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HUMAN LYMPHOBLASTOID CELL LINES AND EPSTEIN-BARR VIRUS: A REVIEW OF THEIR INTERRELATIONSHIPS AND THEIR RELEVANCE TO THE ETIOLOGY OF LEUKOPROLIFERATIVE STATES IN MAN**

The Epstein-Barr virus (EBV), is a herpes-like virus found in continuous cultures of human leukocytic cells derived from certain tumors, from bone marrow and peripheral blood leukocytes of patients with diseases such as leukemia and infectious mononucleosis, and from the blood leukocytes of some normal persons. EBV was discovered in lymphoid cell cultures originating from Burkitt's lymphoma (BL); because it was the first virus to show a regular association with a human malignant tumor, it has received a great deal of attention from experimental oncologists, virologists, epidemiologists, and clinicians in the six years since its discovery.

The hypothesis that viruses are a cause of human malignant disease has been explored with increasing energy since studies in lower animals have demonstrated the oncogenic potential of these agents. The RNA tumor viruses of chickens,[1] mice,[2] and cats[3] have been considered likely models for human leukemia and lymphoma because of the similarity of the diseases in lower animals and in man, and because these agents produce tumors in the species in which they are found in nature. Polyoma and simian virus 40 (SV40), small DNA viruses of the papova group, do not usually produce tumors in their natural host but they are important tools in experimental viral cancerigenesis, because they offer the promise of close genetic and biochemical analysis of malignant transformation *in vitro*. Some types of human adenoviruses, larger DNA viruses, cause tumors in experimental animals and produce malignant transformation of animal cells *in vitro*.[4] Since some adenovirus types are oncogenic and others are not, a great deal of research has been directed toward understanding these differences which, at present, are not fully explained at a molecular level.

More recently a fourth group of animal viruses, the herpes group, which are still larger, more complex enveloped viruses with double-stranded DNA, have been regularly associated with tumors both in lower animals and in man. The herpes virus of the Lucké frog renal adenocarcinoma was dis-

covered in the 1930's[5] and its morphology described in 1956.[6] In the last year its oncogenic capacity was demonstrated experimentally by the induction of tumors on the mesonephric ridge of tadpoles.[7] A herpes virus has been found in Marek's disease, a lymphoma of chickens; the agent cultivated in tissue culture is able to reproduce the disease upon inoculation of susceptible birds.[8] Preliminary field trials of an attenuated virus vaccine suggest that this commercially important avian tumor can be prevented by immunization.[9] In the past year Melendez and his co-workers[10] reported that herpes virus saimiri, indiginous to the squirrel monkey, produces lymphoma and occasionally leukemia on inoculation into cotton top marmosets and owl monkeys, two other South American primates. Finally, two herpes viruses of man, the genital strain, or sub type 2 of herpes virus hominis,[11] and EBV have received attention as possible tumor inducers.

These examples of the close association of herpes viruses with tumors have widened interest in EBV as a candidate human-tumor virus. Furthermore, in early 1968, W. and G. Henle and their collaborators reported that an immune response to EBV developed during the course of infectious mononucleosis (IM).[12] The association of the virus with this long enigmatic disease, which to some appeared as an abortive malignancy, has generated even more interest.[13]

For reasons which will be discussed, it is not possible to state with absolute certainty that EBV is the cause of Burkitt's lymphoma or infectious mononucleosis, the two conditions with which it has been associated most closely. Students of this virus have become polarized into two camps; one group proposes that EBV is the etiologic agent of IM, and perhaps a cocarcinogen with some other factor in BL; the other group considers that EBV is a passenger virus with an attraction for poorly differentiated lymphoblastic cells. It is the purpose of this review to analyze the published information about the virus and to attempt to correlate the available evidence with the central question of whether the virus is pathogen or passenger. No attempt has been made to review the clinical aspects, pathology, or therapy of BL or IM, both of which have been comprehensively described in monographs recently published.[14,15] Instead, attention will be focused on the behavior of EBV virus *in vitro* and on serologic and epidemiologic studies of EB virus infection.

Discovery of EBV and its association with Burkitt tumor cells

In 1958, Burkitt made his original observations on an unusual tumor of the jaw occurring in children of Uganda.[16] The geographic distribution of the disease was of special interest; the disease predominated in hot, wet lowlands. Because this pattern coincided with the distribution of arthropod

vectors, Burkitt considered that a mosquito-borne agent might be the cause. Accordingly, specimens of tumor tissue were submitted to several virologic facilities, where a variety of microorganisms, including reovirus,[17] herpes simplex virus[18] and mycoplasmas,[19] were recovered, although none were isolated regularly. In late 1963, Epstein, Barr, and Achong established several continuously growing *in vitro* cell lines with lymphoblastic features from specimens of tumor tissue.[20-22] Some characteristics of these human lymphoblastoid cell lines (HLCL) were morphology suggesting an immature state of the lymphocyte series, continuous rapid growth after 4-8 weeks in culture, and failure to adhere to glass. Several cell lines examined with the electronmicroscope revealed virus-like particles of a size and structure characteristic of the herpes group.[23] Such particles were generally identified in only a small proportion of the *in vitro* cell population.

Origin of continuous lines of lymphoblastoid cells and presence of EBV therein

Soon after Epstein and his associates established cell culture lines from Burkitt lymphoma, such cell lines were derived from patients with a variety of diseases and examined for viral particles by electronmicroscopy and later for viral antigen by the immunofluorescent (IF) assay on fixed cells (Table 1). Lymphoblastoid cell lines were grown from BL by several workers and nearly all contained EB viral particles.[24-26] The major exception was a BL line designated Raji[27] which, despite extensive examination, has not revealed complete virus.

