

Causation and Disease: Effect of Technology on Postulates of Causation*

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Received September 5, 1991

This paper reviews the technical developments in microbiology that led to the discovery of new infectious agents and the effect of these discoveries on establishing proof of causation. In bacteriology, these advances included the light microscope, bacterial stains, bacterial cultures, and the methods used to isolate clones. In virology, they involved the use of filters to separate viruses from bacteria, the electron microscope, the use of laboratory animals, embryonated eggs, tissue cultures to identify or grow the agent, and the recent development of molecular techniques to detect the presence of antigen in tissues. In immunology, they were based on the discovery of antibodies and of the immune response.

Dedication

This paper is dedicated to the late Arthur J. Viseltear, Associate Professor of the History of Medicine and Public Health. He was a dear friend and colleague, and a frequent luncheon companion on the third floor of the Laboratory of Epidemiology and Public Health. There, his wit and wisdom enlivened our conversation on an almost daily basis. His lectures and writing on the history of public health, as well as his personal encouragement of my efforts, were an inspiration to me in my modest writings on certain aspects of this subject. It is an honor to have this opportunity to express my devotion and indebtedness to him in this memorial issue of *The Yale Journal of Biology and Medicine*.

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

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Abbreviations: CJD: Creutzfeldt-Jakob disease EBV: Epstein-Barr virus ELISA: enzyme-linked immunosorbent assay GSS: Gerstmann-Straussler-Schenker syndrome HBLV: human B-lymphotropic virus HHV-6: human herpes virus, type 6 HIV-1: human immunodeficiency virus, type 1 HVC: hepatitis C virus PCR: polymerase chain reaction

*This article represents pre-publication of a modified version of a chapter from the forthcoming book, *Causation and Disease: A Chronological Journey*, by the author, to be published by Plenum Publishing Company, New York, New York, and is published with their permission.

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TABLE 1
Henle-Koch Postulates

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1. The parasite occurs in every case of the disease in question and under circumstances which can account for the pathological changes and the clinical course of the disease.
 2. It occurs in no other disease as a fortuitous and non-pathogenic parasite.
 3. After being isolated from the body and repeatedly grown in pure culture, it can induce the disease anew.
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Henle-Koch postulates of causation (Table 1) [1,2], which for over 100 years have guided investigators in establishing the causal relationship of an organism to a disease, were limited by conceptual issues that I have previously reviewed [3] and which are summarized in Table 2.

This paper will review the technical developments which preceded Koch, and which permitted him to evolve his evidence of causation, particularly for tuberculosis. I will then present subsequent technical advances, up to the present time, which 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 3.

A few points should be emphasized at the start of this discussion. As has been stated so well by Bulloch in his great and comprehensive book, *The History of Bacteriology* [4], most advances have evolved in a series of small steps based on the work of several successive investigators, and also 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 [4] and of Foster [5] for bacteriology, and from that of Fenner and Gibbs [6] and Hughes [7]

TABLE 2
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 biologic spectrum of disease
 - c. Epidemiologic elements of causation
 - d. Immunologic elements of causation
 - e. Prevention of disease by elimination of putative cause as element of causation
 - f. Multiple causation
 - g. One syndrome having different causes in different settings
 - h. Reactivation of latent agents as cause of disease
 - i. Immunologic processes as cause of diseases
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^aMore properly termed the Henle-Koch postulates

TABLE 3
Technical Developments and the Discovery of Microbial Causes of Disease

Development	Examples
1. Light microscope	<i>M. leprae</i>
2. Laboratory animals:	
Guinea pig	<i>M. tuberculosis</i> , Legionnaires' disease
Ferrets	Influenza
Adult mice	Herpes simplex, yellow fever
Suckling mice	Coxsackie and newer arboviruses
Chimpanzee	Hepatitis B, kuru, Creutzfeldt-Jakob disease
Armadillo	Leprosy
3. Bacterial culture (agar culture)	Most bacteria, especially Legionnaires', Campylobacteria, Yersinia
4. Embryonated eggs	Herpes, smallpox, some influenza viruses
5. Tissue culture:	
Monkey kidney	Enteroviruses, Lassa virus
Adult human	Polio, adeno, RS
Embryonic human:	
Lung WI-38	Cytomegalovirus, rhinoviruses, corona virus
Cord lymphocytes	Epstein-Barr virus
Brain	Papova (JC strain)
6. Electron microscope	Hepatitis A, rotavirus Epstein-Barr virus
7. Fluorescent antibody	<i>M. pneumoniae</i> , etiology of infectious mononucleosis

for virology, in which the original references are cited. Bulloch, in particular, has an excellent reference section and a brief sketch of all the important microbiologists [4].

