CANCER BIOLOGY. III

Herpes Virus Epidemiology, Molecular Events, Oncogenicity and Therapy

Edited By

Carmia Borek, Ph.D.
Donald West King, M.D.

ADVANCES IN PATHOBIOLOGY

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Herpes Virus
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College of Physicians and Surgeons of Columbia University
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Foreword

This symposium on herpes virus was held under the auspices of the National Cancer Institute (grant #5R13 CA15961-02) during the week of August 3–8, 1975 and included 16 faculty members and 60 participants. During this week a diverse group of investigators with wide experience in viruses (both with animal and human cell systems) brought together significant information on the herpes virus implicated in the areas of productive and nonproductive transformation. Various immunologic patterns and serologic factors were reviewed and experimental therapeutic agents were thoroughly discussed.

Donald W. King

Introduction

Herpes viruses have been recognized as infectious entities for many years. The word herpes, derived from the Greek term "to creep," was used in the Hippocratic literature to portray disseminating and usually ulcerative cutaneous lesions [1]. The interest in the herpes viruses which has increased recently can be attributed in part to implications that some of these viruses play a role in the etiology of cancer in man and in a variety of other species.

The purpose of the present symposium has been to assemble the most recent available information on the biochemistry and biology of herpes viruses from experiments using model animals and both animal and human cell systems, and to evaluate the relationship between this information and the clinical-epidemiologic findings on herpes virus infections in man. A central issue in this correlation of data was to determine what are the facts and arguments in favor of herpes viruses being directly implicated in certain malignancies and what are the unanswered questions which might support, impede or modify such an association. Faced with the clinical facts of ubiquitous herpes virus infections in man the most recent advances and approaches to chemotherapy were evaluated. Using the information assembled from the molecular and biologic studies on herpes viruses (structure, antigenicity, host response, latency and recurrent infections) other approaches to therapy and prevention (such as vaccines) were explored.

There are at least five herpes viruses known in man: herpes virus simplex 1 (HSV-1), herpes virus simplex 2 (HSV-2), varicella zoster (herpes zoster virus, VZ, HZV), Epstein-Barr virus (EBV) and cytomegalovirus (CMV). The common use of the term "herpes virus" is usually restricted to HSV-1 and HSV-2. Among these five herpes viruses, HSV-1, HSV-2 and EBV viruses have been associated with cancer in man. Marek's disease herpes virus (MDV) in chickens and herpes virus simiri and ateli in monkeys have been related to oncogenicity in those animals.

The classification of the herpes viruses is based primarily on their structure (see Roizman, Kaplan). These are large enveloped virions with a DNA core which is surrounded by protein layers known as the capsid [2]. Most of the structural studies on herpes virus DNA and on their capsid proteins have been carried out on HSV-1 and to lesser extent on HSV-2 (see Roizman), or similar viruses such as pseudorabies in the pig (see Kaplan). In contrast, the virus-cell interactions of a nonlethal nature (e.g., cell transformation) have been carried out mostly with the EBV viruses (see Henle, Klein, Miller) and more recently with HSV (see Rapp).

Herpes viruses characteristically remain associated with specific cells and tissues, sometimes for the lifetime of the host. Consequently, latency of the

virus, and its persistence in tissues, such as HSV in sensory ganglia, oral-facial (HSV-1) and urogenital (HSV-2) areas (see Stevens, Nahmias, Rapp, Sabin), causing recurrent infections, pose severe clinical problems (see Nahmias, Henle, Alford and Sabin) and are perplexing phenomena on the level of virus-cell interaction (see Klein, Miller, Rapp, Stevens, Nahmias) and on the immunologic level (see Henle, Nahmias, Klein, Miller, Rapp, Sabin), a basic question being whether the ability to cause latent infections plays a role in the induction of cancer.

