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James Nathaniel Dreyfus

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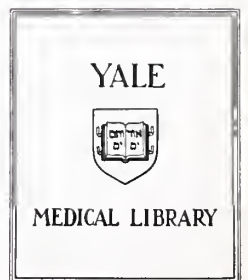
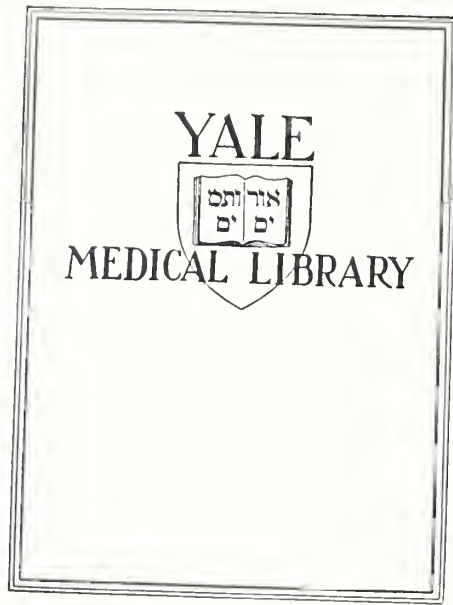
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INTERACTIONS OF EPSTEIN-BARR VIRUS WITH LEUKOCYTES
OF CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS



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INTERACTIONS OF EPSTEIN-BARR VIRUS WITH LEUKOCYTES
OF CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS

James Nathaniel Dreyfus
A.B., Harvard College

A Thesis Submitted to the Yale University School of Medicine
In Partial Fulfillment of the Requirements for the Degree
Of Doctor of Medicine

May, 1978

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Acknowledgments

I would like to record my deepest appreciation to those individuals whose assistance made possible this thesis:

Dr. James Robinson, thesis advisor and friend, who offered me enthusiasm, ideas, encouragement, and much of his time.

Dr. George Miller, who stimulated my interest in EBV, supported my efforts, and provided critical advice.

Dr. Sally Marchesi, Dr. Paul Lebowitz, and the attending staff of the Department of Hematology who facilitated the obtaining of samples.

Lee Heston and the other members of the research group who furnished valuable technical assistance and advice.

My wife, Ellen, who was understanding and encouraging throughout this long process.

The patients.

INTRODUCTION

Statement of the Problem

Epstein-Barr virus (EBV), a member of the herpes-virus group, is the etiologic agent of infectious mononucleosis and is a candidate as the cause of Burkitt lymphoma and nasopharyngeal carcinoma. (Reviewed by Miller, 1975; Miller, 1974 .) Discovered in 1964 in a cell line from a Burkitt lymphoma patient, EBV was the first virus to be regularly linked with a human malignant tumor. This finding together with the serendipitous observation in 1968 that infectious mononucleosis is associated with EBV sero-conversion (Henle et al., 1968), has stimulated a vast amount of research on the virus and its relationship to a spectrum of disease from inapparent infection to malignancy.

In cell culture, EBV strains demonstrate two biologic properties. The first is "transformation" or what Miller has alternatively termed "immortalization" (Henle et al., 1967; Pope et al., 1969; Gerber et al., 1969; Miller et al., 1971). EBV causes normal human lymphocytes and certain non-human primate lymphocytes with a limited life span in vitro to form continuous, i.e., immortal, cell lines with a lymphoblastoid appearance. Preceding the establishment of cell lines, EBV stimulates cellular DNA synthesis. This stimulation can be quantified and used as an indicator of immortalization. (Robinson and Miller, 1975).

The second property is termed "superinfection". (Miller et al., 1974). When the P3JHR1 strain of EBV is added to established lymphoblastoid lines which contain the EBV genome but do not produce mature virus particles, an intra-cellular antigen complex appears. (Henle et al., 1970) This antigen has been termed "early antigen" (EA) since cellular DNA synthesis is not required for its induction. (Gergely et al., 1971) Since the superinfected cells do not become virus producers, the superinfection is also abortive.

The correlate of EA in vivo is unclear; however, high titers of antibody to EA are regularly found in the sera of patients with acute EBV infections or EBV-associated tumors such as Burkitt's lymphoma and nasopharyngeal carcinoma. (Horwitz et al., 1975).

The events leading to lymphocyte immortalization are poorly understood. Research has been hampered by the inability to identify a subpopulation of cells uniformly susceptible to EBV-induced transformation. Immortalization has been carried out on a variety of human leukocyte populations including leukocytes of umbilical cord, infant, and adult blood, fetal spleen, lymph node, and thymus. (reviewed by Miller, 1971). The calculated fraction of cells immortalized by EBV, known as the efficiency of transformation, varies in different populations from approximately 0.0003 in adult cells to 0.03 in umbilical cord cells. (Henderson et al., 1977a).

Evidence from several studies suggests that the susceptible target lymphocyte in vitro bears a specific EBV receptor as well as the complement receptor and surface immunoglobulin which are the characteristic markers of the bone marrow-derived lymphocytes (B-cells) (Jondal et al., 1973; Pattengale et al., 1974; Greaves et al., 1975; Robinson et al., 1977). In contrast, thymus dependent lymphocytes (T-cells) with sheep red blood cell receptors lack the EBV receptor and are not immortalized.

The purpose of this study was to explore the feasibility of using chronic lymphocytic leukemia (CLL) cells as a relatively uniform population of target cells for immortalization. CLL is a lymphoproliferative disorder characterized by the accumulation of large numbers of small, mature-appearing, functionally abnormal lymphocytes usually bearing the surface immunoglobulin and complement receptor markers of B-lymphocytes. (reviewed by Astaldi et al., 1975). (CLL with a T-lymphocyte predominance has been described but will not be discussed further here. (Brouet et al., 1975) Studies of surface

immunoglobulins on the neoplastic cells have demonstrated that CLL results from a monoclonal proliferation (Aisenberg et al., 1973; see below). Theoretically, therefore, in a given patient the CLL cells might be uniformly susceptible to EBV infection.

As will be reviewed in further detail below, the CLL cell is abnormal with altered ultrastructure, metabolism, and response to mitogenic agents. The effect of EBV on the CLL cell, however, has scarcely been documented in the literature. It is unclear whether the CLL cell can be immortalized. Chang et al., (1976) in a study on EBV-induced transformation in various cell populations, examined lymphocytes from four CLL patients. Transformation frequency was extremely low ($< 10^{-6}$), and the cellular origin (i.e. leukemia vs. normal lymphocytes) of the rare transformants he obtained was unknown. In a brief discussion note, Gergely et al. (1974) claimed that they have observed EBV stimulation of DNA synthesis and immortalization of CLL lymphocytes. However, their data have never been published or corroborated.

In preliminary experiments in this laboratory, Robinson, using the DNA stimulation assay, noted that EBV stimulated DNA synthesis in lymphocytes from one of two CLL patients (unpublished). This result suggested that this assay might be a useful tool to evaluate the susceptibility of CLL cells to EBV infection as it had been for cord and adult cells (see review below). An advantage of this method is its speed compared to the conventional morphologic assay for immortalization.

With adult cells, in contrast to immunologically naive cord cells, it is necessary to distinguish the stimulation of DNA synthesis caused by EBV transformation from that resulting from an anamnestic response to antigen by lymphocytes of EBV-immune individuals. This latter response is transient and is induced by EBV preparations without immortalizing activity, e.g.,

ultraviolet light irradiated EBV.

In summary, an attempt was made in this study to determine whether CLL cells were capable of undergoing stimulation of DNA synthesis and immortalization by EBV. During the study, the stimulation data suggested that several patients were immune to EBV. To confirm this hypothesis, EBV specific antibody titers were performed. Unexpectedly, high titers of antibody to the early antigen of EBV, consistent with ongoing infection with EBV, were found. The significance of these titers and the possible relation to the clinical disease are discussed. Prior to the presentation of the data, the stimulation assay, EBV-specific antigens and antibodies, and the nature of the CLL cell will be briefly reviewed.

EBV Stimulation of Cellular DNA Synthesis

In 1971, Gerber and Hoyer demonstrated that EBV infection of normal human leukocytes induces cellular DNA synthesis. Leukocytes from EBV sero-negative adults were inoculated with EBV with demonstrated immortalizing activity and were pulsed with tritiated thymidine ($[^3\text{H}]$ Tdr) at various times thereafter. DNA synthesis was measured by the incorporation of the isotope into acid-insoluble material. An increase in ^3H Tdr incorporation in inoculated cultures compared to controls was detectable three days after virus inoculation and a progressive increase in ^3H tldr incorporation was observed over the following nine days. Infected cells showed definite morphologic evidence of immortalization about a week after DNA stimulation was seen. Both DNA stimulating and immortalizing activities of the virus were eliminated by heat or ultraviolet radiation.

The density of the labelled DNA on a cesium chloride gradient was the same as that of cellular DNA rather than of EBV-DNA implying that the virally

stimulated DNA was predominantly cellular. (Gerber et al., 1971; Henderson et al., 1977^b).

Using cord blood leukocytes, Robinson and Miller (1975), demonstrated that the stimulation of DNA synthesis by EBV could be used as an assay for virus, since the rate of ³H-Tdr incorporation in infected cells was quantitatively related to the initial virus inoculum. Stimulation of DNA synthesis by EBV was eliminated by neutralizing antibody or by exposure of virus to ultraviolet irradiation. Robinson et al., (1977) recently showed that only B-lymphocytes with complement receptors are susceptible to transformation by EBV. In addition, when T-cell-depleted lymphocytes are fractionated on the basis of size, the homogeneously small lymphocytes found in the peak fractions are transformed by EBV. (Henderson et al., 1977^b) Since these cells have a very low level of spontaneous DNA synthesis, it appears that resting B-lymphocytes are preferentially transformed by EBV. Furthermore, the subpopulation in which EBV stimulates DNA synthesis is the same which is transformed (Robinson et al., 1978).