Itakawa and Grace[28] and Foley and co-workers[29] found that HLCL could be established from peripheral blood leukocytes of leukemia patients. Eventually HLCL were derived from the peripheral blood of patients with acute leukemia of the myeloblastic[30] and lymphoblastic types[31] and from patients with acute and chronic myelogenous leukemia.[32,33] HLCL originating from patients with acute leukemia often, though not invariably, contain EBV virions. Moore and his co-workers found herpes-like particles in lines from acute leukemia;[30] three lines derived in our laboratory from children with leukemia contain EBV antigen. Certain HLCL from leukemia patients do not contain EBV antigen or particles, such as the CCRF-CEM line, which has been extensively studied by Foley and co-workers.[29]

Lymphoblastoid lines were also derived from lymph node biopsies of reticulum cell sarcoma, mycosis fungoides, Hodgkin's disease, multiple myeloma, carcinoma of the lung, and from a spleen removed because of hereditary spherocytosis.[34,35]

Continuous culture of cell lines from the blood of patients with IM was reported by Pope in 1967,[36] by Glade and co-workers in 1968,[37] and in 1969

TABLE 1. ESTABLISHMENT OF HUMAN LYMPHOBLASTOID CELL LINES FROM PATIENTS WITH DIVERSE DISEASES AND FROM NORMALS

Disease	Reference	No. individuals studied	No. HLCL established	No. donors of HLCL with EBV antibody	Presence of EBV in HLCL	
					By EM	By IF
Burkitt lymphoma*	20, 21, 22	NS	3	NS	3/3	3/3†
Infectious mononucleosis	38	32	29	29	NS	29/29
Infectious mononucleosis	39	23	9	NS	4/8	8/9
Leukemia	33	23	7	NS	0/3	2/2†
Hepatitis	40	6	3	3	3/3	2/3
Hepatitis	41	35	21	NS	NS	NS
Normal	45	8	6	6	4/6	3/6
Normal	41	16	3	NS	NS	NS

NOTE:
HLCL = Human lymphoblastoid cell lines.
EM = Electronmicroscopy.
IF = Indirect immunofluorescence on acetone fixed cells.
NS = Not stated.
* In Burkitt lymphoma HLCL were established from tumor biopsies; in other instances from peripheral blood leukocytes.
† See Ref. 53.

by the Henles.[12] Diehl[38] and Moses, et al.[39] demonstrated in a series of cases
that blood leukocytes from patients with IM grew readily in the acute phase
of the disease and frequently, though not invariably, during convalescence
or many years later. Most HLCL derived from patients with IM contain
detectable EBV.[38,39]

Lymphoblastoid lines have been grown from peripheral blood of patients
with other viral illnesses associated with circulating "atypical" lymphocytes.
Glade and his associates and Stevens, et al. found that blood leukocytes from
about half of patients with presumed viral hepatitis grew into HLCL.[40,41] All
patients in Glade's series had EBV antibody when their leukocytes were
cultivated in vitro. Two of the lines established by Glade from hepatitis pa-
tients showed evidence of EBV. Minnefor and co-workers established long-
term suspension cultures of leukocytes from patients with viral diseases
such as herpes simplex, mumps, herpes zoster, and measles, but did not pro-
vide data about EBV antibody in their patients.[42] Klemola, et al. found that
in 3 of 12 adult patients with cytomegalovirus infection HLCL have been
obtained from 6 months to several years after appearance of the clinical
disease.[43]

In few series has a large control group of normal individuals been studied
in order to compare the frequency of formation of HLCL in a particular
disease and in normals. Such information as is available, including results
from our laboratory, suggests that when small inocula of cells (10^5-10^6 cells/
ml.) and small volumes of cells (10-20 ml.) are used, normal blood leuko-
cytes do not spontaneously form HLCL. By contrast, white blood cells from
patients with IM and from some patients with leukemia form lines readily
under these conditions. However, under special conditions white blood cells
from healthy individuals who possess EBV antibody can be grown as
HLCL. Long-term cultures of leukocytes from normal individuals were first
described by Moore[44] and later by Gerber and Monroe.[45] Both workers ob-
served EBV in some of these cell lines. The experimental method in large
part influences whether or not lymphocytes from normal persons can be
grown as HLCL. Both Moore and Gerber and Monroe initiated normal
blood cultures with white blood cells from 500 ml. of blood, and frequently
adjusted the leukocyte concentration so that at least 5×10^5 viable cells/ml.
were always present.

Several reports of serial attempts to cultivate HLCL are summarized in
Table 1. HLCL have been derived from patients with a variety of diseases,
malignant and benign, and under special cultural conditions from normals.
The majority of such leukocyte suspension cultures have contained EB virus,
and whenever it has been studied the donor of the leukocyte line has had
detectable EBV antibodies. A reasonable general hypothesis under which to

assemble these observations is that a very small number of cells with the capacity to divide continuously *in vitro* is present in the peripheral blood of normal individuals with EBV antibodies and that in diseases such as leukemia or IM the number of such cells competent to initiate DNA synthesis *in vitro* increases.

Significance of EBV "free" lines

A line "free" of detectable EBV poses many interesting questions, such as "Was EBV initially responsible for its growth?" or "Is EBV present in the line in some form not detectable by our current limited assay techniques?" Detection of EBV is difficult since viral particles or viral antigens are present in a small proportion of cells. Detection of virus by electronmicroscopy represents a serious sampling problem. In IF tests unwanted fluorescence in certain lines due to production of gamma globulins makes a low level of immunofluorescence difficult to interpret. Another problem in detection of EBV is the downward fluctuation with time in the level of immunofluorescent cells due to unknown causes.