Herpes virus infection can be productive in permissive cells, resulting in virus replication, host cell alteration and ultimate lysis (see Roizman, Courtney, Rapp, Kaplan, Henle). The herpes viruses vary in their ability to grow in various test systems. HSV-1 and HSV-2 can replicate in both human and animal fibroblasts (see Rapp), while EBV growth is confined to human and primate lymphoblast cell lines and its replication in those cells is restricted or can be induced only under specific circumstances (see Henle, Rapp).

The nonproductive infection is of great interest in evaluating the role of herpes viruses in malignancy. The infection of appropriate cells with inactivated or defective herpes viruses may lead to cell transformation (see Rapp, Sabin, Henle, Klein, Miller). The transformed cells to varying degrees, depending on the virus and the host cell, carry the genetic information of the virus detectable biochemically; this information is expressed by the presence of new nuclear and surface antigens in the host cells and is detectable by immunologic methods (see Henle, Klein, Miller, Rapp, Sabin).

EBV transforms cells *in vitro* by endowing lymphocytes with the capacity to proliferate continuously to produce lymphoblastoid cell lines (see Klein, Henle, Miller, Rapp). There is no evidence that these EBV "immortalized" lines are oncogenic *in vivo* in an immune-competent animal (see Miller), yet they have neoplastic potential in immunologically deficient nude mice (see Klein). The factors determining whether the EBV "immortalized" cells are neoplastic may depend on a specific genetic constitution of the cell and/or its state of differentiation (see Klein).

Infection of rodent or human cells by defective virus mutants (produced by UV-irradiation or photodynamic inactivation) of HSV-1 and HSV-2 and possibly CMV (see Rapp) results in neoplastic transformation similar to that induced *in vitro* by known oncogenic viruses (see Sabin). The transformed cells acquire a variety of properties characteristic of neoplastic cells, and in tested cases (e.g., mouse and hamster transformed fibroblasts) they can give rise to tumors upon injection into syngenetic hosts (see Rapp).

Herpes viruses have been implicated in cancer on the basis of clinical-epidemiologic findings in man (see Henle, Nahmias, Klein, Sabin, Rapp). EBV has been consistently associated with two human malignancies, Bur-

kitt lymphoma (BL) and nasopharingeal carcinoma (NPC). EBV is considered also as the cause of infectious mononucleosis (IM), a benign disease in which lymphocytes have the capacity to proliferate continuously, reminiscent of the lymphocyte "immortalization" *in vitro* by EBV. The oncogenicity of a virus *in vivo* which occurs in some situations but not in others may be attributed to deficient immune surveillance mechanisms (see Klein, Henle). Findings of consistent specific chromosomal abnormalities in BL cells but not in benign EBV-infected cells alternately suggest that a certain cellular genetic makeup might be necessary in order for the virus to induce malignant transformation in certain cell populations, similar to the situation *in vitro* (see Klein, Rapp). It is of interest that IM is a multiclonal disease, while BL is uniclonal [3]. The mode of involvement of EBV in NPC mentioned above has been recently questioned, following findings that the virus is carried by the epithelial cells of the carcinoma rather than by the infiltrating lymphocytes, as believed heretofore (see Klein, Henle, Rapp).

The complex immunologic factors and patterns of antibody synthesis involved in various EBV-associated diseases have been extensively discussed (see Henle). Antibodies are made preferentially against certain subcomponents and components of the antigen complex, depending on the disease. Among the various antigens (membrane and nuclear), the EBV early intracellular antigen is of particular importance since antibodies against it are formed in high titers in EBV-associated diseases but relatively rarely and in low titers in healthy individuals.

The oncogenic potential of HSV in man has been more difficult to evaluate than that of EBV. HSV-2 has been associated with squamous cervical carcinoma on the basis of seroepidemiologic studies (see Nahmias, Sabin, Rapp). Attempts to demonstrate herpes virus DNA or antigens in human cervical carcinomas have been frustrating and inconclusive (see Sabin, Rapp).

Several schemes suggest the relationship between infectivity by the herpes viruses, transformation and malignancy (see Sabin, Nahmias, Miller, Rapp). Discussions by various participants emphasized that there are still many open questions on the defined interactions between the viruses and their hosts as pertaining to latency and malignancy and the answers are not yet at hand.