Gerber also demonstrated that the stimulation assay could be used to detect cell-mediated immunity to EBV antigens. (Gerber and Lucas, 1972^b) He examined EBV stimulation of DNA synthesis in lymphocytes from EBV seropositive donors (complement-fixing antibody titers of 1:8 to 1:512), and found that increased ³H-Tdr incorporation occurred after inoculation with either active or UV-inactivated virus (UV-EBV). This finding suggested an immunologic response of sensitized lymphocytes to specific antigen. The time course of UV-EBV stimulation differed from that caused by active virus in cells from non-immune donors. UV-EBV induced synthesis appeared on days 3 to 4 and increased rapidly, reaching a peak on day 7 with a sharp decline thereafter. The antigenic stimulatory effect of UV-EBV was not inhibited by

preincubation of the antigen with EBV antibody whereas the immortalizing activity of live virus was. Recently, Gergely et al. (1977) showed that purified T-cells from sero-positive but not seronegative individuals were stimulated by EBV.

In summary, EBV stimulates cellular DNA synthesis in human leukocytes by two mechanisms: (1) infection of B-lymphocytes, and (2) antigenic stimulation of sensitized T-lymphocytes. The first reaction requires biologically active virus and is independent of the EBV-antibody status of the cell donors. In the second mechanism, only lymphocytes from EBV sero-positive donors are reactive. Virus which has been inactivated by exposure to UV-light or neutralized by antibody remains stimulatory. Furthermore, antigenic stimulation is transient while infection induces a permanent stimulation.

EBV Specific Antigens and Antibodies

Since prior immunity to EBV serves to distinguish the mechanisms of in vitro viral stimulation of DNA synthesis, EBV specific antibodies were measured in the patients in this study. Four major groups of EBV-specific antigens have been described. (Henle and Henle, 1975; Miller, 1975). This section will review the antigen-antibody systems of clinical significance and the presence of EBV antibody in CLL patients.

Viral capsid antigen (VCA) is an intracellular antigen detected only in those cells which are producing EBV. VCA is not usually found in fresh tissue specimens from Burkitt lymphoma or infectious mononucleosis (IM) but appears in cells of lymphoblastoid lines which produce virus (Henle and Henle, 1966). The source of antigen in vivo for stimulation of antibody synthesis is not known but is postulated to derive from a few productively infected cells which degenerate and release their contents. (Henle and Henle, 1975).

Although IgM antibodies to VCA have been described (Schmitz and Scheier 1972), only IgG anti-VCA antibodies, detected by indirect immunofluorescence are routinely measured for epidemiologic studies. In infectious mononucleosis, anti-VCA antibodies with titers usually ranging from 1:80 to 1:320 are found early in the disease. These levels decline over approximately six months to titers of 1:20 to 1:160 and appear to persist for life. No correlation exists between the peak titer of antibody and the clinical severity of the disease. (Niederman et al., 1968).

Anti-VCA antibodies are present in patients with Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC). Although there is no significant difference in prevalence of these antibodies in patients and control groups in the endemic area, the majority of patients will have geometric^{mean} titers of anti-VCA 8 to 10 times higher than that of controls (Henle et al., 1969, 1973^a). In NPC, the titer of anti-VCA correlates with advancing disease and declines with successful eradication of the tumor. (Henle et al., 1973^b).

Epstein-Barr nuclear antigen (EBNA) is detected in all EBV-genome containing lymphoblastoid cells by the method of anticomplement immunofluorescence. (Reedman and Klein, 1973). Unlike VCA, EBNA is found in vivo and has been described in cells from BL, NPC, and experimental EBV-induced lymphoma of marmosets. Presence of the antigen does not depend on the production of virus. (Lindhall et al., 1974).

Anti-EBNA antibodies arise after all primary EBV infections and persist for life. (Miller, 1975). However, determination of anti-EBNA levels offers no advantage over anti-VCA levels.

Early antigen (EA), as noted in the introduction is an intracellular antigen appearing after superinfection by certain EBV strains of lymphoblastoid lines which contain the EBV genome but do not produce virus, e.g., Raji and RPMI 64-10. (Henle et al., 1970). EA can also be induced in the same lines

by halogenated pyrimidines such as 5 iododeoxyuridine and bromodeoxyuridine (Gerber and Lucas, 1972a).

EA is detected by indirect immunofluorescence with sera empirically shown to be reactive in superinfected cells. The antigen is presumed to exist in virus producer cell lines but cannot be specifically identified since these cells also contain VCA. No sera are available which have anti-EA activity but lack anti-VCA antibody. (The location of EA in vivo is not known.) The Henles have described two components of EA: (1) the diffuse component (D) which is cytoplasmic and intranuclear, and resists methanol fixation; (2) the restricted component (R) which is present only in the cytoplasm and is methanol labile. (Henle et al., 1971^a). The two components are difficult to distinguish from one another since they can exist in the same cell.

Antibodies to EA are associated with IM, BL, and NPC. In IM, approximately 70 percent of patients develop anti-EA titers of 1:10 to 1:320. The peak titer of anti-EA occurs 2 to 4 weeks after anti-VCA. Unlike anti-VCA and anti-EBNA, anti-EA is said to disappear within several months of primary infection and is therefore useful as an indicator of ongoing or recent infection. (Henle et al., (1971^b). The Henles have suggested that patients with high titers of anti-EA ($>1:80$) more often have clinically severe disease requiring steroid therapy than do patients with lower titers. Since EA is an intracellular antigen, the Henles propose that high titers of anti-EA result from greater cellular necrosis.

In BL as well, approximately 70 percent of patients have anti-EA. High titers of correlate with poor prognosis. All patients with a short course of six months between diagnosis and death had titers of anti-EA greater than 1:160 whereas

only 44 percent of patients surviving greater than 2 years had equally high titers. (Henle et al., 1969). In treated patients in remission, anti-EA declined and even disappeared. Similarly in NPC, anti-EA is present with the height of the titer correlating with stage of the disease and prognosis.

As with anti-VCA, the titer of anti-EA antibodies declines with eradication of the tumor (Henle et al., 1973^b).

As a rule, the anti-EA present in IM and NPC is usually anti-D whereas anti-R is the predominant anti-EA antibody in BL. Since the biologic differences between the diffuse and restricted antigens are not known, the reason for disease specificity is matter of speculation. The Henles have proposed that anti-D might be related to lymph node involvement based on the observation that lymphadenopathy is more prominent in IM and advanced NPC than in BL. (Henle et al., 1973^b).

In general, anti-EA is rare in control populations (3 to 5%) and, if present, is of low titer ($<1:25$). (Henle and Henle, 1973). Whether there is a correlation between persistent anti-EA and the prolonged oropharyngeal excretion of virus known to occur in some IM patients has not been investigated (Miller, Niederman, et al., 1973).

Other EBV antigen-antibody systems which have been described include membrane antigen (MA) which is present on living Burkitt lymphoma cells. Anti-MA is analogous to anti-VCA in its clinical occurrence. (Gunven et al., 1974). Complement fixing antibodies to soluble EBV antigens are comparable to anti-EBNA. Of all the EBV antibodies extensively studied, anti-EA has a special significance since it appears to correlate with clinical course and prognosis.

EBV-Specific Antibodies in CLL

Following reports of the association of EBV with IM and BL, antibodies

to EBV were sought in other lymphoproliferative diseases including CLL. Four major studies have been published. Johansson and his colleagues (1971) compared anti-VCA titers in 96 sera from 59 CLL patients with sera from 47 normal controls. The geometric mean titer in the CLL sera was 1:79 compared to 1:43 in the control sera, although the percentage of CLL sera with high titers, i.e., greater than 1:160, exceeded that of controls, 45% to 17%. Subdivision of the CLL sera by the patients' presenting symptoms, gamma-globulin levels, and survival failed to correlate with titer. Sera from patients with higher peripheral leukocyte counts tended to have higher titers.

The study is flawed, since it is unclear which patients furnished multiple serum specimens. If samples were obtained more frequently from patients with high titered antibodies, the mean titers would be misleadingly elevated.

In another study on anti-VCA titers in CLL, Levine et al., (1971) found significantly higher titers (geometric mean titer = 1:444) in 34 patients than in controls (mean 1:90). There was no correlation between titer and lymphocyte count or gamma globulin level. Insufficient clinical information is presented to determine how these patients differed from Johansson's series. The reason for the discrepancy in titers is therefore not clear.

In a more recent study (Hesse et al., 1973) of 14 CLL patients, the mean titer of anti-VCA (1:195) and anti-EA (1:12) were significantly elevated compared to control titers, 1:45 and 1:3, respectively. A hypothesis that the titers of antibody might correlate with impaired delayed hypersensitivity was not corroborated. Two patients had high titers of anti-EA (1:160 and 1:2560) but their clinical courses were not described.

In the largest series to date, the Henles examined sera from 235 patients with CLL (Henle and Henle, 1973). Ninety-three percent had anti-VCA titers greater than 1:10 (mean titer, 1:110) compared to 91% of "old adults" (mean titer 1:51). Forty-six percent of the CLL patients had anti-EA (mean titer 1:46) compared to 5% of controls (mean titer 1:25). Clinical data on these patients were not presented.

There have been no satisfactory explanations why anti-VCA and anti-EA are elevated in CLL (or in Hodgkin's disease or lymphomas where increases are also observed). There is no evidence that EBV is causally related to CLL. The Henles have proposed that defective cellular immunity associated with malignancy somehow disrupts the balance between cellular and humoral immunity thereby permitting exacerbation of a persistent EBV infection and therefore increased anti-EBV antibodies.

