begun when Weigert showed that methyl violet stain can reveal cocci in tissues (Bulloch, 1938). Robert Koch improved on the method by preparing thin films on cover glasses, drying and fixing them in alcohol, and then using various stains, of which methyl violet 5B, fuchsin, and aniline brown were the most successful. Paul Ehrlich introduced methylene blue, and by adding an alkali to the dye, which allowed it to penetrate bacilli, Koch was able to identify the tubercle bacillus in 1882. This technique was also the basis of Löffler's methylene blue stain in 1884 (Löffler, 1884). Ehrlich improved the method of staining the tubercle bacillus by heating the slide and using aniline dye in a technique that was named the Ziehl–Nielsen acid-fast stain. Based on these observations, in 1884 Christian Gram, a Dane, developed (some say by accident) the stain that bears his name. This was made by adding Lugol's solution of iodine, followed by alcohol, and the Ehrlich stain (aniline–water–gentian violet). This stain is still

used routinely today: every laboratory applies it to identify an unknown organism. Indeed, the first question in bacteriological classification is whether an organism is Gram-positive or Gram-negative.

Pure Cultures of Bacteria

In order to study specific bacteria, it was necessary to find both an appropriate medium for their pure culture and a technique to separate out individual organisms or clones to determine the homogeneity of the bacteria. Many workers were involved in these tasks, including Pasteur, Cohn, Löffler, and Klebs. Edwin Klebs (1834–1913) had worked with the tubercle bacillus and with anthrax at the same time as Koch, or even slightly before, and apparently made the first attempts to obtain separate cultures by a technique he termed “fractional method.” Oscar Brefeld, a great mycologist, laid down the basic principles of obtaining pure cultures based on studies of fungi. These observations were published in 18 volumes. Joseph Lister, the Scottish surgeon who introduced aseptic techniques into the operating room, invented a specially constructed syringe with a graduated nut that could deliver volumes as small as 1/100 of a minum. After a millionfold dilution, he was able to deliver a drop containing a single bacterium. In this way he separated single colonies of *Bacterium lactis* in 1878. Other workers such as Nagele, Fitz, Hansen, and Salomonsen also used this dilution technique to obtain pure cultures. Parenthetically, it was this dilution technique by which Albert Sabin, some 100 years later, was able to obtain attenuated strains of poliomyelitis viruses that formed the basis of his oral vaccine. In bacterial cultures, it was Robert Koch who devised methods of isolating pure cultures that are still used today. His method was based on the use of clear nutrient gelatin with 1% meat extract. He prepared sterile slides, over which the medium was poured, inoculated with a platinum wire or needle, and then placed in a test tube with the sterile gelatin on a slant or upright. This method was demonstrated in 1881 with great acclaim before a very distinguished audience in London that included Lister and Pasteur. By 1883 Koch had improved the technique by mixing the bacterial inoculum with melted gelatin and pouring it over cold sterile glass plates. Students from all over the world flocked to Koch’s laboratory in Berlin to learn the method. The ability to separate out and grow a single organism lent great specificity to the search for the causative agent of a disease. In virology, molecular techniques now permit identification of specific viruses, their genetic variability in human passage, and the genomic properties that determine pathogenicity and clinical response patterns. Epidemiologically, these tools in both bacteriology and virology have permitted tracing of epidemics due to a specific strain of the organism, to differentiate between reactivation of an agent and exogenous reinfection, and to identify many molecular charac-

teristics of the microbial agent responsible for pathogenicity, virulence, and the pattern of host response. For example, a change in even one amino acid may alter a virus so that it is pathogenic, as is the case of rabies virus.

Virology

The technical developments discussed above for bacteriology had little impact on the field of virology because viruses differ from bacteria in at least two essential ways: (1) except for the large vaccinia virus, most viruses are smaller than bacteria and cannot be seen under the light microscope, which permits identification of bacteria; viruses thus require an electron microscope for their visualization. (2) Viruses cannot be grown in bacterial media but depend on living tissues for their multiplication, either in a living organism or in tissue cultures derived from human or animal tissues. Thus, the major developments that permitted virology to emerge as a separate discipline were based on filtration methods, by which viruses could be separated from bacteria and other large parasites, the discovery of the electron microscope, and the development of susceptible animal models and of tissue cultures.