A final answer to the question, "Do all HLCL contain at least part of the EBV genome?" must await development of more refined techniques to demonstrate presence of the viral genome. A recent study by Zur Hausen and co-workers,[48] employing techniques of nucleic acid hybridization, suggests that viral genome is present in some HLCL, such as the Raji line, which do not reveal particles or viral antigen. Zur Hausen and collaborators showed that the Raji line contained DNA with homology to purified EB viral DNA. Furthermore, in preliminary experiments they found an RNA in Raji cells which hybridized with EB viral DNA. This result suggests that at least part of the EB viral DNA in Raji cells is transcribed into RNA.

Characteristics of the EB virion—Morphological and biophysical

EBV in electronmicrographs resembles herpes simplex virus, though it is smaller.[47] Encapsidated forms with or without an electron dense nucleoid are seen in the cell nucleus. The approximate diameter of the electron dense central nucleoid is 45 nm, and of the capsid plus nucleoid, 70 nm. Some enveloped forms are found in the cell cytoplasm, but rarely extracellularly. The diameter of the completed enveloped virion is approximately 115 nm. Most particles seen in an infected cell are imperfect; they either are unenveloped, or contain no nucleoid core; occasionally free nucleoids are seen. In early pictures of virus from Burkitt tumor cells, extracellular virions were enclosed in a hazy cloud of amorphous material, thought to be specific antibody coating since these early HLCL cultures contained human serum.

Cells harboring the herpes-like particles are usually degenerated, although occasionally particles are found in morphologically altered but intact cells. The nuclear membrane of virus-infected cells frequently shows segments of reduplication, a morphological sign of infection with many other herpes viruses. In addition, infected cells demonstrate collections of tubular structures located in the cytoplasm.[47]

EBV particles were partially purified from infected cell lines by Toplin and Schidlovsky[48] who submitted homogenates of a large number of cells to rate zonal centrifugation. Several grossly visible bands were isolated that contained viral particles in various stages of maturation, similar to those present in thin sections of infected cells.

The DNA of EBV has been purified and its density determined.[49-51] Isotopically labelled virus was first isolated either from supernatant fluids or cell extracts by rate zonal centrifugation in sucrose gradients. The virus was then extracted further, and the labelled DNA was placed on an equilibrium buoyant density gradient of cesium chloride. In three laboratories the density of EB viral DNA was between 1.716 to 1.720 gm/cm.^{3} This value is similar to that obtained for herpes simplex DNA, and significantly different from cell DNA (1.695 gm/cm^3). DNA of the density corresponding to that of EBV was not obtained when the Raji-line, free of EBV particles, was treated in parallel, although, as previously mentioned, EB viral DNA can be demonstrated in the Raji line by nucleic acid hybridization.

Assays for the presence of EBV

Sensitive quantitative assays for the biologic activity of EBV, such as the plaque assay employed for other animal viruses, are not yet available. For this reason, little is known of the replicative cycle of the virus. However, several existing assays detect the presence of EBV.

Electronmicroscopy: Hinuma and co-workers[52] studied growth of EBV in a BL line by sampling the cells and supernatant fluids at intervals and counting particles by electronmicroscopy. Their results suggested that the majority of the virus was cell-bound but that some was released into the fluid phase of the culture as the cells were kept without refeeding.

Intracellular EBV antigen: Assay for the virus was simplified by the finding that most adult human sera contained anti-viral antibodies detectable by the indirect IF test on cells previously subjected to acetone fixation.[53] The frequency of fluorescent cells correlated with the number of cells containing viral particles in electron micrographs and certain lines without viral particles were also free of viral antigen by the IF test. Increased specificity of the IF test was achieved by preparation of anti-EBV sera in rabbits and conjugation of the sera with fluorescein isothiocyanate.[54] When individual cells

were studied both by electronmicroscopy and by the IF test, cells which contained viral antigens also contained particles.[55] The IF test is at present the most widely employed assay both for determination of EBV antibody levels in human sera and also for detection of the presence of EBV in various HLCL.

Membrane antigen: Another assay detects an antigen on the cell membrane of unfixed HLCL cells and is called the membrane immunofluorescence (MIF) test.[56] MIF antigen has been found on the surface of cells in HLCL derived from patients with Burkitt's lymphoma, IM, leukemia, and from normals. By contrast to the intracellular IF antigen, which has only been found in cells grown for a time *in vitro,* the MIF reaction can be demonstrated on cells taken directly from biopsy material as well as those grown in culture.

The MIF antigen is distinct from antigens responsible for leukocyte typing; it can be demonstrated by using a patient's own serum to test his own tumor cells.[57] The MIF reaction is probably due to more than a single new antigen at the cell surface.[58] Evidence for multiple antigens comes from experiments in which sets of sera with MIF reactivity are compared for their capacity to block the MIF reaction of other sera.

The MIF antigen and the intracellular EBV antigen are distinct antigen-antibody systems, for MIF antibody can be removed by absorption with whole cells from an HLCL; after absorption, antibody against intracellular antigen persists.[59] In contrast to the low level of intracellular antigen, often as many as 50% of cells may contain surface antigens. However, there appears to be some correlation between the extent of the MIF reaction and the extent of IF staining for intracellular viral antigen in a given HLCL.[60] These results suggest that the extent of expression of the viral genome varies from one HLCL to another and that regulation of membrane antigen and of viral antigen may be coordinated to some extent.

The implication of studies to date is that the MIF detects an antigen coded by viral genome. However, this point has not been proved conclusively and the alternative, that it represents an "unmasked" cellular antigen, must be explored.[61]

Complement fixation (CF): Extracts of certain BL or leukemia cell lines contain antigens that fix complement in the presence of certain human and primate sera.[62,63] At least two CF antigens are present in EBV-infected HLCL.[64,65] One antigen (V antigen) is associated with viral particles; the other antigen (S or soluble antigen) is present in supernatant fluids from which virus has been sedimented by ultracentrifugation. The CF test appears to be specific for EBV, since positive sera do not fix complement with anti-

gens prepared from normal human leukocytes, a variety of non-leukocytic tissue cultures, or antigens prepared from other herpes-group viruses.