Control of herpetic infections in man involves recognition of their biochemical nature as large DNA viruses and of their distinctive biologic behavior, such as life cycle, latency, chronicity and recurrence of infection. The approaches have been immunologic and chemotherapeutic (see Alford). The most effective approach has been chemotherapy (see Alford, O'Connor, Cohen). Compounds such as 5-iododeoxyuridine (IUDR) which are directed against virus-coded enzymes induced in the infected cells and

essential for viral replication have been effective; or inhibitors of DNA synthesis, some of which inhibit viral replication faster than killing the host cell, have been widely used (e.g., D-arabinosyladenine—ara A). The effectiveness of ara A, which is also an antitumor agent, varies with the infecting herpes virus (see Alford) and is decreased after administration due to metabolic inactivation by deamination. The use of deaminase inhibitors is being explored. These inhibitors must be used with caution in man since they increase the toxicity of the arabynosyl compound (see Cohen).

In discussions among the participants, the possibility of herpes virus vaccines was evaluated on the basis of the assembled information. The problem of heterogeneity of the herpes viruses was raised (e.g., to date 50 strains of HSV alone have been identified). The use of attenuated virus is unlikely, since a follow-up of many years would be required to test for oncogenicity. The use of inactivated virus would not be feasible since the viral DNA is still oncogenic (see Rapp). The answer may lie in the use of vaccines containing subunits, such as DNA or purified protein. Little is known, however, about the immune response to these viruses, since the predominating studies in the herpes virus field have been serologic.

The Editors

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The Sero-Epidemiology of Epstein-Barr Virus

Werner Henle, M.D., and Gertrude Henle, M.D.

Isolation of a seemingly new virus from a patient must be followed, among others, by several immunologic test procedures to ascertain (a) whether the virus is indeed new or can be identified; (b) whether it was truly isolated from the patient by demonstration of antibodies to the virus in the patient's serum; (c) whether other patients with a similar disease also have antibodies to the virus; and (d) whether, and at what fequency, antibodies are found in the general population—which, in turn, will indicate whether the virus is widely disseminated or rare, and whether the disease involved might be an unusual manifestation or the inevitable result of infections by the virus. These procedures are routine for the diagnostic virologist but, unfortunately, they are not generally applied to viruses derived from human malignancies and proclaimed as "first human cancer viruses." The immunologic approaches mentioned might have prevented false hopes and embarrassment in several recent instances.

The validity of the required immunologic studies is well illustrated by the results obtained with the Epstein-Barr virus (EBV). This herpes group virus was detected by electron microscopy over 11 years ago in a small proportion of lymphoblastoid cells cultured from Burkitt's lymphoma (BL) biopsies by Epstein and his co-workers. It was readily assumed by most observers that it was one of the then known human herpes group viruses; i.e., herpes simplex virus (HSV), cytomegalovirus (CMV) or varicella-zoster virus (VZV) and, since the virus was subsequently found also in lymphoblast cultures of non-BL origin and no herpes virus had as yet been shown to be oncogenic, it was considered of no particular importance and relegated to a passenger role. However, the virus could not be transmitted to cell cultures or other host systems known to be susceptible to HSV, CMV, or VZV,

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For detailed references the reader is referred to recent reviews of this and related topics listed at the end of this chapter. Only references to new observations are presented in the text.

which thus provided the first clue that it was a heretofore unknown member of the herpes group and soon to be named the Epstein-Barr virus.

Immunologic studies were hampered by the fact that EBV could be maintained only in continuous lymphoblast lines, such as the original BL cultures, in which at best only about 10% of the cells produce a largely defective viral progeny or merely virus-specific antigens; i.e., abortive cycles of replication. This situation still holds at present, since no cells have been found which are fully permissive for EBV. The lymphoblast cultures are divided into "producer" and "nonproducer" lines, depending on whether or not virus is synthesized in some of the cells, although all harbor EBV genomes. Virus separated from producer cultures can either superinfect nonproducer cells, which results, as a rule, in an abortive infection with synthesis of early but not late antigens and thus no virus particles, or it transforms lymphocytes *in vitro* into permanently growing cultures of lymphoblasts, all carrying EBV genomes. The lines so established again are divided into producers and nonproducers.

