Characterization of Epstein-Barr Virus-carrying Cell Lines Established from Chronic Lymphocytic Leukemia

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ABSTRACT

Attempts were made to establish lymphoid cell lines from the cultured peripheral blood lymphocytes of six patients with chronic lymphocytic leukemia. In only one case was cell growth obtained following the addition of exogenous transforming Epstein-Barr virus, and those cell cultures proved not to have acquired the ability to proliferate permanently. In the same case, cell lines were established spontaneously from the peripheral blood without addition of Epstein-Barr virus. The cells which grew spontaneously were large, were occasionally weakly surface adherent, and grew in suspension as loose clumps or as single cells. They were negative for surface immunoglobulins and spontaneous rosette formation with sheep erythrocytes and positive for intracytoplasmic immunoglobulins (Fc and C3 receptors). At an early passage, the spontaneous lines had an aneuploid karyotype with some triploid and some tetraploid cells. Structural chromosomal aberrations include a 14q+. Electron microscopy of the chronic lymphocytic leukemia lines revealed relatively smooth surfaces with numerous mitochondria, widespread vacuolization, and unusual ‘myelin’ figures. Five to 10% of the cells were phagocytic as detected by internalization of latex particles; however, they were Epstein-Barr nuclear antigen positive. The nature of these cells and their possible relationship to the etiology of chronic lymphocytic leukemia are discussed.

INTRODUCTION

EBV (8) is a lymphotropic herpesvirus, capable of transforming B-lymphocytes, which are the only cells permissive for EBV proven to possess EBV receptors (23, 28, 38, 41). In general, 2 phenotypically distinct types of cell lines positive for EBNA (34) have been observed. The first cell type is derived from nonmalignant tissue, while the second type is that of tumor cell lines which are most frequently represented by the cells of B-lymphoblastoid origin which grow out of BL tumor cell lines which are most frequently represented by the cell lines grown spontaneously were large, were occasionally weakly surface adherent, and grew in suspension as loose clumps or as single cells. They were negative for surface immunoglobulins and spontaneous rosette formation with sheep erythrocytes and positive for intracytoplasmic immunoglobulins (Fc and C3 receptors). At an early passage, the spontaneous lines had an aneuploid karyotype with some triploid and some tetraploid cells. Structural chromosomal aberrations include a 14q+. Electron microscopy of the chronic lymphocytic leukemia lines revealed relatively smooth surfaces with numerous mitochondria, widespread vacuolization, and numerous unusual ‘myelin’ figures. Five to 10% of the cells were phagocytic as detected by internalization of latex particles; however, they were Epstein-Barr nuclear antigen positive. The nature of these cells and their possible relationship to the etiology of chronic lymphocytic leukemia are discussed.

MATERIALS AND METHODS

Patient Information. Blood was obtained by venipuncture from 6 patients with a clinical diagnosis of CLL made at Temple University Hospital, Philadelphia, Pa. Of the 6, none had been previously transplanted, but one patient, Patient 2, had been treated previously with Leukeran (chlorambucil), an alkylating agent, and prednisone, a corticosteroid immunosuppressant, and one, Patient 3, had been treated previously with Leukeran alone. The patient from whom the cell lines were obtained was untreated and had normal serum immunoglobulin levels both quantitatively and qualitatively as determined by serum protein electrophoresis and immunoelectrophoresis, respectively. The patient remains asymptomatic and has required no therapy. Seventy-five % of his lymphocytes had B-cell markers, with 71% of those being positive for surface IgM. All of the patients in this study were found to be seropositive for VCA (1:40 to 1:640) and EBNA (1:2 to 1:80).