Clinical Description of CLL

CLL is a chronic disorder characterized by the accumulation in the lymphoid organs, bone marrow, and peripheral blood of long-lived, functionally abnormal cells resembling small mature lymphocytes. (Reviewed by Durant, 1972) Afflicting older people, CLL is most common after the age of 50, and occurs slightly more frequently in males (ratio 3:2).

Certain manifestations of the disease are a direct consequence of the infiltration of organs by leukemic cells. Lymphadenopathy, splenomegaly, and hepatomegaly commonly occur. The skin (leukemia cutis) and gastrointestinal tract may be involved. Bulky disease produces secondary complications, e.g., mediastinal adenopathy may cause partial bronchial occlusion or superior vena caval syndrome. In advanced cases, replacement of the marrow with CLL cells ("packed marrow") interfer^{es} with other hematopoietic precursors

and leads to anemia, thrombocytopenia, and neutropenia.

Additional findings are secondary to the disordered B-cell function of the CLL cell. Hypogammaglobulinemia resulting in an increased susceptibility to infection is present in advanced CLL. Production of abnormal antibodies as demonstrated by the increased incidence of Coombs positive hemolytic anemia, cold agglutinins, and monoclonal gammopathies can also occur.

Diagnosis of the disease is based on the findings of a sustained lymphocytosis greater than $15,000/\text{mm}^3$ in the peripheral blood and of greater than 30% of the nucleated white cells in the marrow. In cases of doubt, for example where the absolute lymphocytosis is less than 15,000, clinical follow-up for development of increasing adenopathy, splenomegaly and bone marrow infiltration can confirm the suspected diagnosis.

CLL displays a wide variety of activity. Some patients will have a prolonged indolent course (>10 years) not requiring therapy. Other patients will have more aggressive disease. In patients with active disease, the response to therapy is variable. Research on clinical staging systems which categorize patients according to the presence of adenopathy, splenomegaly, anemia, and thrombocytopenia, has demonstrated that median survival time is significantly shorter in patients who present with or develop anemia and/or thrombocytopenia (19 months) than in patients with lymphocytosis with or without adenopathy (100 to greater than 150 months). (Rai, et al., 1975; Binet, et al., 1977).

Current thinking on the nosology of CLL has emphasized that the term does not encompass a single disease entity but rather a spectrum of disease of morphologic diversity. (Kass, 1976). In addition there has been speculation that the lymphocytic leukemias and lymphomas are different clinical expressions of the same neoplastic process. (Jaffe, 1976). Thus,

similar to the classification system for the lymphocytic lymphomas based on the stage of differentiation of the neoplastic cell, CLL may be subdivided into categories of well- to poorly-differentiated disease. This latter category, also termed chronic lymphosarcoma cell leukemia (CLSL) is characterized by the predominance of large atypical lymphocytes with clefted nuclei, prominent nucleoli, reticulated nuclear pattern and scant to abundant cytoplasm. (Schwartz et al., 1965). The clinical significance of this subdivision is that patients with CLSL are reported to have a poorer response to therapy, more aggressive disease, and decreased survival. (Schnitzer et al., 1970; Zacharski et al., 1969). Recent studies, however, have contradicted this finding, and the issue remains unresolved. (Peterson et al., 1975; Knospe et al., 1977).

In this study, cells were obtained from one patient carrying a diagnosis of CLSL in order to compare whether the stage of differentiation influenced the susceptibility to EBV stimulation and immortalization.

The CLL Cell

Ease of obtaining large numbers of CLL cells from peripheral blood has facilitated extensive study of the structure and function of this abnormal cell population. (review articles: Perera et al., 1974; Theml et al., 1977). The poorly differentiated lymphocytic leukemia cell, the lymphosarcoma cell, has not been as thoroughly characterized. (Ouaglino et al., 1964; Knospe et al., 1977). In this section, the chief differences between the normal lymphocyte and well differentiated leukemia cell, and the evidence for monoclonality will be reviewed.

Structural and Metabolic Differences

The tendency for CLL cells to form smudge cells or damaged cells on

routine smears is an easily observed consequence of the abnormal structure of the CLL cell membrane. By electron microscopy, the membrane is thicker than in normal lymphocytes. (Catovsky et al., 1971). Decreased membrane fluidity and turnover of constituents have also been observed. (Holt et al., 1972; Lewis et al., 1976). Ultrastructural studies have shown a predominance of smooth nuclei, small mitochondria, and decreased amounts of cytoplasm which correlate with diminished metabolic activity. (Schrek, 1972).

Glycogen metabolism is abnormal: excessive amounts of intracellular glycogen accumulate. Turnover is diminished presumably because of a relative deficiency of glycolytic enzymes. (Brody et al., 1969). The number of lysosomes and the level of lysosomal enzymes are diminished. (Douglas, 1972). The activity of DNA synthetic and catabolic enzymes is diminished. (Loeb et al., 1973, Ambrogi et al., 1977). The CAMP of the CLL cell level is low. (Astaldi et al., 1975). The overall significance of any of these abnormalities is a matter of speculation.

Surface Markers and the Origin of the CLL Cell

As noted in the introduction, the conclusion that the neoplastic cell in CLL is commonly of B-lymphocyte origin is based on the evidence from multiple studies that in most cases the predominant lymphocyte bears membrane-bound immunoglobulin, the sine qua non of the B cell. (reviewed by Astaldi et al., 1975). Rare cases have been described in which the predominant cell type is a T cell which forms sheep red blood cell rosettes (E-rosettes) and is killed by T-lymphocyte specific antiserum. Even more unusual are two cases in which lymphocytes were found with both T and B markers. (Polliack et al., 1973; Shevach et al., 1973).

In the normal adult, approximately 70 to 80 per cent of the peripheral lymphocyte population can be identified as of T-cell origin on the basis of

ability to form E-rosettes. Another five to 15 percent, identified as B-cells, have surface immunoglobulin and usually receptors for complement and the Fc moiety of IgG. The remaining 5 to 15 percent of the lymphocyte population have some other constellation of markers and remain largely unclassified. In contrast, in one series, typical of CLL patients, the percentage of E-rosette forming cells ranged from 3 to 31% with the majority of patients having less than 15% T-cells, whereas the percentage of cells with surface immunoglobulin ranged from 66 to 95%. (Davis, 1976).

The distribution of B-cell surface markers is not constant from one CLL patient to another. Dissociation of markers occurs in which, the markers on the leukemic lymphocytes of one patient will differ from those on cells of another patient. This variation has been postulated to represent differences in the maturity of the neoplastic cell population in different patients since it is known that the constellation of surface markers changes as the B-lymphocyte matures. (Gelfand et al., 1974).

Monoclonality of the Leukemic B-Lymphocyte

Evidence that the predominance of neoplastic B-lymphocytes in CLL results from a monoclonal proliferation is derived from characterization of the surface immunoglobulins by fluorescence microscopy. In a given CLL a patient, the lymphocyte surface immunoglobulin is predominantly of one heavy chain type (μ or γ) and of one light chain type (κ or λ). (Aisenberg et al., 1973). In patients in which the predominant immunoglobulin is IgG, there is restriction to one IgG subclass and allotype. (Froland et al., 1972). Recently with the development of anti-IgD reagent, it has been shown that many CLL B-cells bear both surface IgM and IgD. The immunoglobulin idiotype has been shown to be identical for the IgM and IgD on different cells suggesting that all the cells arise from one clone. (Salsano et al., 1974.)

Mitogen Responsiveness

Functional abnormalities of CLL cells noted in vitro include decreased responsiveness to various mitogens, impaired cytotoxic activity, and diminished and deranged immunoglobulin secretion. (Perera et al., 1974; Mellstedt et al., 1974; Maino et al., 1977). In reference to the present investigation, the decreased response to mitogens is most pertinent since EBV stimulation causes DNA synthesis and morphologic change as do the plant mitogens. Too little is known about the subcellular mechanisms of EBV stimulation to make comparisons with proposed mechanisms of mitogen stimulation. However determination of which lymphocyte population is abnormal in CLL might illuminate the nature of the EBV response.

Phytohemagglutinin (PHA), a lectin derived from kidney beans, stimulates DNA synthesis and causes morphologic changes selectively in T-lymphocytes. (Greaves et al., 1974). In a mixed population of normal human lymphocytes, PHA causes stimulation of DNA synthesis beginning on day 2, peaking on day 3, and steadily declining to baseline over the next seven days.

Pokeweed mitogen (PWM), stimulates normal B-lymphocytes to synthesize DNA, proliferate, and differentiate into immunoglobulin secreting cells. (Janossy et al., 1975). T-cell participation is required, and the T-cells themselves are stimulated.

Numerous studies have shown that in lymphocytes from CLL patients, the DNA synthetic response to these mitogens is diminished in magnitude and delayed in onset. (Rubin et al., 1969; Abell et al., 1970; Smith et al., 1972). However, studies performed prior to 1971 failed to distinguish the contributions made by T and B cell subpopulations. Wybran hypothesized that the delay and diminution of the synthetic response to PHA were a consequence of dilution by non-responding leukemic cells of a small but normally reactive

T-cell population. (Wybran et al., 1973). By the method of E-rosette centrifugation, a T-lymphocyte fraction was isolated from the peripheral blood of CLL patients. In these cells, the PHA response was normal. In contrast, the remaining T-depleted population, consisting largely of immunoglobulin-bearing cells, had virtually no response to PHA. Although these results do not prove that all the T-cells in CLL are normally responsive, the data did show that a normally reactive subpopulation does exist. Controversy remains, however, with some investigators believing that the T-cells are abnormal as well.