The poxviruses represent the prototype virus type and smallpox the prototype disease on which most major developments in virology were first made. The large size of the virus, its ease of cultivation in the laboratory, and the characteristic features and epidemic importance of the disease it produced were the major reasons for this. The historical importance of the virus becomes very apparent when one reads Frank Fenner's chapter on "The Poxvirus" in the book *Portraits of Viruses*, which was edited by him and Adrian Gibbs (1988). The story of the conquest of smallpox has also been told in magnificent detail in a beautifully illustrated book, *Smallpox*, edited by Fenner, D. A. Henderson, and others (1989).

Viral Filters

In 1892, Dimitri Isoifirch Iwanowski, a graduate student at the University of St. Petersburg, discovered that tobacco mosaic virus could pass through a filter that held back bacteria. Six years later, and without knowledge of the Russian's work, Martines William Beijerinck also found this virus to be filterable, and called it "contagium vivum fluidum" (Hughes, 1977). Much later, but of fundamental importance to the sizing of viruses, was the development of graded membrane filters by Elford in 1931. The earliest classification of viruses was based on sizing by this method. Later, morphological and biochemical methods became available for classification.

Today, these are being replaced by molecular techniques by which the genomic properties and amino acid sequences can be determined.

Electron Microscope

The first electron microscope was built in 1932 by Knoll and Ruska, and the first pictures of a virus, that of tobacco mosaic virus, were shown in 1939 by Kausche *et al.* Over time, and with improvement in techniques, almost all human and animal viruses have been visualized with the electron microscope. The exceptions to this are the slow or unconventional viruses, which are now referred to as “prions” because the infectious particle appears to be a protein rather than a nucleic acid (RNA or DNA), which characterizes conventional viruses. These prion agents cause a number of diseases of animals and man, including a spongiform encephalopathy of sheep (scrapie), mink, and cattle, and kuru, Creutzfeldt–Jakob disease, and Gerstmann–Straussler–Schenker syndrome in humans.

Through the development of methods in preparation, such as metal shadowing, negative staining, freezing, and osmic acid fixation, the morphological appearance of the external surfaces of conventional viruses and bacteria became well characterized under the electron microscope. Plaque counting techniques permitted quantitation of their numbers, and the addition of specific immune sera, which resulted in clumping, allowed specific identification of viral groups (termed “immunoelectron microscopy” or “direct virology” when applied to clinical specimens). Most of these studies involved known viruses that had been identified by other techniques.

The electron microscope has also been the means of discovering new viruses, or at least, of first visualizing viruses suspected of causing a disease, but in which the web of causation was indirect. Two examples of the former will be given and one of the latter.

In 1964, Epstein, Achong, and Barr reported the presence of viral particles under the electron microscope in cultures of lymphoblasts derived from Burkitt’s lymphoma, a childhood tumor of African children first described in detail by Denis Burkitt, an English surgeon. The particles were found to be a new herpesvirus, distinct from herpes simplex, varicella/zoster, and cytomegalovirus. It was eventually called Epstein–Barr virus (EBV). While identified in Burkitt tumor tissue, its causal relationship to this tumor remained unclear until added means of identifying the presence of EBV or its antibody became available. The latter technique was provided by Gertrude and Werner Henle in 1966 when they developed an immunofluorescence test for identifying IgG antibody to the viral capsid antigen. This led to both case–control and prospective studies that implicated EBV as an initiator of the pathogenesis of the tumor by creating a prolifera-

tion of B lymphocytes. This was augmented by malaria, which is also a B-cell mitogen and also impairs the immune system in its responses to such proliferation. The rapid multiplication of B cells resulted in a chromosomal translocation (from chromosome 8 to chromosome 14, 2, or 22), and with this the activation of an oncogene, *c-myc*, which was the final step in the appearance of a malignant cell whose multiplication constituted Burkitt's lymphoma (Evans and de The, 1989).