Immunodiffusion: Precipitating antigens are found in highly concentrated preparations of aged cultures of the Jijoye line of BL lymphoblasts.[65] In further applications of this technique as many as three lines of reaction between various sera and the Jijoye line were found.[67] Agar gel immunodiffusion correlates well with the IF method on fixed cells, and with CF, for detection of EBV.[68] However, of the three methods, immunodiffusion is the least sensitive.

Biologic activity of EBV

Two biologic properties of EBV can be studied in cell culture. First, materials containing EBV cause normal leukocytes to undergo transformation into continuous cell lines of lymphoblastoid character. Second, highly concentrated, partially purified preparations of EBV may superinfect HLCL that are initially devoid of EBV antigens.

Transformation of normal leukocytes into continuously growing cell lines by EBV was discovered by Henle and co-workers.[69] As a source of EBV, Henle used lethally x-irradiated cells of the BL Jijoye line, and as a source of normal leukocytes the peripheral blood white cells of infant girls. (The Jijoye line maintained a diploid male karyotype and thus the origin of cells growing in co-cultivation could be identified by examination of chromosomes.) After two to three weeks of co-cultivation of the x-irradiated Jijoye cells and the female leukocytes, in the presence of a human diploid fibroblast "feeder layer," lymphoblastoid cell lines developed. These lines had female sex chromosomes (thus were derived from the infant leukocytes) and contained EBV antigen. Under the conditions of the experiment, the x-irradiated Jijoye cells in separate culture did not survive, nor did the female leukocytes. As a control, x-irradiated cells of the Raji BL line, without EBV antigen, were incapable of promoting continuous growth of normal leukocytes. Using a similar co-cultivation system, Miller and co-workers showed that a factor capable of inducing continuous growth of normal adult leukocytes was also present in the x-irradiated cells of an EBV-infected HLCL from a leukemic child.[70] Similarly a leukemic cell line without EBV antigen was incapable of inducing normal white blood cell transformation.[71] Further studies by Diehl and co-workers and by our laboratory with this system have shown that transformed normal leukocytes (TNL) are capable of inducing continuous growth of other normal leukocytes.[72] Since EBV antigen appears in the TNL, the hypothesis considered most likely is that the transformation results from infection of the normal leukocytes by EBV and alteration of the cell's growth regulation by the virus.

This thesis is supported by the findings of J. H. Pope and his co-workers in Brisbane, Australia, who showed that cell-free filtrates prepared from extracts of a leukemic HLCL, containing EBV, were capable of inducing continuous growth of leukocytic cells from human fetal thymus or spleen.[73] Appropriate controls indicated that the fetal lymphocytes did not proliferate in the absence of filtrates containing EBV. Sera that contained EBV antibodies inhibited the transformation, and sera devoid of such antibodies had no effect.[74] Neutralization of EBV-induced transformation of adult leukocytes by immune sera has recently been demonstrated in our laboratory.[71] Gerber, *et al.* have found[75] that EBV concentrated and purified from supernatant fluids of aged cultures induced continuous growth of leukocytes obtained from an adult who had no evidence of past experience with any member of the herpes group. The mechanisms of EBV-induced leukocyte transformation remain to be elucidated.

Extracts of infected HLCL have been placed in a wide variety of tissue culture systems and a large number of experimental animals, without consistent cytopathic or other effects.[47] In preliminary reports it appeared that EBV might be cytopathic for dog thymus cells[76] or that EBV-infected cells might cause an encephalitis in newborn thymectomized hamsters;[77] however, these reports have not been confirmed.

Horoszewicz found that concentrated and purified virus obtained from the fluid phase of infected cell lines when transferred to other HLCL apparently devoid of EBV, produced IF antigen in the recipient cells.[78] Various cell lines free of antigen have been used as receptors for the virus.[79,80] It has not been resolved whether viral antigen that appears in the "negative" lines is due to the superinfecting agent or to reactivation of latent virus in the recipient line, although the former explanation appears most likely. The activity of virus preparations used to "superinfect" is very low (10^2 to 10^3 ID50/ml.) despite concentration of up to 1,000-fold in some instances; the reasons for this have not been adequately explained.

Superinfection of the Raji line with concentrated partially purified EBV forms the basis for another serologic test, called the early antigen assay.[81] When the superinfected Raji line is treated with certain normal human sera in an immunofluorescence assay a very small number of EBV-infected cells is detected ($< 1\%$). By contrast, when sera of comparable anti-EBV titer from patients with BL or nasopharyngeal cancer and from some IM patients are used, a larger number of EBV-superinfected cells (as many as 50%) are detected. The superinfected Raji cells are said to be "abortively infected" because the EBV infection does not spread in the culture. Sera from some patients detect this abortive or early antigen while sera from other persons

such as convalescent IM patients or apparently healthy people do not detect the antigen.

Relationship of EBV genome to individual cells of HLCL

Viral particles or viral antigens detectable by the IF test are present in few cells of any HLCL, usually not more than 5% and often less. The number of fluorescent cells in some HLCL can be increased by cultivation of the cells at 32° C.,[82] or by cultivation of the cells in a medium deficient in L-arginine.[83] No published information is available on the effect of other techniques that induce replication of temperate bacteriophage or that induce replication of SV_{40} in transformed cells such as x-ray, Mitomycin C, or cell fusion.