(Utsinger, 1975; Schultz et al., 1976). Wybran applied the same experimental model to PWM responsiveness and found that the T-cells from CLL patients were stimulated although mixed or T-cell depleted populations were not. This data together with similar work by others (Smith et al., 1972; Catovsky et al., 1972; Mellstedt et al., 1974) point to a defect in B-cell responsiveness in CLL. Since the failure to respond to PWM could also be a result of insufficient numbers of helper T-cells, experiments are now in progress investigating the effect of different normal T-cell subpopulations on CLL cell mitogen responsiveness (Moino et al., 1977).

In summary, current evidence suggests that in CLL B-lymphocyte function is impaired while certain T-lymphocyte functions appear to be normal.

Lymphocyte and Serum Donors

Patients with a diagnosis of CLL or chronic lymphosarcoma leukemia served as donors. With the exception of one inpatient, all patients were selected at random from the population of CLL patients followed in the Hematology Outpatient Clinic. Healthy adults served as donors of normal lymphocytes and sera. Informed consent was obtained with a permit approved by the Human Investigations Committee (see appendix).

Medium

The medium for all leukocyte cultures and cell lines was RPMI 1640 (Grand Island Biological Co.) supplemented with heat inactivated fetal calf serum (20% v/v), penicillin (50U/ml), streptomycin (50 μ g/ml), and amphotericin B (1ug/ml). Cells were washed with Hanks Balanced Salt Solution (HBSS) unless otherwise stated.

Isolation of Lymphocytes

Lymphocytes were separated from heparinized venous blood on a Ficoll-Hypaque gradient using a modification of the method of Boyum (Boyum, 1968).

Blood was obtained by venipuncture in sterile heparinized glass tubes. Routine smears were made, stained with Giemsa, and a differential count performed. Following centrifugation of the sample at 400 xg for 10 minutes, the plasma was removed and stored at -20 C for later antibody studies.

To isolate the lymphocytes, five ml of buffy coat and packed cells were diluted with 15 ml of HBSS and then layered on 10 ml of Ficoll-Hypaque. The gradient was centrifuged at 400 xg at room temperature for 40 minutes. The interface cell layer was carefully removed by pipette, resuspended in HBSS, and washed twice. The cells were resuspended in complete medium and counted in a hemocytometer. The cell concentration was adjusted to 2×10^6 /ml.

Ficoll-Hypaque separation resulted in a greater than 95% pure mononuclear cell suspension.

The cells were placed in Corning plastic culture flasks, gassed with 5% CO₂, and incubated overnight at 36 C.

Lymphocyte Markers

A. Assay for lymphocytes binding sheep red blood cells (E-Rosettes).

The percentage of T cells was measured by enumerating the proportion of lymphocytes with receptors for neuraminidase treated sheep red blood cells (SRBC) (Weiner et al., 1973). One ml of a 5% suspension of washed SRBC in HBSS mixed with 0.05 ml Neuraminidase (500 U/ml; Calbiochem) and incubated for one hour in a 37 C water bath. The cells were then washed x 3 with HBSS and resuspended in one ml HBSS.

Lymphocytes (0.1 ml containing 2×10^5 cells) were mixed with 0.05 ml of SRBC and centrifuged at 200 xg for 10 minutes at room temperature. The pellet was left undisturbed at 4 C for one hour, then gently resuspended and examined under the microscope. Each sample was tested in duplicate. At least 200 cells were observed for rosette formation; a E-rosette was defined at a lymphocyte to which three or more SRBC were attached.

B. Assay for complement-receptor bearing lymphocytes (EAC rosettes).

The percentage of B cells was estimated by measuring the percentage of lymphocytes with complement receptors. (Bianco et al., 1970). One ml of 5% SRBC was mixed with an equal volume of 19S-rabbit anti-SRBC. The mixture was incubated for 30 minutes at 37 C. The cells were washed twice in Gelatin-Veronal Buffer (GVB) and resuspended in 1 ml GVB. An equal volume of the complement source (mouse serum diluted 1:10 with GVB) was added and the mixture was incubated at 37 C for 30 minutes. The cells were again washed twice and resuspended in 1 ml GVB.

To make the EAC rosettes, 0.1 ml of lymphocyte suspension was mixed with 0.05 ml of 1% EAC and incubated for 30 minutes at 37 C. The suspension was gently resuspended and examined in a hemocytometer for rosette formation. EAC rosettes were defined as a cell^s binding 3 or more treated SRBC.

Isolation of Lymphocyte Subpopulations

T-cell enriched (T_p) and T-cell depleted (T-) lymphocyte fractions were prepared from the separated lymphocytes by mixing the cells with SRBC to form E rosettes and centrifuging the mixture on a Ficoll-Hypaque gradient. Rosette forming T-cells with SRBC receptors pelleted through the gradient whereas non-rosette forming cells (non-T) remained at the Ficoll-medium interface. (Wahl et al., 1976). 10 ml of a 1% suspension of washed SRBC were mixed with an equal volume of lymphocytes at a concentration of 1 to 2×10^7 cells/ml. The mixture was centrifuged at 200 xg for 10 minutes at room temperature and then placed at 4C for 60 minutes. The mixture was gently agitated, layered on 10 ml of Ficoll-Hypaque, and centrifuged for 30 minutes at 400 xg at room temperature. Following centrifugation, the interface cells (T-) were collected, washed twice, and resuspended in complete medium. The pelleted cells (T_p + SRBC) were washed once and resuspended in 5 ml HBSS. To lyse the SRBC, 10 ml of a solution containing 155 mM NH₄Cl, 10 mM KHCO₃ and 0.1 mM EDTA (pH 7.4) were added. The cells were immediately pelleted at 4°C, washed twice and resuspended in medium. Lymphocyte markers (E, EAC) were tested on the fractionated cells.

Preparation of Virus Stocks (Robinson and Miller, 1975).

The B95-8 strain of EBV was prepared from a marmoset cell lines (B95-8) originally immortalized with EBV from a case of transfusion-induced infectious mononucleosis. (Miller and Lipman, 1973). The infected marmoset cells were seeded at a 3×10^5 cells/ml and incubated at 36 C for 7 days. Supernatant culture fluid was then centrifuged to remove cells, frozen and thawed

three times and passed through a 0.8 μ m membrane filter (Millipore Corp.). Infectivity of the stock was 4.5 \log_{10} 50% transforming doses (TD_{50}) per 0.1 ml as assayed by the ability of virus dilutions to transform umbilical cord cells. The stocks contained approximately 10^6 to 10^7 physical particles/ml as determined by virus particle counts of negatively stained hanging drop preparations. (Miller and Lipman, 1973^b). Stocks were stored at -70 C.

As a control for non-specific cellular metabolites which might stimulate cellular DNA synthesis, a virus free supernatant was prepared. Supernatant culture fluid from Raji cells, a non-producer lymphoblastoid line derived from a Burkitt lymphoma patient, was frozen and thawed three times and filtered. This fluid lacks the ability to transform leukocytes and was shown in preliminary experiments to have no stimulating activity.

Preparation of UV irradiated EBV (UV-EBV).

One milliliter aliquots of virus stock were placed in 60 mm petri dishes. UV light was delivered by a 15 watt GE 15T8 low-pressure mercury germicidal lamp. UV dose rate was 600 ergs/mm² per min for 10 minutes. This dose has been shown to eliminate the ability of EBV to stimulate DNA synthesis in human cord leukocytes. (Robinson and Miller, 1975).

Phytohemagglutinin

Phytohemagglutinin, (PHA, Difco Laboratories), a selective T cell mitogen, was prepared as a stock solution of 1 mg/ml and used in lymphocyte cultures at varying concentrations of 2.8 to 50 μ g/ml.

Lymphocyte cultures

Lymphocyte cultures were set up in accordance with one of two methods. In method one, a single 25 ml Corning culture flask was used for each test

parameter. For each 4 ml of cell suspension (2×10^6 cells/ml), one ml of supplemented medium and one ml of test stock (EBV, UV-EBV, PHA, or medium control) were added. The final volume was either 6 or 9 mls with a final cell density of 1.3×10^6 /ml. The cultures were gassed with 5% CO_2 and incubated at 36 C. The medium was partially changed weekly one or two days before the [^3H]Tdr pulse.

In method two the lymphocyte suspension was distributed to sterile stoppered tubes (13x100 mm) in 0.4 ml aliquots. 0.1 ml of supplemented medium plus 0.1 ml of test stock were added to make a total volume of 0.6 ml. Multiple tubes were set up for each parameter such that each [^3H]Tdr pulse could be performed in duplicate on each test day.

After several initial experiments, method 1 was used exclusively since it was less time-consuming and minimized pipetting errors.

Pulse Assay for DNA Synthesis (Robinson and Miller, 1975)

[^3H]Tdr incorporation into acid-insoluble material was measured in control and EBV, UV-EBV, and PHA inoculated cultures.

When leukocytes were cultured by method 1, 0.6 ml aliquots of cell suspension were removed from the culture flasks and placed in 13x100 mm stoppered tubes. 0.5 ml of [^3H]Tdr (specific activity 6.7 Ci/mm; New England Nuclear) at a concentration of 5 $\mu\text{Ci/ml}$ was added to each tube. The final concentration of the isotope was 2.3 $\mu\text{Ci/ml}$. The suspension was then incubated for 60 minutes in a 37 C. water bath. The reaction was stopped by the addition of 4 ml of cold HBSS, and the suspension was centrifuged at 200 xg for 10 minutes at 5 C. The supernatant was removed and the pellet was resuspended in 3 ml of 5% trichloroacetic acid (wt/vol) in water. The tubes were kept at 4°C for a minimum of 20 minutes.