Because of the restricted growth and maintenance of the virus, the more usual immunologic technics, such as neutralization and complement fixation tests, were not immediately applicable to studies of the virus. EBV- or viral antigen-synthesizing cells in producer cultures were readily detectable, however, by indirect immunofluorescence with many human sera known to be devoid of antibodies to HSV, CMV or VZV. These results, as well as the absence of immunofluorescent staining by HSV-, CMV-, or VZV-specific immune sera proved beyond doubt that EBV was a new virus.

Although sera were not available from the patients who provided the first BL cultures, there is no doubt that they would have contained antibodies to EBV since to date all sera from African BL patients have yielded positive immunofluorescence, usually at high titers. Thus, EBV appeared to be associated with African BL.

Serologic surveys with this first immunofluorescence test, which was shown to detect mainly antibodies to viral capsid antigen (VCA), indicated that EBV has a world-wide distribution in that anti-VCA was present in sera from children at variable but substantial frequencies, depending on hygienic and socioeconomic conditions, and in the great majority of sera from adults. These observations clearly implied that BL, if caused by EBV, would be an unusual manifestation rather than the inevitable result of infections by the virus.

In a search for other diseases possibly caused by EBV it was noted that several individuals seroconverted from anti-VCA negative to positive status in the course of infectious mononucleosis (IM). This provided the first clue that EBV might be the cause of IM, which by now has been firmly es-

tablished. It was also observed that patients with anaplastic or poorly differentiated nasopharyngeal carcinomas (NPC) uniformly had antibodies to VCA, usually at high titers. Thus three diseases (BL, IM and NPC) were shown to be closely associated with EBV on serologic grounds. Compared to controls, an over-representation of high anti-VCA titers, but not as striking as in IM, BL or NPC, was noted also in several other malignant and nonmalignant diseases, such as Hodgkin's disease, chronic lymphatic leukemia, sarcoidosis, systemic lupus erythematosus and others. Since some of these types of patients have no antibodies to EBV, an etiologic relationship of EBV to these diseases seems unlikely.

It was evident that merely the incidence and titers of anti-VCA were insufficient for discrimination between causal and other relationships of EBV to given diseases. More refined serologic technics, to be discussed below, and other types of evidence, to be reviewed by Drs. Klein and Miller, were needed for this purpose.

EBV-related Antigen-Antibody Systems

Four groups of antigens have been differentiated in lymphoblasts per se or after various manipulations by immunofluorescence technics with selected human sera (Table 1). They are (a) the viral capsid antigen (VCA) already noted; (b) EBV-determined cell membrane antigens (MA), which comprise several early and late components; (c) the EBV-induced early (intracellular) antigens (EA), which are subdivided into the D (diffuse) and R (restricted) components on the basis of immunofluorescent staining patterns; and (d) the EBV-associated nuclear antigen (EBNA), which is present in all EBV-transformed cells and resembles a T antigen. In addition, EBV neutralization tests have been developed which are based on the prevention of either EA synthesis in, or death (no colony formation) of, nonproducer cells after superinfection by EBV, or on the prevention of transformation of cord blood lymphocytes by the virus. The neutralizing antibodies appear to be identical with antibodies to some MA components. Also soluble (S) complement-fixing antigens have been extracted from cells of producer as well as nonproducer cultures which seem largely identical with EBNA. Among the various antigens, the EA complex is of particular importance, since the corresponding antibodies are found at high frequencies and often high titers in EBV-associated diseases but relatively rarely and at only low levels in healthy donors. Anti-D predominates in many IM and NPC, and anti-R in most BL patients. The antibody spectra and titers in these diseases will be discussed below.