Several interpretations may be considered to explain the constant low level of EBV infection in HLCL. The first is that HLCL are "carrier cultures" for EBV; that is, a few infected cells in the culture produce virus that infects the other cells. The low level of infection is explained by the presence of interferon which may protect the cells.[84] The second is that the cultures consist of two distinct populations of cells: EBV-infected and -uninfected; both populations give rise either to EBV-infected or -uninfected cells. These two schema each assume that a very small proportion of cells are actually infected at any one time. A third model, which has received experimental support recently,[85-87] is that all cells in an EBV-infected culture contain viral genome but only a small proportion of cells spontaneously make viral particles or viral antigens. Experimental proof depended on methods for growing mass cultures of HLCL from isolated single cells. Single cell clones were derived from various HLCL, each of which contained approximately 1% of cells with viral antigen. The clones were treated with EBV anti-serum to neutralize any virus that might be present at the cell surface. Each clone tested showed evidence of EBV, thus suggesting that EBV in some form is associated with all the cells in the culture.

Are HLCL tumor cells?

It has not yet been determined whether the cells that comprise a HLCL are tumor cells or whether they are "activated" normal lymphocytes. The question is difficult to resolve for definite morphologic, biologic, or biochemical markers among HLCL derived from normal blood, from patients with IM, and from patients with malignant lymphoma or leukemia have not been recognized. Attempts to study the problem of tumorigenicity has involved transplantation of HLCL into heterologous hosts, such as newborn rats[88] and newborn hamsters, which have been immunologically crippled with antilymphocyte serum.[89,90] After several passages in hamsters, the tumor cells

retain human surface antigens. Whether heterotransplantation represents oncogenic potential or adaptation of the cells to an *in vivo* culture system is not yet clear.

SEROLOGIC AND EPIDEMIOLOGIC STUDY OF EBV INFECTIONS

Three aspects of the human immune response to EBV have been studied; namely, circulating antibodies to surface membrane antigens on intact virus-infected cells, cell mediated reactions and circulating antibodies to the virus *per se* or to virus-associated antigens.

Antibodies against surface antigens of HLCL have been found in sera of BL patients and IM patients.[91] In a single reported case of BL, membrane antibodies were examined sequentially. They were elevated during remission induced by chemotherapy, after which they fell to a low level before recurrence of the disease.[92] Whether recurrence was related to disappearance of the membrane antibodies is speculative. The actual biologic function of membrane antibodies is uncertain, and it is not known whether or not the membrane antibodies are cytotoxic *in vivo*.

Recently Fass and co-workers[93] found that some patients with BL developed delayed type hypersensitivity skin reactions to autologous tumor cell extracts. In only one of 12 patients was the skin test positive before treatment, but in 7 of 12 patients delayed cutaneous hypersensitivity to extracts of their own tumors was evident after clinical remission was induced by chemotherapy. In several patients who failed to improve with treatment, delayed skin reactions did not develop. The nature of the antigens responsible for the skin reaction is not known.

Very little published work concerns *in vitro* tests for cell-mediated immunity to EBV. Recently Steel and Hardy[94] and Junge, *et al.*[95] reported that extracts of cell lines formed from the peripheral blood of patients with IM stimulate short-term DNA synthesis in primary cultures of lymphocytes from the same patient. The nature of this mitogenic factor is not clear but preliminary studies by Junge suggest that it is not virus, but has the characteristic of a membrane-bound antigen.

When serological methods for detection of EBV antibodies became available in the mid-1960's, it was of greatest interest to determine whether the presence of antibodies to EBV correlated with those disease states, specifically BL and leukemia, in which EBV could be frequently found in long-term leukocyte cultures. Some results from these early serologic surveys, done with various techniques, gave in general the same over-all picture, as illustrated by the accompanying Table 2. A high frequency of EBV was found with all methods in patients with BL. Similarly, nearly all sera of African, Chinese, and American patients with nasopharyngeal cancer con-

tained EBV antibody. The frequency of antibody titers in sera of leukemia patients was much lower than in the other two diseases. A most surprising finding from initial studies was that antibody was widely distributed in the sera of healthy individuals, both African and American, although patients with BL and IM, and nasal tumor have usually had significantly higher antibody titers than normal individuals.

Age and geographic patterns of antibody

In a study of complement-fixing antibodies to EBV, Gerber[96] showed that the age-distribution was similar to the pattern observed for other acute viral diseases of childhood such as measles or poliomyelitis in the pre-vaccine era. Nearly all sera from newborn infants contained antibody, presumably maternal in origin. Less than 20% of sera from infants aged 6 months to 1 year contained antibody. With increasing age, the percentage of sera with antibody rose from 30% in the 1 to 2 year group to more than 60% in the 3 to 5 year group. Porter and associates demonstrated a similar increase in the prevalence of EBV antibodies with increasing age.[97]

Henle and Henle have examined sera taken prospectively to determine whether the acquisition of antibody in the younger child was accompanied by an identifiable clinical illness such as "non-bacterial tonsillitis."[98] In most cases seroconversion to EBV was clinically silent.

The age at which antibody to EBV is acquired is influenced by geographic and by social factors. Eighty percent of the African children studied by Henle, *et al.*[99] possesed EBV by age 2, while a majority of 10 year old middle-class children from Cleveland, studied by the same workers, had no antibodies.[98] In their studies of Yale college students, Niederman, Evans, and collaborators[100-102] found approximately 60-75% of freshmen to be susceptible to EBV. This figure, significantly higher than predicted by Gerber's earlier serologic survey using sera from low-income families, was attributed to the social-class background of college students. By contrast to their findings in Yale undergraduates, Niederman and co-workers found only 25% of University of Philippines undergraduates without EBV antibodies.

Although large-scale geographic surveys have not yet been done, antibodies have been found in sera collected from several isolated primitive peoples from New Guinea, Micronesia, and South America.[96]

A most important point which has received preliminary study[103] is the relationship between the distribution of Burkitt's tumor and the distribution of EBV antibodies. EBV antibodies are no more frequent in residents of the lowlands of Uganda where BL is endemic than in those living in other geographic areas where the tumor is much less frequent.