The discovery of the immunofluorescence test for EBV also led in 1968 to the discovery by the Henles and Volker Diehl (1968) that EBV was the cause of infectious mononucleosis. It is ironic that Werner Henle was the grandson of Jacob Henle, who proposed the first criteria for causation of an infectious disease in 1840 (see Henle, 1938), yet none of these criteria were met by his grandson in establishing the proof that EBV caused infectious mononucleosis. Indeed, neither EBV nor hepatitis B virus fulfilled the preexisting postulates of Henle-Koch (Henle, 1938; Koch, 1882a; 1936), Rivers (1937), or Huebner (1957). They required new ones based on immunological evidence (Evans, 1974) consisting of (1) the appearance of specific antibody to the agent during the course of the disease, (2) that such immunity protected against primary infection, (3) that only persons lacking the antibody were susceptible to the infection, and (4) that no other antibody could induce similar immunological events.

Another important group of viruses discovered by means of the electron microscope were the rotaviruses. These were identified in duodenal cells and stools of children with acute gastroenteritis in Australia by Bishop and associates (Bishop *et al.*, 1973, 1974). Proof of causation rested on regular identification of the virus in sick children and the appearance of virus-specific antibody. Experimental reproduction of the clinical disease in animals was not possible, and even initial isolation in tissue cultures was extremely difficult. The demonstration by immunoelectron microscopy of both virus and antibody was necessary for viral identification until easier methods, such as radioimmunoassay and ELISA (enzyme-linked immunosorbent assay), were developed. It is now recognized that this group of rotaviruses is the cause of 30–50% of worldwide cases of acute gastroenteritis in children under 3 years old. Vigorous attempts to develop a vaccine with attenuated human strains or animal rotaviruses (the so-called Jennerian approach) are under way. Unfortunately, the diversity of rotavirus strains and their poor antigenic properties are making this a difficult task. Enough evidence of homotypic protection from a vaccine is at hand, however, to indicate fulfillment of Huebner's criterion for causation, i.e., that a vaccine prepared from the putative cause should decrease the incidence of the disease (Huebner, 1957).

For hepatitis A, the electron microscope was the means of establishing visually the presence of a virus in stool samples by Feinstone *et al.* (1973), as well as providing a means of diagnosis by immune electron microscopy. This

confirmed epidemiological evidence derived from human volunteer experiments that the virus was present in the stool of infected persons. Attempts to reproduce the disease in nonhuman primates had failed (although infection occurred), as had many early attempts to grow the virus in tissue culture. Later, and with much difficulty, it was successfully adapted to growth in tissue culture by Provost and Hilleman (1979). This has paved the way for development of a vaccine, a task now being vigorously pursued with both killed and attenuated viral preparations. At present the outlook looks very good for an effective vaccine that would protect against infection with HAV. Evidence that such a vaccine decreases or eliminates the disease would also provide a final step in the causal proof that HAV causes infectious hepatitis.

Embryonated Eggs and Tissue Cultures

Tissue cultures of rabbit and guinea pig cornea had been shown by Steinhardt *et al.* (1913) to sustain the growth of vaccinia virus, and in the same year, poliomyelitis virus was reported to grow in cultures of spinal ganglia by Levaditi (1913). Neither of these methods was widely adapted, however, because of the difficulty of maintaining the cultures free of bacterial contamination. Control of this problem awaited the discovery of antibiotics. In the meantime, the chicken embryo was discovered to be an important way to cultivate viruses. For example, Woodruff and Goodpasture (1931) showed that fowlpox virus replicated on the chorioallantoic membrane of developing chick embryos. Subsequently, other poxviruses and herpes simplex viruses were cultivated in a similar way, each producing characteristic plaques on the membrane. Many other viruses, such as the myxoviruses (influenza, Newcastle disease virus, and mumps), were also found to grow in the amniotic sac or allantoic cavity of the chick embryo, and many rickettsiae multiplied in the yolk sac. For these microbial agents, the chick embryo was not the initial means of discovery, but was an important medium for diagnosis and for their growth for vaccines.