TABLE 1. EBV-Related Antigen-Antibody Systems

Antigens		Antibodies	
Designation	**Optimal Test Cells**	**Test Procedure**	**Human Sera**
Viral Capsid Antigen (VCA)	Producer Lines (acetone-fixed)	Indirect Immunofluorescence	All donors with past EBV experience
Cell Membrane Antigens (MA)	Producer Lines (live)	Blocking of direct Immunofluorescence*	Most donors with past EBV experience
Early and Late Early Antigens (EA)	Nonproducer Lines EBV-superinfected IdU-induced		
Diffuse (D) component	(acetone or methanol-fixed)	Indirect Immunofluorescence	Mainly IM or NPC patients
Restricted (R) component	(acetone-fixed)	Indirect Immunofluorescence	Mainly BL patients
Soluble CF Antigen (S)	Nonproducer Lines (extracts)	Complement Fixation	All donors with past EBV experience
Nuclear Antigen (EBNA)	Nonproducer Lines (acetone/methanol-fixed)	Anti-Complement Immunofluorescence	All donors with past EBV experience
Enveloped Virus Particles (VP)		Neutralization	All donors with past EBV experience
Lytic	Nonproducer Lines	Prevention of EA Synthesis Reversal of Colony Inhibition	
Transforming	Cord Blood Lymphocytes	Prevention of Transformation	

IM, Infectious Mononucleosis; NPC, Nasopharyngeal Carcinoma; BL, Burkitt's Lymphoma; * Conjugate free of isoantibodies.

Infectious Mononucleosis

As shown schematically in Figure 1, prospective studies have shown that IM occurs only in individuals who previously had no antibodies to EBV. In the acute phase of the disease, IgM and IgG antibodies to VCA are regularly found, the former disappearing again within a few weeks, the latter persisting at readily detectable levels for life. Anti-MA (not shown) and EBV neutralizing antibodies also arise in all patients and they too persist, usually for life, but anti-D appears only transiently in about 80% of the patients. All these antibodies reach peak titers by the time of onset of illness or shortly thereafter, with anti-VCA and anti-D slightly preceding anti-MA and neutralizing antibodies. In contrast, anti-EBNA and anti-S, as a rule, develop only several weeks or even months after onset. This difference in appearance of the two groups of antibodies has implications which will be discussed later. The heterophil antibody response, which with few exceptions is specific for IM, is seen in about 90% of young adult patients but with decreasing frequency in consecutively younger age ranges.

These results show (a) that absence of antibodies to EBV denotes susceptibility to IM, and the simultaneous persistence of anti-VCA and neutralizing antibodies explains why anti-VCA, which is not a protective antibody, nevertheless serves as a dependable indicator of immunity to the disease; and (b) that serodiagnostic evidence for current primary EBV infections in single acute phase sera is provided by detection of VCA-specific IgM antibodies, presence of anti-D, and absence of anti-EBNA. Confirmation is obtained by demonstration in subsequent sera of a decline and disappearance of the IgM antibodies and of anti-D and appearance of anti-EBNA. While EBV-specific serodiagnostic tests are not needed for typical heterophil antibody-positive cases of IM, they are needed for HA-negative cases or patients with non characteristic or unusual manifestations of the disease, whether observed as complications or as the only clinical signs of infection.

During the acute phase of IM, EBV is uniformly found in the oropharyngeal secretions, as determined by transformation of cord blood lymphocytes. Furthermore, continuous, EBV-positive lymphoblast lines can be established regularly from peripheral lymphocytes of the patients. Virus excretion into the oropharynx may persist for many months after onset and may recur, presumably intermittently, for life. Also, lymphoblast lines can be established at considerable frequencies long after overt or silent primary infections from the peripheral blood and almost invariably from lymph nodes. These observations provide clear evidence that primary EBV infections lead regularly to a persistent viral carrier state in the lymphoreticular system which, in turn, explains the life-long maintenance of antibodies to certain of the EBV-related antigens.