The precipitates were collected on 2.5 cm membrane filters (millipore Corp., type HA, 0.45 μm) and washed twice with distilled water. The filters

were placed in vials, dried, and 4 ml of Econofluor (New England Nuclear) was added. Incorporated radioactivity was determined in a liquid scintillation counter.

For leukocytes cultured initially in tubes (method 2), the 0.5 ml of isotope solution was added directly and the remainder of the pulse carried out as above.

Immortalization (transformation)

All lymphocyte cultures, whether EBV inoculated or control, were observed weekly for evidence of immortalization (or transformation) into lymphoblastoid lines. Cultures were evaluated for the following:

1. Change of morphology of cells from small, round cells to larger ameboid cells with pseudopodia;
2. Acid production as demonstrated by a color change of the pH indicator of the medium;
3. Clumping of cells;
4. Proliferation and ability of the culture to grow following subdivision.

All viable cultures without evidence of transformation were observed for a minimum of six weeks.

Fluorescent Antibody Tests for Antibodies to EBV Viral Capsid Antigen and Early Antigen

Antibodies in patient sera to VCA and EA were measured by indirect fluorescent antibody tests. (Andiman and Miller, 1976). The general method was to prepare smears of cells containing the appropriate antigen, incubate with test sera of increasing dilution, and then add fluorescein conjugated anti-human IgG to stain cells with bound IgG. All sera were heat inactivated by incubation for 30 minutes at 56°C.

A. Preparation of VCA smear.

Antigen smears were prepared from suspensions of an EBV-producer cell line (B95-8). 3ml at 10^6 cells/ml were pelleted and the cells resuspended in 0.1 ml phosphate-buffered saline (PBS). The suspension was smeared on coverslips or glass slides, allowed to dry, and then fixed in acetone for 10 minutes. Smears of non-producer cells (Raji or transformed umbilical cord leukocytes) which are VCA negative were used as controls for non-specific anti-cellular antibodies. The smears were stored at -20 C until use.

B. Preparation of EA smears.

"Early antigen" appears in non-producer lymphoblastoid lines (Raji, 64-10) following superinfection with virus concentrates of the P₃J-HR1 Burkitt cell line. Superinfection was performed by adding 3 ml of supernatant fluid derived from cultured P₃J-HR1 cells to an equal volume of Raji cells at a cell density of 10^6 cells/ml. Four days after superinfection, the cells were washed, pelleted, and smeared on coverslips on slides as described above. Raji cells which had not been superinfected were used as negative controls.

C. Indirect Fluorescence Antibody Test

Sera were serially diluted ten-fold with PBS in a microtiter tray. One drop of serum dilution was added to each smear. Known positive and negative sera were used as controls. The slides were then incubated for one hour at 37 C in a moist chamber. The slides were rinsed with cold PBS and washed twice for 5 minutes. One drop of fluorescein-isothiocyanate conjugated goat antihuman IgG (Antibodies, Inc.) diluted 1:10 with PBS was placed on each smear. The slides were again incubated for one hour, washed five times for 3 minutes, and dried with a hair dryer. The coverslips were mounted with buffered glycerol (pH 9) on acetone cleaned slides and examined under a Leitz-Wetzlar indirect illuminating fluorescence microscope.

RESULTS

Patients

Eight patients (7 males, 1 female) with the diagnosis of CLL were entered in this study. The clinical data are summarized in Table I. Six patients (#1-6) had well differentiated CLL with a predominance of small, mature lymphocytes. Two patients (#7,8) were considered to have poorly differentiated CLL because of the large proportion of poorly differentiated lymphoblasts (20-40% of nucleated cells) present in the peripheral blood and bone marrow. The cells, classified as lymphosarcoma leukemia cells, had increased diameter, prominent nucleoli and clefted nuclei, and an open reticulated chromatin pattern. (Zacharski et al., 1969).

The ages of the CLL patients ranged from 48 to 78 years old with a median age of 66 years. Duration of disease from presentation to entrance into the study ranged from less than one month to 13 years with a median duration of 5 years.

According to the clinical staging system proposed by Binet et al., (Table I) the distribution of patients at the time of initial presentation was as follows: Stage 0: 2; Stage II: 2; Stage III: 3; Stage IV: 1. Over the period of observation at Yale, the patients' clinical courses have varied. Patient #5 presenting in Stage II has spontaneously improved with a decrease in lymphocytosis and disappearance of splenomegaly within three months of diagnosis. Patient #2, presenting with serendipitously discovered lymphocytosis in 1965, has had a very slowly progressive course, but has never been treated. Patients #1 and #4, each with CLL for eight years and evidence of disordered immunity, have had indolent disease controlled with chemotherapy. In contrast, patients #3 and #6 have had more active disease with constitutional symptoms and hypersplenism requiring chemotherapy and, for patient #6, splenic irradiation.

The two patients with CLL with poorly differentiated cells have had clinically aggressive disease with complications and will be discussed briefly.

Patient #8, hospitalized in February, 1976, with a two month prodrome of night sweats, dyspnea, lethargy and 30 pound weight loss was diagnosed as having CLL with multifocal adenopathy and massive hepato-splenomegaly. The white count was $520,000/\text{mm}^3$ with 83% lymphocytes and 14% abnormal precursors. Anemia and thrombocytopenia were present. The bone marrow biopsy revealed hypercellularity and infiltration with lymphoid cells (40% lymphoblastic forms). Treatment with vincristine, cyclophosphamide, and prednisone produced a drop in white count to 100,000 but anemia and thrombocytopenia persisted. Agranulocytosis developed, and the patient developed sepsis despite antibiotics. The white count plummeted to 17,000. On the 30th hospital day, the patient died. Post-mortem examination revealed multiple fungal abscesses (*Candida albicans*) as well as the underlying widespread lymphoproliferative disease with diffusely infiltrated lymph nodes.

Patient #7 was admitted to the hospital in May, 1976 because of asthenia and left upper quadrant pain. Splenomegaly and adenopathy were noted. The white count was $8600/\text{mm}^3$ with 47% lymphocytes. The marrow was infiltrated with lymphocytes. Prednisone therapy was begun. In November, 1976, the patient was hospitalized because of severe anemia and weakness. The spleen was palpable at the iliac crest. The white count had risen to 55,900 with 79% lymphocytes of which more than half were large immature cells with prominent clefts and nucleoli. Thrombocytopenia and hypogammaglobulinemia were present. The diagnosis of chronic lymphosarcoma cell leukemia was made. A single course of vincristine and splenic radiation produced mild temporary improvement in the lymphocytosis and splenomegaly. However,

the patient was readmitted in March, 1977, for splenectomy and radiation of a retropharyngeal mass, believed to be a lymph node but not biopsied. Since then the white count has continued to climb and the patient has had constitutional symptoms. Chlorambucil therapy has produced no response.

Enumeration of T and B Lymphocytes (Table II)

The percentage of EAC-rosette-forming lymphocytes was elevated in all patients and ranged from 67 to 91.5 percent compared to the normal range of 10 to 30 percent reported in the literature. (Astaldi et al., 1975). The percentage of E-rosette-forming lymphocytes was substantially diminished with a range of less than 1 to 29 percent compared to the normal range of 60 to 70 percent. Thus all patients in this series had a predominance of B-cells in peripheral blood.

The absolute number of E-rosette-forming cells varied among patients. With the exception of patient 6, who had slightly diminished T-cell number, all patients had normal or increased counts.

Immortalization (Table III)

Within two to four weeks of inoculation with EBV, normal adult lymphocytes demonstrated transformation into continuous lymphoblastoid cell lines. In four tubes, each containing 8×10^5 cells, acid production, clumping, and lymphoblastoid cells with "whisker-like" cytoplasmic processes were present. Uninoculated control cells did not survive.

CLL cells, whether from patients with well or poorly differentiated disease were not immortalized upon exposure to EBV. Although several cultures showed clumping and acid production initially, within six weeks most cells were pyknotic. Spontaneous transformation did not occur in uninoculated cultures. Cells from patient #6 were still viable after 6 weeks and formed small clumps but without morphologic change. Two tubes were subdivided

but failed to survive. In conclusion, within the limitations of the culture system employed, CLL cells were completely resistant to transformation by EBV and did not transform spontaneously.

Stimulation of Cellular DNA Synthesis

Stimulation of DNA synthesis in normal and leukemic lymphocytes by EBV, UV-inactivated EBV (UV-EBV), and PHA was determined by comparing the incorporation of ^3H -Tdr in experimental and control cultures. Arbitrarily, a net stimulation of greater than 1000 counts per minute (cpm) was considered to indicate unequivocal stimulation.

A. Stimulation of Normal Lymphocytes

The addition of biologically active EBV to normal adult lymphocytes resulted in increased DNA synthesis which was first detected 5 or 6 days following inoculation (Table IV). In two experiments inoculated lymphocyte cultures which exhibited increased DNA synthesis showed morphologic evidence of immortalization one or two weeks later. UV-inactivated virus had no stimulatory effect, although the donor of the cells was EBV-seropositive. Cells incubated with PHA showed a marked increase in ^3H Tdr incorporation which reached a peak 3 to 5 days after initiating the culture and which declined thereafter. This pattern of rapid induction of DNA synthesis followed by a decline is consistent with the response pattern of normal lymphocytes to PHA described by others (Greaves et al., 1974).

B. Stimulation of Leukemic Cells from Patients with Well-Differentiated CLL.

Serial stimulation assays were performed on lymphocytes from patients with well-differentiated CLL (Patients #1-6). Three patterns of response were observed: 1) No stimulation of DNA synthesis by EBV or UV-EBV; 2) Stimulation by EBV alone; or 3) Stimulation by both EBV and UV-EBV. The results are shown in Table V.