In 1945, a major breakthrough came in the use of tissue cultures when Enders *et al.* (1945) reported the growth of poliovirus in tissue cultures derived from various human embryonic sources, for which work they were awarded the Nobel Prize in 1954. Primary kidney cultures were also shown capable of supporting the growth of poliovirus and were the main source of cells for polio vaccines. The addition of antibiotics to their cultures had prevented bacterial and fungal contamination. The use of tissue cultures rapidly expanded from this point onwards. However, these two early tissue culture cells did not grow continuously, and thus required fresh material for every new viral passage. Other cell lines derived from human (HeLa, Hep-2, WI-38) and animal sources (Vero) were then developed and shown to multiply continuously in culture. They provided

appropriate substrates for growth of many viruses and for the production of many vaccines. The WI-38 cell line, in particular, was used for many vaccines because it was derived from normal human embryonic lung tissue and did not pose the potential oncogenic problem of introducing cell lines derived from a human malignancy or from an animal source into humans.

Tissue cultures of various sorts also led to the discovery of many new viruses. These included several viruses that cause acute respiratory infections, such as rhinoviruses, adenoviruses, parainfluenza viruses, and respiratory syncytial virus, and also of viruses causing acute infections of the central nervous system, such as the large group of enteroviruses, and of some Cocksackie viruses. The tissue culture systems also provided a method of isolating and propagating exanthem viruses, such as measles and rubella for vaccine production. Vero cells from green monkey kidneys were also an important growth medium for arboviruses.

The evidence for causal associations of these new viruses with the clinical conditions from which they had been isolated came primarily from such viral isolation, as well as the specific antibody responses to them. Reproduction of these diseases in laboratory animals proved very difficult, except for polioviruses, arboviruses, and measles virus [which had been shown capable of producing a rash in monkeys as early as 1921 (Blake and Trask, 1921)].

In 1967 I published a list of "Five Realities of Acute Respiratory Disease" (see Table 3.3) (Evans, 1967) to indicate that (1) the same syndrome could be produced by several agents, (2) the same virus could produce several clinical syndromes, (3) the cause of the syndrome varied by geographic area, age, and other factors, (4) the causes of only about one-half of the common acute respiratory and intestinal syndromes and of about one quarter of acute viral infections of the central nervous system have been identified, and (5) diagnosis of the etiological agent could rarely be made on clinical grounds alone and required laboratory methods such as isolation of the virus and/or demonstration of an antibody response. These same concepts were found later to apply to many syndromes of infectious and chronic diseases as well as to many malignancies. These observations meant that a given virus or bacterium might be established as the cause of a given disease in one setting, but that in another geographic area or another age group, some other infectious agent might result in the same clinical picture.

More recently, the growth of human B and T lymphocytes in suspension cultures has led to the discovery of several important groups of viruses. B-type lymphocytes, derived from lymph node biopsies of cases of Burkitt's lymphoma, were successfully grown *in vitro* in both by Epstein and Barr (1964) and by Pulvertaft (1964). As discussed above, examination of such cultured cells under the electron microscope led to the discovery of Epstein-Barr virus (EBV) (Epstein *et al.*, 1964). Indeed, EBV was found to be necessary for the continual growth of B lymphocytes in the laboratory, their so-called "immortalization."

This virus infects B cells at a receptor site on the cell similar to the receptor of C3 complement.

T-type lymphocytes were more difficult to culture, but were found to multiply in the presence of T-cell growth factor. This led to the discovery of five human retroviruses, originally designated as human T-cell lymphotropic viruses. The first of these, HTLV-I, was isolated by Poiesz *et al.* (1980) and is the cause of adult T-cell leukemia, as well as of tropical spastic paraparesis (Gessain *et al.*, 1985). It occurs primarily in Japan and in the Caribbean islands.

HTLV-II was isolated in 1982 from T cells of a patient with a T-cell variant of hairy cell leukemia (Kalyanaraman *et al.*, 1982). Its full clinical spectrum is unknown.

HTLV-III (now called human immunodeficiency virus or HIV-1) was isolated from lymphocytes of patients with AIDS by Montagnier and his group in France in 1983 (Barre-Sinoussi *et al.*, 1983) and in 1984 by Gallo and associates at the National Institutes of Health in the United States (Gallo *et al.*, 1984). The virus has been propagated in large amounts in special lymphocyte cultures, also developed in Gallo's laboratory in 1984 (Popovic *et al.*, 1984). This has permitted extensive work on the biological, biochemical, and genetic makeup of the virus.