The Light Microscope

This instrument evolved at the end of the sixteenth or beginning of the seventeenth century and was based on much older knowledge of the art of making convex and concave lenses. The Dutchman, Antony van Leuwenhoek, was said to be the first to apply the light microscope to (1) the identification of living protozoa, in 1675, and (2) to bacteria, in 1675 [4]. This remarkable man ground his own lenses and made some 400 single biconvex microscopes (really more like magnifying glasses), which were capable of enlarging objects some 300 times. In addition to 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 other various morphological bacterial forms, such as cocci and spiral forms. His description of a motile animalcule which he found in his own feces was, in all probability, *Giardia lamblia* and, if so, was the first parasitic protozoa to be observed in man [4,8].

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. The staining of bacteria as an art is said to have begun, however, when Weigert showed that

methyl violet stain can reveal cocci in tissues [4]. 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 anilin 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 Loeffler's methylene blue stain in 1884 [10]. 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, which 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 both to find 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, Loeffler, 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 the “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 some 18 volumes. Joseph Lister, the Scottish surgeon who introduced aseptic techniques into the operating room, invented a specially constructed syringe with a graduated nut which could deliver volumes as small as $\frac{1}{100}$ of a minim. 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 percent 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 distinguished audience in London, which 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 the 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 characteristics of the microbial agent responsible for pathogenicity, virulence, and the pattern of host response. For example, the change in even one amino acid may alter a virus so that it is pathogenic, as in the case of rabies virus.

VIROLOGY

The technical developments discussed above for bacteriology had little influence 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 in size than bacteria and cannot be seen under the light microscope; viruses 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 pox viruses represent the prototype virus 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 beginning. The historical importance of the virus becomes clearly 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 [10]. The story of the conquest of smallpox has also been told in magnificent detail in a beautifully illustrated book, *Smallpox and its Eradication*, edited by Fenner, D.A. Henderson, and others [11].

Viral Filters

In 1892, Demetri 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, Martine William Beijerinck also found this virus to be filterable, and called it "contagium vivum fluidum" [7]. 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 [12], and the first pictures of a virus, that of tobacco mosaic virus, were shown in 1939 by Kausche et al. [13]. Over time, and with improvement in techniques, almost all human and animal viruses have been visualized under the electron microscope. The exceptions to this statement are the slow, or unconventional, viruses, which are now referred to as "prions" because the infectious particle appears to be protein in nature, rather than nucleic acids (RNA or DNA), which characterize 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 (CJD), and Gerstmann-Straussler-Schenker syndrome (GSS) 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 "immune electron microscope" 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 for 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 [14] 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 [15]. The particles were found to be a new herpes virus, 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 [16], when they developed an immunofluorescence test for identifying IgG antibody to the viral capsid antigen of the virus. This advance led to both case-control and prospective studies that implicated EBV as an initiator of the pathogenesis of the tumor by creating a proliferation of B lymphocytes. This rapid multiplication of B lymphocytes was augmented by malaria, which is also a B-cell mitogen, and which also impairs the cytotoxic T-cell response 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 clonal multiplication constitutes Burkitt's lymphoma.

The discovery of the immunofluorescence test for EBV also led in 1968 to the discovery by the Henles and Volker Diehl [17] that EBV was the cause of infectious mononucleosis. It is ironic that Werner Henle was the grandson of Jakob Henle, who proposed the first criteria for causation of an infectious disease in 1840 [1], yet none of these criteria were met by his grandson in establishing the causal proof that EBV caused infectious mononucleosis. Indeed, neither EBV nor hepatitis B virus fulfilled the pre-existing postulates of Henle-Koch [1,2], Rivers [18], or Huebner [19]. These viruses required new ones based on immunological evidence [20], 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 in 1973–74

[21,22]. Proof of causation rested on regular identification of the virus in sick children and the appearance of viral-specific antibody. Experimental reproduction of the clinical disease in animals was not possible, and even initial isolation in tissue cultures was extremely difficult. The diagnosis by immune electron microscopy of both virus and antibody was a major technique used for viral identification until easier methods, such as radioimmunoassay and the ELISA (enzyme-linked immunosorbent assay) tests were developed. It is now recognized that this group of rotaviruses is the cause of some 30–50 percent of cases of acute gastroenteritis in children under three years old worldwide. 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 research a difficult task. Enough evidence of homotypic protection from a vaccine is at hand, however, to indicate fulfillment of one of Huebner's criteria for causation, i.e., that a vaccine prepared from the putative cause should decrease the incidence of the disease [19].