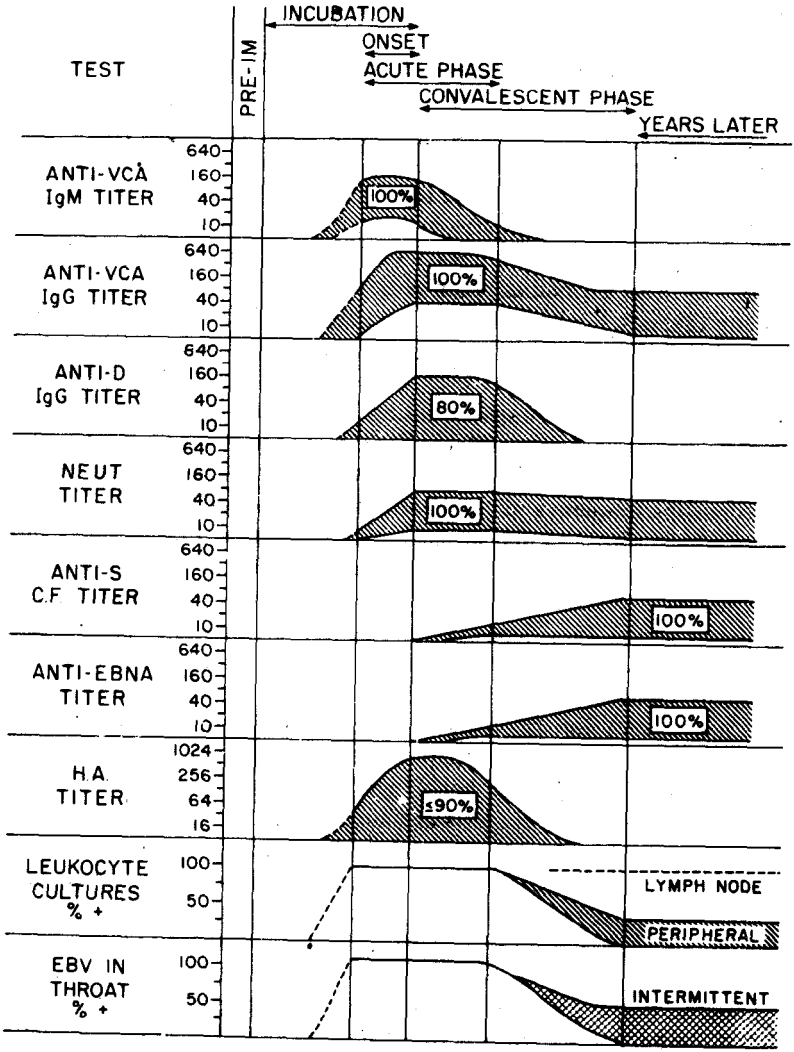

FIG. 1. Scheme of antibody responses, leukocyte cultures and EBV assays in throat washings during the course of infectious mononucleosis.

Typical IM is not a regular consequence of primary EBV infections. In young adults of socioeconomically advanced nations, about one-third to one-half of the infections remain silent, or cause such mild illnesses that IM is not suspected. No or limited clinical responses are seen even more often in young children; in pediatric cases with otherwise typical signs, a diag-

nosis of IM has been rejected in the past when heterophil antibodies were not detected. For these reasons, IM is practically unknown in those parts of the world where primary EBV infections generally occur very early in life because of poor hygenic or low socioeconomic conditions.

The early appearance of anti-VCA, anti-D and neutralizing antibodies in the course of IM and the late development of anti-EBNA and anti-S suggest that the two groups of corresponding antigens become available for antibody stimulation under different conditions [1]. The first group is obviously derived from lytically infected cells which shed the various components during degeneration (Table 2). While synthesis of EBNA probably occurs in the lytic cycle, it is produced in insufficient quantities, as a rule, for induction of early antibody responses. It is likely that aside of lytic infections, EBV also transforms lymphoid cells *in vivo*. Indeed, virus excreted into the oropharynx is mainly of the transforming type. Such transformed cells would be EBNA-positive, viable and presumably capable

TABLE 2. Suggested Scheme of Primary and Continuing Antibody Responses to EBV-related Antigens