1) Cells from three patients (#1,3,4) were completely unresponsive to either EBV or UV-EBV; net stimulation did not exceed 64 cpm. Furthermore, in contrast to normal lymphocytes, baseline DNA synthesis in this group did not increase with time in vitro. The response to PHA was variable. Cells from patients #1 and 3,, both having a diminished percentage of T-cells but a normal absolute number, were stimulated by PHA. In comparison to normal lymphocytes, the onset of the response was delayed (day 6 vs. day 3) and the net stimulation was lowered (approximately 1000 cpm vs. 4300 cpm in the normal). By contrast, cells from patient #4, who had an absolute increase in T-cells, displayed no response to PHA.

The results with cells from patient #2 were equivocal. Net increase of 263 and 476 cpm were present respectively in EBV and UV-EBV exposed cultures on day 6. However, the results in this experiment are marred by a wide variability in replicate values (greater than 30%), apparently a consequence of uneven distribution of cells in replicate tubes.

2) Cells from patient #5 were stimulated by EBV with a significant increase in isotope incorporation on days 10 and 14. UV-EBV produced no stimulation but appeared to be somewhat inhibitory. Baseline DNA synthesis increased during the time of incubation. The PHA response was intact. The pattern of response, stimulation by EBV but not by UV-inactivated virus, strongly suggested virus stimulation rather than response to antigen. However, since the EBV inoculated cultures failed to immortalize, the process remains undefined.

3) The lymphocytes of patient #6 were stimulated by both EBV and UV-EBV. On day 6 of experiment AA40 (Table V) the net stimulation induced by EBV was approximately 1000 cpm compared to an increase of 3000 cpm in UV-EBV inoculated cultures. PHA had little effect on this patient's cells.

C. Stimulation of Cells from a Patient with Lymphosarcoma Cell Leukemia

Leukocytes from patient #7 who had a significant proportion of poorly differentiated cells were extensively tested on three occasions. (Table VI). In experiments BB42 and AA82, addition of UV-inactivated virus stimulated DNA synthesis with a peak increase of 2000 to 3000 cpm occurring on day 10 with a decline thereafter. In the third experiment (BB71), the peak increase on day 10 was about 900 cpm.

Biologically active EBV also stimulated significant isotope incorporation with a peak increase either on day 10 or day 14. The response to PHA was variable in the three experiments and suggested that the population of responding cells was different in each test. In fact, the greatest response to PHA (4500 cpm in Expt. BB42) correlated with the presence of the greatest number of T cells. (Table II). The degree of stimulation by EBV and UV-EBV did not correlate with PHA responsiveness.

The trend of the data, transitory stimulation by EBV and UV-EBV without immortalization by live virus suggested that the stimulation was a consequence of recognition of antigen. To corroborate this conclusion, an experiment was performed in which human serum containing antibody to EBV (anti-VCA titer of 1:160) was added to virus inoculated cultures on day 0 and the ensuing isotope incorporation compared with that in similar cultures containing human serum without EBV antibody. Addition of specific antibody is known to inhibit immortalization and immortalization-related DNA synthesis but not the DNA stimulation secondary to antigenic stimulation. (Gerber and Lucas, 1972b). On day 10 net ^3H -Tdr incorporation by cells exposed to UV-EBV was 2000 cpm in cultures with and without EBV-specific antibody.

EBV induced net stimulation was not inhibited by the addition of antibody. On day 10, net stimulation was 5500 cpm in antibody-containing cultures compared to 3700 cpm in antibody-negative cultures. The inability of specific antibody to diminish the stimuli effect of EBV and UV-EBV thus suggest the mechanism of stimulation to be immunologic in nature.

Antibody did not inhibit stimulation by EBV. Ten days after inoculation with virus, cultures incubated with antibody showed a net ^3H -Tdr incorporation of 5500 cpm while cultures without antibody incorporated 3700 cpm.

Comparison of Stimulation of T- and B-Lymphocytes

Since the lymphocytes from patient #7 with poorly differentiated CLL were consistently stimulated by both EBV and UV-EBV, an attempt was made to determine whether the stimulation was occurring primarily in cells with B- or T-lymphocyte markers. Mixed leukocytes were incubated with sheep red blood cells to form E-rosettes and then separated on a Ficoll-Hypaque gradient to yield E-rosette enriched (T-enriched) and E-rosette-depleted (T-depleted) fractions. Cultures of each fraction were set up, inoculated, and serial pulses performed.

The fractionation procedure was effective in selectively distributing the E-rosette forming cells although the percentage of EAC-rosette-forming cells remained high in the T-enriched fraction. (Table VII).

The response of the lymphocyte subpopulations to EBV, UV-EBV and PHA is summarized in Table VIII. Spontaneous DNA synthesis in uninoculated cultures of the T-cell enriched subpopulation was low in comparison to the T-cell depleted subpopulation or the unfractionated cells (data shown in Table VI, Expt. BB42). Cells depleted of T-lymphocytes were not stimulated by PHA. An increase in DNA synthesis in the E-enriched fraction was observed (800 cpm net); however, this stimulation was far less than that observed in

the unfractionated cells (4500 cpm, Table VI). The data suggest that the fractionation procedure may have altered the ability of the T-lymphocytes to respond.

EBV inoculation produced significant stimulation of the T-cell enriched fraction but had an equivocal effect (500 cpm) on the T-depleted population. UV-EBV inoculation resulted in slight stimulation of the T-enriched fraction but moderate stimulation of the T-depleted fraction. These results are inconsistent with one another given the reasonable assumption that antigen recognition of EBV and UV-EBV involves the same lymphocyte subpopulation. Further experiments, particularly auto-radiography, are necessary to identify the cell type stimulated by UV-EBV.

EBV Antibody Titers

Anti-VCA antibodies were present in the sera of all CLL patients. (Table IX). The titers ranged from 1:10 to greater than 1:160. Anti-EA antibodies were also present in five CLL patients and ranged in titer from 1:20 to 1:400. On the basis of the presence or absence of antibodies to EA, the patients could be divided into two categories:

1. Patients #1,2, and 3 had no detectable anti-EA. Patient #2 did have an anti-VCA titer of 1:80 to 1:160, whereas the other patients had low titers. Although paired sera were available in only one case, a reasonable explanation for the presence of anti-VCA antibody would be previous EBV infection.

2. Patients #4,5,6, and 7 had antibodies to EA perhaps indicating ongoing or recent EBV infection. Paired sera, obtained four to six weeks later, were available from patients #6 and 7 and showed no change. As expected, the anti-VCA titers were also elevated.

The presence in patient #7 of a very high titer of anti-EA (1:400),

comparable to that observed in EBV-associated malignancy, was intriguing. This patient unlike the others had CLL with poorly differentiated cells. Serum obtained from patient #8, also with a poorly differentiated CLL, was tested and found to have an elevated anti-VCA and an anti-EA titer of 1:400. Further samples from similar patients were not available.

There was no strict correlation between the presence of antibody stimulation of lymphocytes by either EBV or UV-EBV (Table X). Lymphocytes from patients #6 and #7, who had anti-EA and anti-VCA, were stimulated. However, cells from patient #4 with a titer of anti-EA of 1:80 were not stimulated. Failure to observe antigenic stimulation in patients (2, #4) with EBV antibody may be a consequence of impaired T-lymphocyte function. Consistent with this hypothesis is that PHA did not stimulate the cells of these patients. However, it should be noted that the normal leukocytes obtained from a seropositive donor (anti-VCA of 1:20) did respond to UV-inactivated EBV. These data are in conflict with those of Gerber and Lucas (1972b) who reported stimulation of DNA synthesis by UV-treated virus in leukocytes of seropositive individuals.

Discussion

Failure to Stimulate or Immortalize CLL Cells with EBV:

The purpose of this study was to determine whether lymphocytes from patients with chronic lymphocytic leukemia were susceptible to EBV induced stimulation of DNA synthesis and immortalization. Peripheral mononuclear cells from seven CLL patients were inoculated with EBV. In no case could lymphoblastoid cell lines be established. By contrast normal adult leukocytes invariably form lymphoblastoid cell lines when cultured in the presence of EBV. In four of the seven patients stimulation of DNA synthesis by EBV was not observed. In another two patients stimulation of DNA occurred in cells exposed to EBV. However, this increase in isotope incorporation was transient and was not accompanied by morphologic changes suggesting lymphoproliferation. Since DNA synthesis in these cells was also induced by UV-inactivated virus, it appeared that stimulation resulted from a response of sensitized cells (presumably T-cells) to EBV-antigens. Only in one patient (#5) did an increase DNA synthesis occur in the presence of biologically active EBV but not in the presence of UV-treated virus. The results in this case are problematic. Conceivably the stimulation of this patient's cells was due to a direct effect of the virus, e.g., an "abortive transformation". However, such an abortive infection has never been described in any EBV system. It is more likely that a technical error was responsible for the failure of UV-inactivated EBV to stimulate the cells of this patient. The cells of this patient deserve further study. It would be of interest for instance to look for the induction of EBV related antigens (EBNA, EA) in these cells.

In general, the CLL cells appeared resistant to EBV infection.

Henderson performed a conventional microwell transformation assay on lymphocytes from patient #2 and did not observe any transformation. (Henderson, unpublished data.) Addition of PHA and lipopolysaccharide which have been shown to potentiate transformation in cord blood leukocytes had no effect. (Henderson et al., 1977a).

Since the mechanisms of immortalization are poorly understood, the reasons for the failure of the CLL cells to respond to EBV are a matter of speculation. EBV receptors have been described on the surface of lymphocytes bearing surface immunoglobulin and complement receptors. (Jondal and Klein, 1973; Greaves et al., 1975). Whether CLL cells have receptors is controversial. Gergely et al. (1974) found that EBV-producer productive cells which are known to bear virus envelope-related membrane antigen were capable of forming rosettes with CLL cells. They concluded that CLL cells must, therefore, have receptors for EBV. However, binding by some other receptor (e.g. immunoglobulin receptor) was not ruled out. Greaves et al. (1975) were unable to demonstrate receptors on CLL cells using an indirect immunofluorescence assay.