The demonstration of hepatitis A virus in stool samples under the electron microscope by Feinstone et al. [23] not only established visually the presence of the virus in the intestinal tract but also was the basis for the immune electron microscope test for diagnosis. This diagnostic test confirmed epidemiological evidence derived from human volunteer experiments that the virus could be transmitted by the fecal-oral route. Attempts to reproduce the disease in non-human 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 in 1979 [24]. This success 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 appears very good for an effective vaccine. Evidence that such a vaccine decreases or eliminates the disease would also provide a final step in the causal proof that hepatitis A virus causes infectious hepatitis.

Embryonated Eggs and Tissue Cultures

Tissue cultures of rabbit and guinea pig cornea had been shown by Steinhardt et al. [25], as early as 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 [26]. Neither of these methods were widely adopted, 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, in 1931 Woodruff and Goodpasture [27] showed that fowlpox virus replicated on the chorioallantoic membrane of developing chicken embryos. Subsequently, other pox viruses and herpes simplex viruses were cultivated in a similar manner, 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 provided an important media for diagnosis and for their growth for vaccines.

A major breakthrough in the use of tissue cultures occurred in 1949 when Enders, Robbins, and Weller reported the growth of poliovirus in tissue cultures derived from various human embryonic sources [28], for which work they were awarded the Nobel prize in 1954. Primary kidney cultures were also shown to be 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 onward; 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 the 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 Coxsackie 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 a causal association 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. Measles virus had been shown capable of producing a rash in monkeys as early as 1921 [29].

In 1967, I published a list of "Five Realities of Acute Respiratory Disease" [30] 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 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 bacteria 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 1964 both by Epstein and Barr [31] and by Pulvertaft [32]. As discussed above, examination of such cultured cells under the electron microscope

TABLE 4
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 [39].

led to the discovery of Epstein-Barr virus (EBV) [14]. 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 finding led to the discovery of four human retroviruses, originally designated as human T-cell lymphotropic viruses.

The first of these, HTLV-I, was isolated in 1980 by Poiesz et al. [33] and is the cause of adult T-cell leukemia, as well as of tropical spastic paraparesis [34]. It occurs primarily in Japan and in the Caribbean islands. The second, HTLV-II, was isolated in 1982 from T cells of a patient with a T-cell variant of hairy cell leukemia [35]. Its full clinical spectrum is unknown. The third, HTLV-III, now called human immunodeficiency virus, type 1, or HIV-1, was isolated from lymphocytes of patients with AIDS by Montagnier and his group in France in 1983 [36] and by Gallo and associates at the National Institutes of Health in the United States in 1984 [37]. The virus has been propagated in large amounts in special lymphocyte cultures, also developed in Gallo's laboratory in 1984 [38], which has permitted extensive work on the biological, biochemical, and genetic make-up 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 to this view is Peter Duesberg, a distinguished molecular virologist at the University of California, Berkeley [39,40]. Duesberg's major objections, included in his first paper in 1987, are listed in Table 4, to which I have replied in detail [41]. Although much argument back and forth has been published [42–45], he remains firm in his belief that HIV is not the cause of AIDS. In fact, he feels it has nothing to do with the disease, even as a co-factor, and continues to publish detailed virological and epidemiologic papers in support of his view [46,47].

A fourth human retrovirus, HTLV-IV, now designated as HIV-2, has also been isolated in lymphocyte cultures by Clavel et al. [48] and by Kanki et al. [49] and is another cause of AIDS, although not as pathogenic to the immune system as HIV-2 [48].

In addition to the retroviruses, a new herpes virus, 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 [50,51]. The virus can multiply in both B- and T-type lymphocytes. The prevalence of antibody to this virus has been of the order of 60 percent in healthy donors, indicating that 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 [52,53]. The virus is easily reactivated, and antibody has been found in high titer in 80 percent or so of a number of chronic and malignant conditions, such as the chronic fatigue syndrome, Hodgkin's disease, African Burkitt's lymphoma, and acute lymphocytic leukemia [52]. In most of these disease settings, the presence of high antibody titers appears to represent 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, Burnett 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 [54]. The latter is of particular importance because that was the means by which this group of viruses was first found, and it raised the problem of causal association in patients with a non-paralytic 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.