TABLE 2. SUMMARY STUDIES OF PREVALENCE OF SERUM ANTIBODIES AGAINST EBV IN VARIOUS POPULATIONS

Principal investigators	Serologic techniques	Healthy persons				Patients, children			Patients, adults			
		Afr. child.	Afr. adults	Amer. child.	Amer. adults	BL	Leuke-mia	Solid tumors	Leuke-mia	Solid tumors	Cancer Post-nasal space	IM Het. +
Henle[53,99]	IF	282/359*	98/105	NS	26/33	17/17	5/16	3/7	5/10	14/15	241/241	NS
Niederman[100,108]	IF	NS	NS	NS	387/873	NS	NS	NS	NS	NS	NS	135/135
Porter[97]	IF	NS	NS	240/505	NS	NS	NS	NS	NS	NS	NS	NS
Hirshaut[106]	IF	NS	NS	NS	NS	30/30	NS	NS	NS	NS	NS	45/45
Gerber[62,96,104]	CF	NS	NS	NS	47/61	25/29	NS	NS	NS	NS	7/7	21/21
Old[95]	ID	NS	NS	NS	8/82	31/55	NS	NS	6/41	8/68	33/39	1/17
Stevens[97]	ID	NS	19/30	38/121	85/141	67/73	6/14	NS	11/17	NS	NS	17/28

NOTE:

* Number shown = Number with antibody/number studied.

IF = Indirect immunofluorescence on acetone-fixed cells. A significant antibody titer is a 1:10 or greater dilution of serum.

CF = Complement fixation. A significant antibody titer is a 1:30 or greater dilution of serum.

ID = Immunodiffusion. Antibody determined on undiluted serum.

NS = Not specified or group not studied.

371

Relationship of EBV to infectious mononucleosis

In late 1967 Henle, Henle, and Diehl discovered a relationship between EBV and IM through observations during the illness of their laboratory assistant, Elaine H.[13] Elaine H.'s leukocytes, used as controls in experiments before she acquired IM, failed to proliferate for more than a short time. After IM, her leukocytes grew continuously and contained EBV. Her serum, free of EBV antibodies before her illness, contained EBV antibodies after her illness. A small series of pre- and post- IM sera obtained from Dr. Niederman at Yale, confirmed the observation that EBV antibodies were absent in pre-illness sera and present in sera taken after the illness. Also with Niederman's sera, Gerber and co-workers demonstrated with complement fixation that EBV antibodies were absent in pre-IM sera and that they appeared during the disease,[104] while in the same paired sera no consistent response to herpes simplex, cytomegalovirus (CMV), or reovirus, was observed. Absorption of sera with sheep red blood cells had no effect on EBV antibodies, although heterophile antibodies were removed. Niederman and his collaborators[105] showed that EBV antibodies appeared during the clinical illness and persisted thereafter, while heterophile antibodies declined. In an elegant epidemiologic analysis[100] made possible through a long-term prospective collection of sera, Evans, *et al.* showed that the absence of EBV antibody correlated well with susceptibility to IM, and conversely that college students who possessed EBV antibody at entrance did not develop IM. In this investigation they established that occasional cases of heterophile-negative IM were associated with antibody rises to EBV. Their data suggested, furthermore, that inapparent infection with EBV was considerably more frequent than clinical IM in persons younger than college-age, thus confirming the hypothesis proposed ten years earlier by Evans.[101] By contrast, in the college-age group clinical IM was approximately twice as frequent as inapparent infection with EBV. Other investigators have confirmed that EBV antibodies develop during clinical IM,[106] though they disagree about the significance of these antibodies. The complex question of their significance will be treated in the DISCUSSION.

Klemola and Kaariainen[107] found rising complement-fixation antibody titers to CMV in certain clinical cases of heterophile-negative cases resembling IM, but usually distinguished by the absence of sore throat and widespread lymphadenopathy. It was subsequently shown that CMV could frequently be isolated from these patients. Klemola, Von Essen, Henle, and Henle[108] recently studied 44 cases diagnosed as heterophile-negative IM. Their results showed that CMV and EBV each cause some cases diagnosed

as heterophile negative IM and that possibly other agents as well are responsible for a proportion of the cases.

Transmission of EBV infections

The route of transmission of EBV infections is in large part unresolved. It has been presumed that IM is transmitted by the oral or respiratory route but EBV has not been isolated from these sites. The failure to isolate EBV from the upper respiratory tract may reflect the insensitivity of available methods for cultivation of infectious virus. Attempts to isolate EBV from mosquito pools in Africa have not met with success. This also may be a matter of technique. There is no evidence of congenital EBV infection in the form of isolations of the virus from embryonic sites or from leukocytes of newborn infants. Limited data collected by the Henles in the Cleveland family study failed to detect individual infants whose maternal antibodies did not disappear.

Transmission of EBV through blood transfusion has been documented.[109-111] Gerber called attention to rising EBV antibody titers in five individuals of a large series of patients who were treated with multiple blood transfusions during and after open heart surgery. EBV was cultivated in the leukocytes of one patient who developed an illness similar to IM, with fever, atypical lymphocytes, and a positive heterophile test. Blacklow and co-workers studied a similar case of post-transfusion mononucleosis in a very elderly patient who ocquired an IM-like illness with heterophile antibodies five weeks after receiving three blood transfusions for a hip fracture. EBV was isolated from this patient and Ig-M anti-EBV antibodies were present in her acute phase serum. Henle and others showed in a large prospective series of patients receiving multiple blood transfusions for heart surgery, that of 18 without pre-operative antibodies, six acquired EBV antibodies, although none of the patients in this series developed illness clinically similar to IM. Parenteral transmission of the virus probably occurs in association with infected leukocytes in blood; however, other routes will have to be sought for transmission of the majority of EBV infections in which there is no evidence for parenteral exposure.