Preparation of Blood, Cell Lines, and Virus. The mononuclear cells were isolated from whole body by the Ficoll-Hypaque method of Böyum

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The cells were maintained following isolation in Roswell Park Memorial Institute Medium 1640 (Flow Laboratories, McLean, Va.) to which had been added 20% fetal bovine serum (Flow Laboratories) as well as penicillin (80 units/ml; Grand Island Biological Co., Grand Island, N. Y.), streptomycin (80 μg/ml; Grand Island Biological Co.), and fungizone (1 μg/ml; Flow Laboratories). Within 3 days of isolation (usually within the first 24 hr), the cells were plated into 96-well, flat-bottomed microtiter dishes (Linbro Scientific, Inc., Hamden, Conn.). In each well was placed from 8.0 × 10^4 to 6.0 × 10^5 cells in a final volume of 0.2 ml. Most experiments utilized 1.0 to 2.0 × 10^5 cells/well. To each well was added 0.1 ml of an appropriate dilution of viral supernatant or 0.1 ml of complete medium. Due to the occasional appearance of "cytolytic factors" associated with nondiluted EBV-containing supernatants, diluted EBV was also used in each experiment. The cells were resupplemented with complete medium weekly. After 4 to 5 weeks at 37°C and 5% CO_2, the wells were observed with an inverted tissue culture microscope and scored for morphological transformation. Wells which displayed colony formation or which otherwise showed cell growth were established into cell lines by being transferred into 25-cm^2 plastic tissue culture flasks (Corning, Medfield, Mass.) and maintained on Roswell Park Memorial Institute Medium 1640 plus 10% bovine calf serum, as well as penicillin, streptomycin, and fungizone. As a precaution, we routinely added tylosin (10 μg/ml; Flow Laboratories) at this stage to prevent Mycoplasma contamination.

The EBV stocks used in these experiments were prepared from cottontop marmoset producer LCL supernatants (B95-8) (29). The supernatant was filtered through a 0.45-μm filter (Nalge Co., Rochester, N. Y.) prior to use, in order to remove any possible contaminating live marmoset cells. The spent supernatant contained transforming virus as determined by limiting dilutions using human umbilical cord lymphocytes.

Assays for EBNA, Early Antigen, and VCA. Immunofluorescence assays were performed to determine the presence of EBNA, early antigen, and VCA. EBNA was determined essentially by the method of Reedman and Klein (35); early antigen both, diffuse and restricted, by the method of Henle et al. (17, 18); and VCA by the method of Henle and Henle (16).

Electron Microscopy. Transmission electron microscopy was performed by a variation of standard published procedures for free cells (15, 22). Briefly, the cells were fixed for 1 hr in 3% glutaraldehyde (Tousnimis Research, Rockville, Md.) and then postfixed in 1.33% osmium tetroxide (Electron Microscopy Sciences, Fort Washington, Pa.). Following en bloc staining in 2% uranyl acetate (Fisher Scientific Co., Pittsburgh, Pa.), a few drops of 2% agar (Flow Laboratories) were added. Dehydration was accomplished with graded ethanol (Pharmco, Publcker Industries, Philadelphia, Pa.), and propylene oxide (Eastman Kodak Co., Rochester, N. Y.) infiltration was completed with propylene oxide and EMbed (Electron Microscopy Sciences), after which the cells were left in complete EMbed overnight. The next day, the cells were changed to a fresh EMbed mixture and embedded and incubated in a pellet at 60°C for 72 hr. Thick and thin sections were cut on a Porter-Blum Sorvall MT-2-B microtome. Electron micrographs were taken on a Phillips 300 electron microscope.

Scanning electron microscopy was also performed by published procedures (31). Briefly, the cells were fixed for 2 hr in suspension in 2% glutaraldehyde (Polysciences, Inc., Warrington, Pa.) postfixed in 1.33% osmium tetroxide, and gently filtered onto 0.45-μm-pore-size Millipore filters. They were then dehydrated in graded ethanol. Critical-point drying was accomplished versus liquid CO_2 in a Sorvall critical-point drying system. The preparation was mounted on stubs and sputtered with approximately 200 Å of 60% gold:40% palladium (Ernst F. Hahne, Inc., Schenectady, N. Y.). The cells were observed and photographed on an Elec Autoscan scanning electron microscope and classified according to the predominant surface feature. By comparing to similarly prepared PL, it was calculated that as much as 50 to 65% shrinkage occurred during the preparation.