In preliminary experiments, mitomycin treated T-cell depleted CLL lymphocytes were incubated with EBV. Free virus was washed off and the prepared cells placed in culture with autochthonous T-enriched cells. The T-lymphocytes from this sero-positive individual were stimulated. (Robinson, unpublished data). This result suggests that B-lymphocytes from a CLL patient are capable of binding virus. Further work with this approach may distinguish whether CLL cells do have EBV receptors.

The failure of CLL cells to be stimulated by virus may also be related to the generally poor response of these cells to mitogens described

in the introduction. Abnormalities of the membrane, energy metabolism, DNA synthetic enzymes and other cellular functions have all been invoked to explain CLL cell unresponsiveness to certain mitogens. (Therl et al., 1976). Any and all of these abnormalities could be relevant to EBV induced stimulation.

Immunologic Recognition of EBV by CLL or Lymphocytes

UV-inactivated EBV stimulated DNA synthesis in lymphocytes from two patients with CLL suggesting that a subpopulation of cells was capable of recognizing EBV as an antigen. The response was transitory and was not inhibited by the addition of EBV specific antibody. Several additional experiments should be done to characterize this stimulation. The nature of the responder population of cells has not been clearly established by the results reported in this study. Autoradiography in combination with rosette tests should distinguish whether cells stimulated into DNA synthesis are T (E-rosette-forming) cells or B (EAC-rosette forming) cells. It would also be of interest to determine whether EBV-related antigens can be induced in CLL cells.

Lymphocyte stimulation assays for cell-mediated immunity to viruses have been described for a large number of viruses including other herpes viruses. (Bellanti et al., 1976). However, the previously quoted study by Gerber and Lucas (1972**b**) is the only published study on the response of lymphocytes from sero-positive individuals to UV-inactivated EBV. The results of the present study are in conflict with their data since normal lymphocytes from a sero positive individual were not stimulated by inactivated virus. However, the stimulation of DNA synthesis by inactivated EBV in lymphocytes from several CLL patients suggests a response to antigenic stimulation and, thus, partially corroborates their data.

Significance of Anti-EA Antibodies in CLL

The most intriguing observation of this study was the finding of elevated titers of EBV specific antibodies, particularly anti-EA in these patients with CLL. As described in the introduction, such titers have been observed before without good explanation. It has been proposed that the elevated titers are a consequence of immunosuppressive effects of the disease or therapy. (Henle and Henle, 1975). However several studies have shown no correlation between depression of cell-mediated immunity and elevated antibodies to EBV. (Hesse et al., 1973; Levine et al., 1975).

The current opinion, repeated in virtually every paper concerning antibodies to EA, is that the antibody is associated only with current or recent infection with EBV and is "rare" in normal individuals. Those normal individuals who do have anti-EA have not been characterized. Are such people inapparently infected or prolonged excretors of EBV and presumably constantly antigenically stimulated or can anti-EA persist without continual stimulation?

Titers of anti-EA greater than 1:160 have not been described in "normal" individuals and are unusual even in patients with acute mononucleosis. Thus the finding of two patients with titers of 1:400 comparable to that seen in BL and NPC was extraordinary. A comparison of white count, stage, treatment, transfusions, and complications failed to show any consistent difference between patients with anti-EA and those without. (Tables I, X). One apparent difference was that both patients with very high anti-EA titers had a significant increase in the proportion of circulating poorly differentiated cells. The failure to observe a correlation of anti-EA with adenopathy (Stages I, III, IV) deserves comment. It has been proposed that the titer of anti-EA (or at least anti-D) correlates with nodal involvement. (Henle

and Henle, 1975). In CLL anti-EA, when present, is said to be primarily anti-D. However, despite an assertion to the contrary (Henle and Henle, 1973) there has been no published evidence that CLL patients with enlarged nodes are more likely to have anti-EA. In this study, it was difficult to determine from a chart review the extent of the adenopathy in each patient. However, patient #2 had adenopathy and massive splenomegaly and yet did not have anti-EA whereas patient #5 lacked adenopathy and did have anti-EA. A larger sample of course is necessary to draw further conclusions.

The apparent correlation of high titers of anti-EA with poorly differentiated CLL requires further investigation. There are no reports on antibody titers in this syndrome. Johansson et al. (1971) in a study have argued that the anti-VCA titers are higher in poorly differentiated lymphocytic lymphoma than in well differentiated lymphoma. However their analysis has been disputed. (Goldman, 1972)

The small number of patients and short follow-up time have made it impossible to correlate severity of disease and survival with anti-EA titer as has been done with NPC. The two patients in this study with high titers of anti-EA and poorly differentiated cells have had severe disease. Elevated antibody titers to EA may identify a sub-group of patients with CLL.

Summary

Lymphocytes from 6 patients with chronic lymphocytic leukemia (CLL) and one patient with chronic lymphosarcoma cell leukemia (CLSL) were inoculated with Epstein-Barr virus (EBV) and observed for evidence of stimulation of cellular DNA synthesis and immortalization. With inocula of undiluted stocks of B95-8, immortalization did not occur. Stimulation as measured by net incorporation of ³Htritiated thymidine occurred in cells from two patients. The increased DNA synthesis appeared to be a consequence of recognition of the virus as antigen since stimulation was also induced by ultraviolet-irradiated virus.

Antibodies to EBV viral capsid antigen were present in all patients and did not correlate with in vitro stimulation. Antibodies to EBV early antigen, (EA), consistent with ongoing EBV infection, were present in several patients. Two patients with poorly differentiated lymphocytic leukemia cells had very high titers of anti-EA comparable to levels seen in EBV-associated tumors. The significance of these titers in CLL as well as the apparent resistance of CLL cells to EBV infection are discussed.

Patient	Expt. #	Age	Sex	Clinical Diagnosis (Date Noted)	Clinical Stage ^a Initial	Clinical Stage ^a Current	Leukocyte Count ^b (% Lymphocytes)	Therapy (Date of last dose)	Transfusions
1	AA28 3/14/77	76	M	CLL (1969) Herpes zoster (1975) Basal cell CA (1977)	II ^c	III	141,900 (94L)	Chlorambucil (1975)	None
	AA81 4/18/77			Hypogammaglobulinemia			187,000 (93L)	Cyclophosphamide (1975) Prednisone (1976)	
2	AA61 4/4/77	78	M	CLL (1965) Hypogammaglobulinemia (1971) Basal cell Epithelioma (1970, 1975)	0	IV	28,700 (93L)	None	None
3	AA83 4/18/77	65	F	CLL (1964) Hypogammaglobulinemia	III ^d	IV	196,900 (96 L/2 atyp.L.)	Prednisone Chlorambucil (4/1/77)	None
4	BB1 4/25/77	66	M	CLL (1969) Basal cell CA Malignant melanoma Auto-immune hemolytic anemia Hypogammaglobulinemia	0	III	178,200 (90L/1atyp.L.)	Prednisone (4/77) Chlorambucil (1974)	None
5	AA71 4/11/77	62	M	CLL (1/77) Monoclonal gammopathy (1/77)	II	0	17,300 (76L)	None	None

<u>Patient</u>	<u>Expt. #</u>	<u>Age</u>	<u>Sex</u>	<u>Clinical Diagnosis</u> <u>(Date Noted)</u>	<u>Clinical Stage</u> <u>Initial</u>	<u>Clinical Stage</u> <u>Current</u>	<u>Leukocyte Count</u> <u>(% Lymphocytes)</u>	<u>Therapy</u> <u>(Date of</u> <u>last dose)</u>	<u>Transfusions</u>
6	AA40 3/21/77	48	M	CLL (10/74) Fever of unknown origin (1/77)	III	III	7300 (84L)	Chlorambucil (1/77)	2/77
	BB41 5/16/77			Pancytopenia (1/77)			9500 (78L/2 atyp.L.)	Cytosan (1976) Prednisone (1976) Vincristine(1975) Spleen irradiation (4/77)	
7	A82 4/18/77	65	M	Chronic lympho- sarcoma cell leukemia(5/76)	III	IV	80,300 (53L/14 cleft L./ 28 atypical 2 lymphoblasts	Splenectomy (3/77)	3/30
				Skin Ca face				Radiation to Waldyers ring (4/77)	
				Retropharyngeal mass ? node					
	BB42 5/16/77						111,000 (68L/13 cleft L/ 3 atyp L./ 15 immature L.)	Chlorambucil Prednisone(4/18/77)	
8	JR1 2/26/76	65	M	CLL (2/26/76) Death from condial sepsis (3/29/76)	IV	IV	520,200 (83L/14 abnormal L.) ^c	Vincristine ^d Prednisone (2/26/76)	
				Hypogammaglobulinemia					

TABLE I. PATIENT DATA (continued)

- a). Clinical stage at times of initial presentation and of experiment using system of Binet et al. (1977).
- | | |
|------------|---|
| Stage 0: | Peripheral lymphocytosis (greater than $4000/\text{mm}^3$) + bone marrow lymphocytosis (greater than 40% infiltration) |
| Stage I: | Stage 0 + Lymph node enlargement |
| Stage II: | Stage 0 + Splenomegaly |
| Stage III: | Stage I + Stage II |
| Stage IV: | Stage III + Anemia (hemoglobin $<10\text{g } 1100 \text{ ml.}$) and/or thrombocytopenia (platelets $<1000,000/\text{mm}^3$) |
- b). Leukocyte count (cells/ mm^3) at time of experiment
- c). Stage in 1975; earlier data not available
- d). Stage in 1972.
- e). Lymphoblasts and prolymphocytes

TABLE II. ABSOLUTE NUMBER OF EAC AND E ROSETTE FORMING CELLS.