While HIV is widely regarded as the cause of AIDS, and this belief is the basis of our national prevention campaign, a vigorous and outspoken opponent of this view is Peter Duesberg, a distinguished molecular virologist at the University of California, Berkeley (Duesberg, 1987, 1989a). Duesberg's major objections, included in his first paper in 1987, are listed in Table 7.3, to which I have replied in detail (Evans, 1989a). Duesberg then responded to my article in a letter to the journal (Duesberg, 1989b), stating that we agreed on six areas, but disagreed on two, to which I answered that we still disagreed on the concept that HIV led to AIDS and that interruption of virus transmission should decrease the

Table 7.3
Duesberg's Objections to the Concept That HIV Causes Aids^a

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1. Infections with no or low risk for AIDS indicate the virus is not sufficient to cause AIDS.
 2. The long incubation period of AIDS is incompatible with the short latent period of viral replication.
 3. Levels of AIDS virus expression and infiltration appear too low to account for AIDS or other diseases.
 4. AIDS virus is not directly cytotoxic.
 5. AIDS virus is an indicator of a low risk for AIDS.
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^aDerived from Duesberg (1987).

incidence of the disease (Evans, 1989b). Other scientists have also presented evidence against Duesberg's hypothesis (Blattner *et al.*, 1989; Ginsberg, 1988), but he remains firm in his belief that HIV is not the cause of AIDS (in fact, has nothing to do with it, even as a cofactor) and continues to add detailed virological and epidemiological "evidence" in support of his view (Duesberg, 1990, 1991).

HTLV-IV, now designated as HIV-2, has also been isolated in lymphocyte culture by Clavel *et al.* (1986; see Blattner, 1989) and by Kanki *et al.* (1986), and is another cause of AIDS, although not as pathogenic to the immune system as HIV (Blattner, 1989).

In addition to the retroviruses, a new herpesvirus, originally called human B-lymphotropic virus (HBLV), and now termed human herpes virus type 6 (HHV-6), has been isolated, initially from lymphocytes of a case of AIDS, and subsequently from healthy donors, infants with exanthem subitum, and patients with the chronic fatigue syndrome (Salahuddin *et al.*, 1986, 1988). It can multiply in both B and T lymphocytes. The prevalence of antibody to this virus has been on the order of 60% in healthy donors, indicating it is a very common and often asymptomatic infection. A true causal relationship to primary infection with the virus appears to exist for exanthem subitum (roseola infantum), a common, febrile rash in young children, and with rare cases of an infectious mononucleosis-like syndrome in young adults (Salahuddin *et al.*, 1986). The virus is easily reactivated and antibody has been found in high titer in 80% or so of a number of chronic and malignant conditions, such as chronic fatigue syndrome, Hodgkin's disease, African Burkitt's lymphoma, and acute lymphocytic leukemia. In most of these disease settings, the presence of high antibody titers appears to represent asymptomatic reactivation of the virus and not a primary causal association.

Laboratory Animals

Animal species of all types and sizes have been used for the induction of infection and disease by bacteria and viruses ever since the early work of Klebs and Koch with tuberculosis and anthrax, and of Pasteur with rabies. In his 1955 book on animal viruses, Burnet presents a table listing the animal species employed in the study of 19 viruses, beginning with the 1879 inoculation of rabbits with rabies virus by Galtier, and ending with the isolation of Cocksackie viruses in 1948 by Dalldorf and Sickles (Burnet, 1955). The latter is of particular importance because this was the means by which this group of viruses was first found, and raised the problem of causal association with patients with a nonparalytic polio-like disease, from which it was first isolated. Members of the Cocksackie group have now been shown to be the cause of a wide variety of clinical syndromes involving the central nervous system, respiratory tract, skin, and diaphragmatic pleura (Burnet, 1955).