RESULTS

Transformation Studies. Despite the fact that CLL is a B-cell leukemia in over 95% of patients diagnosed as having the disease (30), we had a great deal of difficulty in transforming PBL from these cases by the addition of exogenous EBV. This is in contrast to acute lymphocytic leukemia, the PBL of which, in 4 of 6 cases, yielded transformants following addition of exogenous EBV, although at a lower frequency than normal. In our initial studies, we found that, although approximately 87% of the PBL isolated from 2 CLL patients made EAC rosettes, permanent growth could not be induced by addition of exogenous EBV. In only one case of 6 additional patients tested was cell growth actually established, transformation being successful in 2 of 3 bleeds, between 1979 and 1981 (Table 1). In reality, the nature of these cell cultures as truly transformed LCs is questionable, as they do not appear to be immortal; they tended to die off (senesce) after, at most, 3 to 4 months of continuous culture. Of the 144 wells demonstrating colony formation, 20 with the heaviest growth were subcultured in an

<table>
<thead>
<tr>
<th>Patient</th>
<th>Treatment at time blood was obtained</th>
<th>Spontaneous transformation</th>
<th>EBV in vitro transformation of B95-8a (as measured by colony formation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>3/144 b</td>
<td>144/248</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>0/8</td>
<td>0/16</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>0/18</td>
<td>0/32</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>0/304</td>
<td>0/112</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>0/48</td>
<td>0/12</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>0/48</td>
<td>0/12</td>
</tr>
</tbody>
</table>

a B95-8 stocks had a titer ≥10^5 TDL (50% transforming dose) units/0.10 ml as measured on human umbilical cord leukocytes. Virus stocks were used undiluted and at 10^1 dilution.

b Bleed of September 14, 1979.
attempt to establish cell lines. Only 2 of these subcultures were successful; one E78-5 grew for as long as 3 to 5 months, displaying the standard colony morphology of EBV-transformed cell lines. In contrast, 3 of 144 wells from the same untreated patient transformed spontaneously after one of the 3 bleeds, the same as that from which E78-5 was established. Transformation was first noted as round, non-surface-adherent cells that grew singly or in loose clumps which tended to sheet out, eventually covering the entire bottom of the well, unlike the cultures established by the addition of EBV, which initially grew with the normal colony morphology typical of EBV-transformed lymphoblasts, tending to aggregate at the periphery of the well only. Upon transfer, these spontaneous lines appear to be truly immortal, growing in continuous culture for over 12 months.

The cells in each spontaneous lines are very similar in appearance. Although also appearing uniformly round under light microscopy, they tend to vary in size, with some cells having diameters as large as 75 μm or greater. Some cells become very vacuolated (Fig. 1), with vacuoles occasionally becoming as large as nuclei. The spontaneous cell lines derived from Patient 1 have been designated E51-10F, E51-11C, and E55-7G. These cell lines were confirmed as having arisen from the patient’s peripheral blood by HLA typing (HLA-A3, Aw24, Bw62, Bw53, Cw3, Cw4). The best growing of a number of autochthonous cell cultures established by transformation with exogenously added EBV has been designated E78-5 and will be mentioned for comparison at times, although complete data on E78-5 have not yet been attained due to lack of continuous growth. The chemical carcinogen N-methyl-N-nitro-N-nitrosoguanidine, which is capable of increasing the rate of spontaneous transformation from normal EBV seropositives, had no effect on E78-5 have not yet been attained due to lack of continuous growth. The well known. The nature or significance of this apparently secreted substance is not known.

Ultrastructurally, the cells were large, with numerous mitochondria and active Golgi apparatus. They were very vacuolated, with vacuoles with diameters as large as 45 μm often containing microvilli (Fig. 2) and occasionally what appeared to be debris. The cells had abundant cytoplasm. There was considerable nuclear pleomorphism, with dispersed chromat in and many multilobulated nuclei with prominent and multiple nucleoli. We also observed many unusual “myelin” or laminated bodies present in healthy cells, which appear to have evolved from mitochondria. The cell surfaces of the in vitro EBV-transformed culture, E78-5, appeared to be more microvillous (almost 50% of the cells were predominantly microvillous) than are those of the spontaneous CLL cell lines (only 20 to 34% of the cells were microvillous). It was further noted that, when microvilli were present on the spontaneous lines, they were fairly sparse and other surface features were not prominent, giving the cells, overall, a very smooth appearance. The larger cells tended to have a consistently smooth surface and seemed to be shedding or secreting a type of extracellular matrix-like material which appeared to be adherent to other cells (Fig. 3). This extracellular material was not artifactual, because this substance was not observed to be shed from smaller cells. The nature or significance of this apparently secreted substance is not known.