DISCUSSION

Intrinsic biologic interest of EBV

EBV is in many ways a unique virus, and will undoubtedly continue to receive attention from those interested in the mechanisms of viral infections. Thus far it shows strict tropism for primate leukocytic cells of the lymphocytic series. Tropism for cells of certain species *in vitro* and *in vivo* have

been long recognized as a property of animal viruses, for example, in the rather strict preference of poliovirus for primate cells. However, strict preference for one type of differentiated cell is a new phenomenon and deserves further analysis.

Another feature of EBV infection at the cellular and molecular levels of general biologic interest is the regulation of production of complete virus. *In vivo* viral particle production is repressed, but *in vitro* virus is produced. In cloned cell populations a few cells produce viral particles; most do not, even though all cells apparently contain viral genome. The control mechanisms, whether they reside in cell or virus, are closely regulated since approximately the same percentage of cells produce viral particles when the cells are examined at random over a long period of time. A study of the nature and mode of operation of repressors of virus synthesis in EBV infected cells may have general relevance to problems related to cell differentiation.

EBV causes changes in the morphology and growth characteristics of normal leukocytes. It is not yet known whether this phenomenon is analogous to "viral transformation" induced by polyoma virus and SV_{40}, or whether it is a further stage of "blast transformation" produced by substances such as phytohemagglutinin or by specific antigens. The former explanation seems more likely at present than the latter. Whatever the explanation, it will be of great interest to know the details of the mechanism by which the normal lymphocyte with limited potential for *in vitro* growth is stimulated to grow continuously by EBV.

In many ways, except for their *in vitro* immortality, the lymphoblastic cells of HLCL resemble "dedifferentiated" lymphoblasts produced by phytohemagglutinin (PHA). Both PHA-treated cells[112] and HLCL spontaneously release interferon-like viral inhibitors.[42,84] Release of interferon may be related to the presence of viral or other inducers within the leukocytes, or the release of interferon may occur whenever certain lymphocytic cells are in the blast stage. The latter explanation does not account for the observation that various HLCL differ considerably in the amounts of interferon which they produce.[113] The availability of cell lines that continuously manufacture and secrete interferon should allow analysis of the biosynthetic pathways involved in interferon production. Similarly, the availability of a large variety of HLCL which release immunoglobulins spontaneously[114] should permit analysis of the mechanisms of biosynthesis of these important proteins. Specific antibody activity has not yet been assigned to the immunoglobulins synthesized *in vitro* by HLCL.

Does EBV cause infectious mononucleosis?

There are two major hypotheses about the role of EBV in IM. The framework of the first hypothesis follows: EBV is mainly transmitted by the respiratory route. Infection in the young child is associated with either no clinical symptoms, mild febrile illness, or infrequently, IM. In older adolescents and in susceptible adults infection with EBV more frequently causes IM than inapparent infection. Heterophile-positive IM is accompanied by development of EBV antibodies. Antibodies against the complete virus persist for life, and whether they are acquired following IM or following inapparent infection, these antibodies protect against subsequent IM due to EBV. After infection the virus also probably persists in lymphoid cells for life.

Outlines of the second hypothesis may be summarized as follows: EBV is a ubiquitous virus with a predilection for lymphoid cells. Its mode of transmission is not known, but it may be congenitally acquired. It persists in lymphoid cells in defective form, perhaps as viral nucleic acid, only until lymphoid cells are transformed into blastic cells. In its defective form the virus stimulates either a very low level or no antibody. When lymphoblasts appear, due to one of many causes, complete virus proliferates and circulating antibodies to the virus are produced. According to the second hypothesis, EBV antibodies correlate with resistance to IM because they co-exist with antibodies for other agents which are truly responsible for the disease.

The available evidence favors the first hypothesis. The strongest link in the chain of evidence in favor of a causal role for EBV in IM is the demonstration by Niederman, Evans, and colleagues that absence of antibodies against EBV correlates with susceptibility to IM. The evidence is strengthened by the *in vitro* demonstration that EBV stimulates leukocyte proliferation, a basic pathologic change in IM. The reported cases of EBV infection and post-perfusion illness transmitted by blood, after a five-week incubation period, also favor a direct role of EBV in the IM syndrome. Experimental transmission of IM to patients with malignant disease has apparently been accomplished, although published details are few.[115] In general, the major elements lacking to fulfill Koch's postulates are experimental reproduction of disease in man or laboratory animals, and recovery of the organism from experimental disease. These requirements may be very difficult to achieve. For a variety of reasons, especially ethical, it may prove impossible to carry out experimental transmission studies in man. EBV may or may not be ultimately adaptable to an animal host. An objection to the hypothesis of direct causation is that EBV has not been recovered immediately from materials obtained from persons with the disease. It has only

been found in leukocytes which have been cultured *in vitro* for several weeks. However, the intimacy of certain cell-virus relationships may require adjustments of the definition of "viral isolation," as recent experience with measles virus and subacute sclerosing panecephalitis has taught us.[116]

The passenger theory very correctly indicates areas where information is lacking: there is nearly no data on the usual route of transmission of EB virus and no suitable animal model for study of the *in vivo* effects of EB virus. One observation central to the passenger virus thesis is the frequent association of EBV with a number of diseases, besides IM, in which there are either circulating atypical lymphocytes or tissue lymphoblasts. The thesis states that EBV proliferation occurs when such cells are present. However, available evidence suggests that atypical lymphocytes *per se* are not sufficient to activate EBV infection. McCollum and co-workers[117] studied serial serum specimens from children with experimental hepatitis, some of whom developed atypical lymphocytes in the blood. These sera did not show rising EBV antibody titers. In the recent study by Klemola, *et al.* on heterophile-negative IM,[108] patients with CMV-induced IM did not show booster responses in EBV antibodies despite a significant number of atypical lymphocytes in the peripheral blood.