Another example of the first identification of an agent in an experimental

animal is that of kuru, a chronic and fatal degenerative disease of the central nervous system occurring in natives of the Fore tribe in New Guinea. In 1966 Gajdusek and his group at the National Institutes of Health successfully transmitted the disease to chimpanzees (Gajdusek *et al.*, 1966). The transmission of this disease in humans was associated with cannibalism (see Chapter 4). By inoculation of brain material from cases of this disease into chimpanzees, they were able to reproduce the disease clinically and pathologically after a long incubation period. The pathological picture resembled a sponge, so that the name spongiform encephalopathies has been applied to this group of agents. The long incubation period led to the term “slow viruses,” and the lack of DNA or RNA in the agents, their high resistance to heat and chemicals, and their failure to induce a demonstrable antibody response resulted in the designation “unconventional viruses.” The infectious particle appears to be a form of protein called “prion,” and the term “prion” diseases has been suggested for this group, rather than “viruses” because they lack the nucleic acids characteristic of all conventional viruses. The cause of a similar condition, Creutzfeldt–Jakob disease, also produced a similar clinical and pathological disease in chimpanzees and other experimental animals. The finding that these infectious agents could produce chronic degenerative infections of the central nervous system of humans was a key discovery, significant enough for Gajdusek to receive the Nobel Prize. Because these agents could not be grown in tissue culture, nor antibody to them demonstrated, they gave rise to a new set of guidelines for causation. These were published by Johnson and Gibbs (1974) (see Table 4.2). The guidelines were based on the reproducibility and serial transmission of the disease in an experimental animal in several laboratories, and the exclusion of other possible agents contaminating the material.

Other Laboratory Techniques

The agar gel immunodiffusion method for demonstrating precipitin bands that form when antigen and antibody interact was the key technique that permitted Blumberg *et al.* (1965) to discover a new antigen found in the blood of Australian aborigines. Initially, it was called “Australia antigen” because it was not known what disease, if any, was associated with its presence. Subsequent epidemiological and clinical studies by Prince (Prince, 1968) established the virus, hepatitis B virus (HBV), as the cause of a type of hepatitis formerly called “serum” or “transfusion-associated” hepatitis, but now known to be transmitted by parenteral routes, by close contact, and from infected mothers to their offspring. When infection with this virus occurs early in life, as is true in Africa and Asia, the antigen persists in the blood and leads to cirrhosis of the liver and to hepatocellular carcinoma. For his discovery, Blumberg was awarded the Nobel Prize in 1976.

New molecular techniques involving DNA probes, and more recently, the

polymerase chain reaction (PCR), are revolutionizing our basic tools in microbiology (Eisenstein, 1990). They provide very powerful and highly sensitive techniques for identifying antigen in human tissues. Use of PCR, for example, has identified EBV in tissues from Hodgkin's disease (Herbst *et al.*, 1990). The method is being employed to identify the presence of small amounts of viral, bacterial, and parasitic antigens in various tissues, including blood (Eisenstein, 1990). For example, it has permitted the identification of HIV in the blood of 90% of AIDS patients, even well before antibody appears. It is also present in some infants born of HIV-infected mothers, and provides a method to indicate that the infant is infected, since it is impossible to determine for 6 months or so if the antibody in the infant was derived from the mother, or the result of active infection of the newborn. However, the method is currently too complex and expensive for such routine diagnostic tests. The PCR technique is based on amplification of impure DNA by simple chemical proliferation *in vitro* of a predetermined stretch of DNA (Eisenstein, 1990). The method is capable of amplifying specific DNA sequences to more than a millionfold in only a few hours by an automated procedure. At a practical level, it is being applied to the diagnosis of both viral and bacterial infections, as well as to the search for known viral antigens in cancer tissues, such as HTLV-I in T-cell lymphomas, EBV in various lymphomas, hepatitis B and C viruses in liver cancer, and papillomaviruses in cervical cancer (Evans and Mueller, 1990). The high sensitivity of the technique also leads to some false-positive reactions, especially cross contamination from true positive samples that have been previously tested in the same laboratory. Careful washing of equipment is essential. While an extremely important method of seeking known microorganisms, the requirement of prior knowledge of a DNA fragment of the infectious agent under study for amplification limits its application to known agents with known DNA sequences.

Immunology

A key element in establishing that an infectious agent causes a particular disease is the demonstration of the appearance of a specific antibody to the agent, or of a fourfold increase in antibody titer, if antibody is already present when the specimen is taken. The presence of agent-specific IgM antibody is usually indicative of a primary infection, although certain reactivated infections, such as cytomegalovirus, are accompanied by a small IgM response. Another highly important discovery is the development of techniques for producing quantities of highly specific monoclonal antibodies, which was first published by Kohler and Milstein (1975). The application of monoclonal antibodies to viral diagnosis has greatly enhanced demonstration of the specificity of the immune response, per-

mitting differentiation between strains of the same agent and of differentiation of a reactivated infection from one due to exogenous reinfection.