Patient	Expt. #	Lymphocytes/mm ³	% EAC Rosetts (Absolute # /mm ³) ^a	% E Rosettes (Absolute # /mm ³)
1	AA28	133,400	67 (89,400)	4 (5300)
	AA81	173,400	73 (126,900)	<1 (<1700)
2	AA61	119,700	87 (104,100)	2 (2400)
3	AA83	192,900	84 (162,000)	1 (1900)
4	BB1	162,200	91.5 (148,000)	7 (11,400)
5	AA71	13,100	75 (9800)	29 (3800)
6	AA40	6100	87 (5300)	15 (900)
	BB41	7600	90.5 (6900)	9.5 (700)
7	AA82	71,500	76 (54,300)	5 (3600)
	BB42	108,900	76 (82,800)	7.5 (8200)
	BB71	101,200	80 (81,000)	3 (3000)
8	JR-1	504,600		
Normal Adult			35	58.5
			33	57
	Astaldi <u>et al.</u> (1975)		10 to 30	60 to 70
	Han <u>et al.</u> (1976)		23 (350 ± 130)	64 (1600 ± 372)

^a. Absolute # of rosette forming cells = % rosette forming cells X lymphocytes/mm³

TABLE III. IMMORTALIZATION OF NORMAL AND CLL LYMPHOCYTES

		Immortalization (# of lines established/attempts)	
<u>Normal Lymphocytes</u> ^a		<u>FBV-Inoculated</u>	<u>Controls</u>
		4/4	0/4
<u>CLL Lymphocytes</u> ^b			
<u>Patient</u>			
1		0/4	0/4
2		0/2	0/2
3		0/2	0/2
4		0/2	0/2
5		0/2	0/2
6		0/4	0/6
7		0/6	0/6
8			

a. 8×10^5 cells incubated with virus or medium in each tube

b. 2×10^6 cells per culture except #5 where only 10^6 cells were available.

TABLE IV. STIMULATION OF DNA SYNTHESIS IN NORMAL LYMPHOCYTES

Expt. #	Day ^a	Baseline DNA Synthesis ^b	Net Stimulation ^c			
			FBV	UV-EBV	PHA	
BB24	3	585	(-67)	ND	4313 ^d	7637 ^e
	6	2004	817	ND	1646	2155
	13	1476	2002	ND	695	(-810)
JR1	3	563	222	6	-	1413 ^e
	5	1227	1826	103	-	1483
	7	3548	4932	(-313)	-	(-2270)

a.
Days after inoculation

b.
Mean cpm of duplicate cultures. Unless otherwise noted, replicate values were within 10% of the mean.

c.
Mean cpm of inoculated culture minus baseline DNA synthesis

d.
25 μ g/ml of PHA

e.
2.5 μ g/ml of PHA

TABLE V. STIMULATION OF CELLS FROM PATIENTS WITH WELL DIFFERENTIATED CLL

Patient	Expt. #	Day	Baseline DNA Synthesis	Net Stimulation		
				EBV	UV	PHA
1	AA81	3	219	(-12)	58	-21 ^a
		6	230	18	53	1114
		10	208	55	-64	232
		14	160	64	(-17)	(-30)
2	AA61	3	471	-	-	540 ^{b, c}
		6	436	268 ^c	476 ^c	628 ^c
		10	118	179 ^c	100 ^c	-
3	AA83	3	198	(-15)	(-18)	10 ^a
		6	373	(-79)	(-97)	962
		10	404	9	(-203)	288
		14	286	(-43)	(-116)	(-22)
4	BB1	3	150	29	(-4)	52 ^a
		6	249	9	(-27)	118
		10	175	(-26)	(-42)	88
		14	118	14	6	3
5	AA71	3	152	49	43	317 ^a
		6	693	(-62)	(-308)	7182
		10	1338	1091	(-340)	2293
		14	1122	1037	(-310)	2428
6	AA40	1	760	(-239)	155	644 ^b
		3	413	476	532	246
		6	1180	1025	2925	288

a
25 μ g PHA/ml

b
50 μ g PHA/ml

c
Difference in replicates >10% of mean.

TABLE VI. STIMULATION OF CELLS FROM A PATIENT WITH POORLY DIFFERENTIATED CLL

Patient	Expt. #	Day	Baseline DNA Synthesis	Net Stimulation		
				EBV	UV-EBV	PHA
7	AA82	3	432	11	137	(-73) ^a
		6 ^c	2707	(-806)	1545	(-1125)
		10	1384	805	3030	650
		14	2693	1191	(-393)	(-1100)
	BB42	3	344	76	78	70 ^b
		6	1107	57	1165	4442
		10	1233	1574	2179	4689
		14	1005	250	-261	239
	BB71	3	439	(-25)	(-29)	83 ^b
		6	1760	170	894	531
		10	1508	1242	885	188
		14	871	(-266)	(-517)	(-287)
	BB42 (Ab +) ^a	3	283	230	43	-
		6	914	811	255	-
		10	1202	5509	1937	-
		14	2278	3348	(-556)	-
BB42 (Ab -) ^e	3	373	38	47	-	
	6	828	(-82)	220	-	
	10	1225	3665	2151	-	
	14	1962	2526	(-253)	-	

a. 25µg PHA/ml

b. 2.5µg PHA/ml

c. Faulty thermostat permitted increased incubation temperature during pulse

d. Addition of 5% (V/V) human serum with anti-VCA antibody (1:160)

e. Addition of 5% (V/V) human serum with no anti-VCA antibody

TABLE VII. ISOLATION OF LEUKOCYTE SUBPOPULATIONS FROM PATIENT #7

Extp. #	Cell Type	% E-Rosettes	% EAC Rosettes
BB42	Mixed	7.5	76
	T-depleted	1.5	86.5
	T-enriched	52.5	33
BB71	Mixed	3	80
	T-depleted	<<1	78
	T-enriched	50	23

TABLE VIII. STIMULATION OF LYMPHOCYTE SUBPOPULATIONS FROM PATIENT #7

Expt. #	Day	Baseline DNA Synthesis (Mean cpm)	Net Stimulation (Mean cpm)		
			EPV	UV-EBV	PHA
BB42 ^b					
T-depleted	3	198	43	(-7)	(-23)
	6	1289	512	701	(-570)
	10	631	404	594	83
	14	414	573	233	(-90)
T-enriched	3	136	87	13	077
	6	396	256	51	845
	10	256	1411	328	821
	14	200	239	(-46)	279
BB71					
T-depleted	3	234	48	(-7)	(-80)
	6	1396	93	744	(-489)
	10	917	(-57)	738	(-389)
	14	889	(-57)	(-89)	(-731)

T-enriched: Not done because of insufficient number of cells.

^a 2.5 μ g PHA/ml

^b ³H-Tdr incorporation data for the unseparated population is shown in Table VI (Expt. BB42).

TABLE IX. EBV-SPECIFIC ANTIBODY TITERS

Patient	Expt. #	Anti-VCA Titer	Anti-EA Titer
1	AA28	1:20	ND
	AA81	1:20	<1:10
2	AA61	1:80	<1:10
		1:160	
3	AA83	1:10	<1:10
4	BB1	≥ 1:160	1:80
5	AA71	1:40	1:20
			1:40
6	AA40	1:80	1:80
	BB41	1:40	1:80
7	AA82	≥ 1:160	≥ 1:160
			1:400
	BB42		≥ 1:160
8	JR-1	≥ 1:160	≥ 1:160
			1:400
Normal lymphocyte donor	BB24	1:20	<1:10

TABLE X. SUMMARY OF DATA

<u>Patient</u>	<u>Stage</u>	<u>Expt. #</u>	<u>EBV</u> <u>Response</u>	<u>UV-EBV</u> <u>Response</u>	<u>PHA</u> <u>Response</u>	<u>Immortalization</u>	<u>Anti-EA Titer</u>
1	III	AA81	-	-	^a +	-	<1:10
2	IV	AA61	-	-	^b + -	-	<1:10
3	IV	AA83	-	-	+ -	-	<1:10
4	III	BB1	-	-	-	-	1:80
5	0	AA71	+	-	+	-	1:40
6	III	AA40	+	+	+ -	-	1:80
7	IV	AA82	+	+	+ -	-	1:400
		BB42	+	+ -	+	-	>1:160
		BB71	+	+	+ -	-	ND
8	IV	JR1	ND	ND	ND	ND	1:400
Normal donor			+	-	+	+	<1:10

a

+: Net stimulation ≥ 1000 cpm

b

+: Net stimulation ≥ 500 cpm but < 1000 cpm

Appendix

Consent Form for Participation in a Clinical Investigation Project
Yale University School of Medicine
Yale - New Haven Hospital

Description of Project:

We are investigating a virus called Epstein-Barr Virus which is the cause of infectious mononucleosis. This virus appears to infect only lymphocytes (white blood cells). We are interested in learning the response of lymphocytes from patients with your disease to this virus. We ask your permission to draw a sample of blood. Participation in this study will not affect your current therapy and participation is entirely voluntary. You are free to refuse to participate and in the event you do this decision would have no effect on your relationship with your physician or the Medical Center staff.

Authorization: I have read the above and decide that.....
(name of subject)

will participate in the project described above. Its general purposes, the particulars of involvement and possible hazards and inconveniences have been explained to my satisfaction. My signature also indicates that I have received a copy of this consent form.

.....
Signature

.....
Relationship (self, parent, guardian, etc.)

.....
Date

.....
Signature of Principal investigator Telephone
or

.....
Signature of Person obtaining consent Telephone

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