Support for the second hypothesis would consist of demonstration of virus in the leukocytes of a significant number of fetuses or newborn infants who did not possess antibodies to EBV.

Does EBV cause Burkitt lymphomas, nasopharyngeal tumors or leukemia?

EBV-infected HLCL can be derived from individuals with diverse diseases and from individuals without disease. There are at least three different explanations that may account for these facts. First, in some diseases EBV may play a causal role. Second, while causal in some diseases, in other instances the passenger virus theory may be correct; EBV finds an appropriate host cell in which to multiply with the appearance of lymphoblasts from other causes. Third, since any infection with EBV may lead to life-long viral persistence, the appearance of EBV in a given HLCL *in vitro* may merely reflect past experience with the virus as well as the relative ease with which the patient's lymphocytes can be grown *in vitro*.

An unresolved problem is how a single agent (or closely related agents?) might cause a malignant lymphoma (BL) in one population and a benign lymphoproliferative disease (IM) in another. Differences in viral strain, differences in route of transmission, or differences in host factors might account for the high frequency of BL in Africa and its low frequency (compared with IM) in America. Burkitt now suggests[118] that in some way the co-existence of EBV and malaria might account for the high prevalence of

BL in Africa. Epidemiologic evidence that might answer the question of whether BL is caused by EBV is still lacking. A large scale prospective serologic survey might determine for BL as it has for IM whether circulating antibodies are associated with protection from occurrence of the tumor. Some have proposed that the only epidemiologic technique which will determine whether BL is caused by EBV is mass vaccination in the epidemic area against EBV followed by surveillance for BL.[119]

Patients with nasopharyngeal tumors have high antibody titers against EBV[120] and EBV has been found in lymphoid cell lines derived from the tumors.[121] The tumor, although worldwide, is most common in certain parts of Africa and Asia. It is not histologically a lymphoid tumor, but a carcinoma, heavily infiltrated with lymphoid cells. One might suppose that nasopharyngeal tumors call forth proliferation of lymphocytes in which EBV persists. The situation may be analagous in sarcoid, in which high EBV antibody titers have recently been recorded.[122]

Serologic studies have usually shown that leukemic children or leukemic adults have comparable frequency of antibody and comparable antibody titers to age-matched controls (see Table 2). However, data have not been presented to indicate whether the sera tested were obtained from patients under treatment with drugs that suppress the immune response. HLCL have been derived from many patients with acute and chronic leukemia. Some HLCL contain EBV particles and antigen, others have been free of EBV. With the demonstration that EBV-negative cells may still contain viral genome, it is still conceivable that EBV genetic material is associated with leukemia cell lines, but that, for some reason, virion formation does not proceed as well in leukemia cells as in cells from IM or BL. The co-existence of acute leukemia, EBV infection and clinical IM has recently been appreciated,[123] but the significance of this association remains to be determined.

It appears that viral persistence, characteristic of the other members of the herpes group in man, herpes simplex virus, varicella-zoster virus, and cytomegalovirus, is also true for EBV. For example, although CMV may be excreted by many healthy newborn infants,[124] the role of this agent as a pathogen in a certain characteristic syndrome of congenital defects is not questioned. The pattern that emerges from the group of human viral infections due to herpes viruses is one of prolonged association of the host and virus with occasional production of disease.

Prospects for control of EBV infections

Before full-scale efforts to produce a vaccine or other control measures for EBV infection are undertaken, the question whether the virus is indeed

a human tumor virus must first be clarified. The most likely source for this clarification will probably come from an animal system. Should evidence point conclusively to an oncogenic role of EBV, control in the form of vaccination with an attenuated virus might be considered.

Several difficulties are immediately apparent upon consideration of a vaccine against EBV infections of man. It is unlikely that a vaccine could be given to man in the form of infected intact and viable cells which might contain other unknown potential tumor viruses. Hence, one technical prerequisite for an EBV vaccine would be the production of significant amounts of extracellular virus that could be purified. Utilization of an EBV vaccine might be complicated by persistence of the vaccine virus and also, perhaps, by persistence of virulent virus. Persistence of vaccine virus has been a problem with the attenuated virus vaccine against Marek's disease.[125] Whether persistence of these viruses would result in harmful effects could only be learned by long-term surveillance of vaccinated individuals.

As remote and difficult as seems the potential for control of EBV by vaccination, other methods appear equally complicated. Burkitt tumor appears to be sensitive to chemotherapy with cyclophosphamide and longterm remissions have been reported, although drug resistance occurs.[126] The virus might be expected to be sensitive to 5' iododeoxyuridine, a drug which has been used to treat some infections with herpes simplex virus, but it might be anticipated that virucidal doses of the drug given systemically would be highly toxic. It seems unlikely that therapy with interferon or interferon inducers would be of benefit. Cytomegaloviruses, the subgroup of the herpes group of viruses most like EBV, are notably resistant to the action of interferon.[127]

Some novel approach might succeed that involved selective detection and destruction of cells which have been transformed by EBV. One such method, recently proposed by Moolten and Cooperband[128] would involve administration of antibody against EBV-induced membrane antigens. If this antibody were also linked with a toxin capable of destroying cells (diphtheria toxin in Moolten's *in vitro* experiments) all cells with the new membrane antigen would be specifically eliminated. Another speculative approach which might be termed "endogenous oncolysis" would be to devise a method which would induce 100% of EBV-transformed cells to undergo a lytic cycle of viral replication and thus destroy themselves.

NOTE:

The bibliography, chosen to illustrate specific facets of investigations of EBV, is by no means complete and was not compiled with the intention of indicating priority in any aspect of research. A number of important topics have been excluded altogether from the review, largely for lack of space. Such topics include the susceptibility of HLCL to

superinfection with various viruses, the serologic cross-reactions among members of the human and animal herpes viruses, and chromosome abnormalities in HLCL.

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