Another important arm of the immune response is that of cell-mediated or T-cell immunity, which is demonstrable by skin tests, and is useful in the clinical diagnosis of infection due to agents that cannot be grown in culture and/or for which a demonstrable antibody response is not produced. The organism that causes leprosy, *M. leprae*, is an example.

This section will briefly review the history of the key developments and is derived from Bulloch (1938), Foster (1970), and Bellanti (1988). Only key original references derived from these sources will be cited.

Antibodies and the Immune Response

The observation by Edward Jenner that persons who got well after cowpox were immune to smallpox led to his introduction of cowpox vaccine in 1798 (Jenner, 1798). This was an empiric discovery without scientific basis at the time, but the concept on which it was based has withstood the test of time and has eventually led to the complete eradication of smallpox from the world in 1977. About 100 years after Jenner's publication, Louis Pasteur and his associates began the scientific approach to immunology by preparing attenuated strains of microorganisms for protection against infectious diseases, first with fowl cholera vaccine in 1878–1880 (Pasteur, 1880) and then with anthrax in 1881 (Pasteur *et al.*, 1881). Based on Jenner's contribution, Pasteur coined the term “vaccine” (from *vacca*, Latin for cow) for these immunizations. The use of these living attenuated, as well as heat-killed cultures for prophylaxis against infectious diseases constitutes *active* immunization. The proof that this type of immunity, called *humoral* immunity, was due to antibody production was the sentinel observation of Behring and Kitasato (1890). They demonstrated the neutralizing antitoxic activity of sera from animals immunized with tetanus toxin, who were protected against infection. This was followed one week later by a paper by Behring showing the same to be true of diphtheria toxin. In their joint paper, it was also shown that the neutralizing activity of tetanus antitoxin could be transferred by serum from immunized animals to uninoculated animals and to result in protection, a process now known as *passive* immunization. In 1895, Calmette published evidence of the neutralizing property in snake venom antiserum (Calmette, 1895). A mechanism to explain how antibody is produced and how it acts was proposed by Paul Ehrlich in 1897, and was termed the “side chain” or “receptor” theory (Ehrlich, 1897).

After viruses were discovered, it was found that the production of a demonstrable, specific immune antibody response occurred much more frequently in viral than in bacterial diseases, and constituted a critical element of viral diag-

nosis. An important test to demonstrate humoral antibodies was the complement fixation test developed by Bordet and Gengou (1901), but it was much later when Rivers (1937) suggested that the appearance of specific antibody during a viral infection constituted an important element in establishing causation. This was reaffirmed in the guidelines suggested by Huebner (1957) for proof of causation in a viral disease. He also added the concept that longitudinal epidemiological studies were helpful in establishing causation, as was the demonstration that protection against infection followed the use of a vaccine prepared using the candidate causal agent. Later, for infections like EBV in infectious mononucleosis and HBV in viral hepatitis, in which the causative agent could not be grown in the laboratory, the demonstration of an immune response to the candidate agent constituted the major basis for causal inferences. These criteria were published by Evans (1974).

In addition to humoral immunity, an important arm of protection against infection is that termed *cell-mediated immunity*, or in more modern terminology, B- and T-cell immunity. On a practical basis, the most common method to demonstrate the presence of this type of immunity is a skin test, in which a small amount of the antigen is inoculated intracutaneously and followed for 24–72 hours for the appearance of an indurated, red reaction at the site of injection. Perhaps the first demonstration of this phenomenon and of its use as a diagnostic test was the tuberculin test for tuberculosis (Koch, 1890). Cell-mediated immunity (CMI) is important in the control of viruses, fungi, bacteria, and other intracellular parasites. CMI is demonstrable through the skin test, and this is used in the diagnosis of infections, such as tuberculosis and leprosy, in which no antibody response is demonstrable. However, to my knowledge it has not been included as a criterion of causal proof of an infectious disease. For this reason, further historical discussion of its development will not be pursued.

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