slg, clg, Rosette Formation, and Phagocytosis. All 3 of the spontaneous cell lines were negative for slg and E-rosetting, while being positive for clg (IgM, κ chain), weakly positive for EA rosette formation, and strongly positive for EAC rosette formation. Five to 10% of the cells were capable of phagocytosing polystyrene latex particles. The E78-5 LCL was positive for slg and clg, in both cases having IgM, λ chain only, even at very early passage. The association of these in vitro-trans-
formed cell cultures (E78-5), established by addition of exogenous EBV, with a single light immunoglobulin chain is remarkable, since EBV-induced in vitro transformation is usually polyclonal (32). The results are summarized in Table 2.

### DISCUSSION

CLL is a rather interesting disease from the point of view of EBV research. The peripheral blood in CLL contains an increased number of normal-looking, small lymphocytes with condensed-looking chromatin. Based on cell surface and other markers, the CLL lymphocytes are of B-cell origin in almost all cases (30). Thus, based on the increased percentage of B-lymphocytes, a high transformation frequency from CLL PBL following addition of EBV might be expected. However, while it has been reported that EBV is an effective polyclonal B-cell mitogen as measured at 5 days following exposure to virus in CLL PBL (13), it has also been reported that, following addition of exogenous EBV, morphological transformation frequency of PBL from CLL patients is much lower than that from normal individuals (3). Using standard procedures, we have also had a great deal of difficulty transforming CLL PBL by the addition of exogenous EBV. In the single patient from whose blood cell growth was obtained under the above conditions, although the initial apparent transformation frequency with the addition of EBV was high, the cultures apparently were not immortalized, growing for only 3 to 4 months. We believe that it is unlikely that the reason CLL cultures generally fail to transform after addition of EBV is because there are elevated numbers of anti-EBV-memory T-cells in the patients. We base this on our results, which included experiments using cell numbers below $2 \times 10^6$ cells/well, significantly below the optimum cell number needed to elicit "T-cell"-mediated culture regression (36). In addition, the ratio of T-cells (E-rosetting) to B-cells (EAC-rosetting), which is normally approximately 6:1, is usually reversed in CLL. Therefore, considering the foregoing, it appears that the point at which CLL PBL are restricted or the point at which EBV infection occurs is after stimulation of host cell DNA synthesis but before the ability to permanently proliferate is established.

The 3 cell lines that grew spontaneously from the CLL blood may be of clonal origin and perhaps even derived from the same in vivo clone. The evidence for individual clonality is that (a) the spontaneous transformation frequency was very low (approximately 1 in 10^7 cells), (b) all 3 cell lines contained IgM with a k chain, and (c) the karyotypic analysis is strongly suggestive of a monoclonal origin for at least the E51-10F cell line. In addition, the growth of the cell lines in loose clumps or singly rather than as colonies and the fact that the cells are EBNA-positive are similar to observations with monoclonal BL tumor lines (34). Evidence against this conclusion is that it is not unusual to see spontaneous, EBNA-positive cell lines established from seropositive individuals, but these usually resemble the dense colony-forming type of LCL. Cells from the spontaneous lines were distinct from normal LCLs. While they were positive for EA and EAC rosettes and clg, they were slg negative and round. There has been a previous report of cell lines with cells that are uniform in size, EBNA positive, round, slg and clg negative, and EA and EAC positive and that grow singly or in loose clumps (10). Cell lines such as these have been established from both healthy and diseased people and are postulated to originate from B-cell precursors (10). We believe that our cell lines possess certain features which set them apart from the above: (a), they are not uniform in size, and most are vacuolated, some vacuoles being very large; (b), 5 to 10% are phagocytic at any given time. (c), the majority contain intracellular immunoglobulin; (d), and the key point, is that even in very early passage stocks, the karyotypes are aneuploid with numerical and structural chromosomal aberrations not uncharacteristic of those found in CLL lymphocytes stimulated with polyclonal B-cell mitogens (11). At the time that our cell lines were established, the patient had been neither treated nor transfused. True tumor lines are usually aneuploid, while spontaneous transformants from peripheral blood are