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 [55]. The transmission of this disease in humans was associated with cannibalism. By inoculation of brain material from cases of this disease into chimpanzees, the researchers 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 lack of inducing 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 (CJD), 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 systems 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 in 1974 by Johnson and Gibbs [56]. The guidelines were based on the reproducibility and serial transmission of the disease in an experimental animal in several laboratories and on 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. in 1965 [57] 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 in 1968 [58] established the virus as the cause of a type of hepatitis formerly called "serum" or "transfusion-associated" hepatitis, but now known to be transmitted by other 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 in about 90 percent of those infected and leads to cirrhosis of the liver and to hepatocellular carcinoma. For his discovery, Blumberg was also 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 [59]. They provide very powerful and highly sensitive techniques for identifying antigen in human tissues. Use of PCR, for example, has identified Epstein-Barr virus in tissues from Hodgkin's disease [60]. As reviewed by Eisenstein [59], the method is being employed to identify the presence of small amounts of viral, bacterial, and parasitic antigens in various tissues, including blood. For example, it has permitted the identification of HIV in the blood of 90 percent 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 six months or so if the antibody in the infant was derived from the mother or was the result of active infection of the newborn. The method is currently too complex and expensive, however, 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 [59]. The method is capable of amplifying specific DNA sequences 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 papilloma viruses in cervical cancer [61]. The high sensitivity of the technique also leads to some false-positive reactions, especially cross-contamination from true-positive samples, which 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 with respect to unknown agents. Other molecular techniques, however, have enabled investigators to clone the virus of hepatitis C (HCV) and to develop an antibody test for it before it has been grown in tissue culture, seen under the electron microscope, or serologic identification or genome characterization has been possible. This development has been described by Alter [62] under the interesting title, "Descartes before the horse: I clone, therefore I am. The hepatitis C virus in current perspective."

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 in 1975 [63]. The application of monoclonal antibodies to viral diagnosis has greatly enhanced demonstration of the specificity of the immune response, permitting 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 an 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 [4], Foster [5], and Bellanti [64]. 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 [65]. 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 1878–80 [66] and then with anthrax in 1881 [67]. 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 occurred with the sentinel observation of Behring and Kitasato in 1890 [68]. They demonstrated the neutralizing antitoxic activity of sera from animals immunized with tetanus toxin and also that this antitoxic activity protected the animals against infection. This observation was followed one week later by a paper by Behring alone, 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 would result in protection, a process now known as *passive* immunization. In 1895, Calmette published evidence of the neutralizing property in snake venom antiserum [69]. 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 [70].

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 diagnosis. An important test to demonstrate humoral antibodies was the complement fixation test developed by Bordet and Gengou in 1901 [71]. This method is still used as one of the most common diagnostic procedures in viral diagnostic laboratories around the world.

While many tests were developed to demonstrate the immune response, it was not until 1937 that Rivers suggested that the appearance of specific antibody during a viral infection constituted an important element in establishing causation [18], a suggestion reaffirmed in the guidelines suggested by Huebner in 1957 [19]. Huebner 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 infectious agents which could not be grown in tissue culture, the demonstration of an immune response to the candidate agent constituted the major basis for causal inferences. Examples of this type are the role of EBV in infectious mononucleosis and that of hepatitis B virus in viral hepatitis. These criteria, based on the immune response, were published by Evans in 1974 [20].

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 the use of 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 discovery by Koch of the tuberculin test [72]. Cell-mediated immunity is important in the control of viruses, fungi, and other intracellular bacteria and parasites. Although the skin test is used in the diagnosis of many of these infections, 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.

SUMMARY

The technical developments reviewed in this paper have led not only to the discovery of new organisms but also provided methods that have helped to identify their role in the pathogenesis of the diseases with which they are associated. The discovery of each new infectious agent has required evidence to establish the proof that it causes the disease from which it has been isolated. Sometimes, the technology has resulted in identifying agents which do not grow in culture or induce disease in experimental animals, thus failing to fulfill the Henle-Koch postulates for bacterial causation or those of Rivers and Huebner for viral diseases. This type of discovery has resulted in the formulation of new postulates of causation for these infectious agents.

In addition to these microbiological advances, important advances have occurred in immunology, particularly the discovery of antibodies. These advances have led to inclusion of the demonstration of the immune response in viral infections as a criterion for establishing causation.

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