### Table 2

Expression of receptors, phagocytosis and immunoglobulin by CLL lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Establishement with or without addition of exogenous EBV</th>
<th>% positive</th>
<th>Phagocytosis</th>
<th>G</th>
<th>M</th>
<th>A</th>
<th>D</th>
<th>k</th>
<th>λ</th>
<th>λ</th>
</tr>
</thead>
<tbody>
<tr>
<td>E51-10F</td>
<td>-</td>
<td>0</td>
<td>10</td>
<td>79</td>
<td>5-10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E51-11C</td>
<td>-</td>
<td>0</td>
<td>11</td>
<td>81</td>
<td>5-10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E55-7G</td>
<td>-</td>
<td>0</td>
<td>5</td>
<td>83</td>
<td>5-10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E78-5</td>
<td>+</td>
<td>0</td>
<td>3</td>
<td>57</td>
<td>Not done</td>
<td>0</td>
<td>52</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* E, sheep erythrocyte.
almost always diploid (32).

The normal-looking, small B-lymphocytes seen on peripheral blood smears from CLL patients have the same condensed-looking chromatin that other nondividing small PBL have, yet their numbers increase with progression of the disease, without necessarily displaying any blast forms. The possibility must therefore be considered that either a malignant precursor cell is differentiating into CLL PBL or a malignant clone is acting as a “driver” on particular B-cell precursors to advance matura-
tion in some form or another. The chance establishment of the spontaneous cell lines may represent the rare appearance of the truly transformed “driver” CLL cell in the peripheral blood of this patient. Our following 2 experimental observations tend to support the second hypothesis: (a) the expression of different, apparently monoclonal, immunoglobulins by the sponta-
neous cell lines compared to the autologous in vitro-derived lines (Table 2); and (b) the karyotype of the spontaneous cell line E51-1OF, which is also suggestive of a monoclonal origin, compared to the polyclonal appearance of the karyotypes derived from EBV-stimulated autologous PBL.

In summary, we have established and have begun character-
ization of what appear to be unusual tumor lines with both monocytic and lymphoblastic-plasmacytoid characteristics.

There are precedents for the establishment of cell lines from totipotent stem cells in other diseases of the hemopoietic system (6, 9, 27). Because these cells bear no resemblance to non-dividing CLL cells as seen in the peripheral blood, it is concluded that these cells may have a relationship to CLL, possibly even etiological. The nature of this relationship is yet to be determined.

ACKNOWLEDGMENTS

We thank Pat Ford for performing the transmission electron microscopy. We further thank Dr. Walter Ceglovski and Temple University's clinical laboratories for performing the electrophoretic studies and Dr. Robert McAlack and the Albert Einstein Medical Center Tissue Transplant Laboratory for performing HLA stud-
ies. We also thank Drs. Werner and Gertrude Henle and Shiela Kelly for performing immunofluorescence studies.

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Fig. 2. Transmission electron micrographs of E55-7G (A); E51-11C (B); E78-5 (C); and PBL (D). A, large vacuoles with microvilli, multiple or multilobulated nuclei, and what appear to be lipid droplets; B, a large "myelin" figure with several, smaller "myelin" figures developing, as well as abundant mitochondria; C, the different appearance of the in vitro-transformed E78-5; D, unstimulated normal adult peripheral lymphocytes for comparison. A, × 3300; B, × 5600; C, × 4500; D, × 4500.
Fig. 3. Scanning electron micrographs of E51-11C (A), E55-7G (B), E78-5 (C), and PBL (D). A and B, the intracellular matrix being shed or secreted from the large cell, as well as the round, relatively smooth smaller cells; C (E78-5) and D (PBL) are for comparison. Bar, 1.0 µm. Fifty to 65% shrinkage occurred during the preparative procedures.
Fig. 4. Representative karyotype from E51-10F stained with trypsin-Giemsa. The modal number was 48 chromosomes. The karyotype is interpreted as: 48,XY,+7,+20,t(14;16)(q32;q22),4q+,6p−,8q+,8p−,16p (satellited).
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