In Vitro Transformation of Human B-Cell Follicular Lymphoma Cells by Epstein-Barr Virus

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ABSTRACT

Human low-grade B-cell lymphoma cells cannot be readily maintained in long-term tissue culture. In an effort to obtain low-grade B-cell lymphoma cell lines for in vitro study, we used Epstein-Barr virus (EBV) as a transforming agent. T-cells were removed prior to EBV transformation by rosetting with sheep erythrocytes, followed by treatment with anti-T11 monoclonal antibody plus complement. The resulting cell population was cocultured with EBV from tissue culture supernatants of marmoset cell line B95-8. Identical immunoglobulin gene rearrangements of tumor cells and EBV-transformed cells were the criteria used to determine that the transformed cells were of tumor origin. DNA was prepared from both biopsy tissue and EBV cell lines and digested with restriction endonucleases, and Southern blots were prepared by standard methods. B-cells isolated from biopsies of four low-grade B-cell lymphomas of follicular, small cleaved cell type and one of follicular, mixed cell type were transformed by EBV into rapidly growing in vitro tissue culture lines. Two of the five transformed cell lines had immunoglobulin heavy chain and light chain gene rearrangements which were present in cells from the original tumor biopsy, indicating that these EBV-transformed cell lines are of tumor origin.

INTRODUCTION

Most non-Hodgkin’s lymphomas are B-lymphocyte tumors. These lymphomas can be grouped based on morphology and clinical manifestation into high, intermediate, or low grades. The intermediate and high grade lymphomas, such as Burkitt’s lymphoma, can be coaxed into growing in tissue culture, and numerous cell lines are now established from these tumors (3–6). On the other hand, the low grade lymphomas, such as most follicular lymphomas, have not been grown successfully in vitro in long-term tissue culture. This restriction puts constraints on research on the low grade lymphomas, since the amount of material available from any one tumor is limited. In an effort to stimulate the low grade lymphomas to grow in culture, we used EBV 3 as a transforming agent. Exogenous application of EBV has been used by many investigators as a transforming agent to establish lymphoblastoid cell lines of normal origin (7, 8). EBV-transformed lymphoblastoid cell lines also have been derived from patients with chronic lymphocytic leukemia and chronic myelogenous leukemia, and some of these cell lines originated from the tumor cell (9–11). Others have used EBV as a mitogen to induce mitoses in cells from patients with chronic lymphocytic leukemia, although there was no report of long-term cultures as a result of this study (12).

Here we describe the establishment of cell lines obtained by coculturing of EBV with cell suspensions of five primary tumor biopsy samples taken from patients with low grade B-cell lymphomas. Two of these cell lines have immunoglobulin gene rearrangements which are indistinguishable from those detected in cells of the original biopsy.

MATERIALS AND METHODS

Tissues. Neoplastic biopsy tissues from patients with non-Hodgkin’s B-cell lymphoma were obtained immediately after surgery. Lymphomas were biopsied according to the Working Formulation for Clinical Usage (2) and included four follicular small cleaved-cell lymphomas and one follicular mixed-cell lymphoma. A cell line established from biopsy of a patient with osteosarcoma, a nonlymphoid neoplasm, was used as a control in Southern blots to allow us to accurately identify nonrearranged immunoglobulin gene fragments.

Cell Preparation. Tissues were placed in RPMI-1640 tissue culture medium (GIBCO, Grand Island, NY), finely minced with scissors, and passed through a fine meshed screen. Cells in the resulting suspensions were washed 2 times in medium. Elimination of T-cells from these cell preparations was necessary to prevent cytolytic T-cells from killing EBV-infected B-cells. Thus, T-cells were removed prior to EBV transformation of B-cells by SRBC rosetting. SRBC were treated with 0.14 M 2-aminoethylisothiouronium bromide hydrobromide (Sigma, St. Louis, MO), pH 8.0, for 30 min at 4°C. Lymphoid cell suspensions were washed with treated SRBC in RPMI-1640 containing 40% fetal bovine serum (GIBCO, Grand Island, NY). The mixture was spun 5 min, 300 x g, and refrigerated for a minimum of 1 h. The cells were then carefully resuspended, diluted, and centrifuged (10 min, 1000 x g) over lymphocyte separation medium (Litton Bionetics, Charleston, SC). Cells at the interface (containing B-cells) were collected, washed 2 times in RPMI-1640 medium containing 5% fetal bovine serum, and counted using a hemacytometer. To assure removal of all T-cells, the resulting B-cell population was treated with anti-T11 monoclonal antibody (OKT11; ORTHO Diagnostics Systems, Inc., Raritan, NJ) and rabbit complement (Cedar Lane Laboratories, Ltd, Ontario, Canada). Briefly, 10 to 20 μl of OKT11 antibody were incubated with 10 x 10^6 cells on ice for 30 min. The cells were pelleted, the supernatant was discarded, and cells were resuspended in 0.5 ml of rabbit complement. The mixture was incubated at 37°C for 30 min, and the cells were subsequently washed 3 times in RPMI-1640 medium.

Epstein-Barr Virus. EBV from the B95-8 marmoset cell line was obtained either from a commercial supplier (Meloy Laboratories, Inc., Springfield, VA) or by harvesting tissue culture supernatant from B95-8 cells grown to confluence and stimulated with phorbol 12-myristate 13-acetate (Sigma, St. Louis, MO).

EBV Transformation. After removal of T-cells, the remaining lymphoid cells were cultured with EBV at a density of 2 x 10^6 cells per ml in RPMI-1640 medium containing 20% fetal bovine serum, Penicillin G (100 units/ml), streptomycin sulfate (100 μg/ml), L-glutamine (300 μg/ml), all from GIBCO (Grand Island, NY), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (10 mM; Research Organics, Inc., Cleveland, OH), and 2-mercaptoethanol (5 x 10^-4 M; Sigma, St. Louis, MO). Supernatant containing EBV was added in increments varying between 50 and 200 μl per ml of cell culture. Cells were fed by replacing one-half of the spent medium with fresh culture medium every 4 to 5 days until the cultures began to expand. As controls, parallel cultures of T-cell-depleted biopsy cells without EBV were also maintained for each case, although no cell growth was observed in these cultures.

Derivation of Sublines. Sublines were derived from EBV-transformed cells from Case 901 by selecting at random small clusters of cells from the culture. One cluster was drawn into a sterile Pasteur pipet and
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isolated in a well of a 96-well flat-bottomed plate. Each cluster of cells was disrupted into a single cell suspension and then left undisturbed for 24 to 48 h. After this time, new clusters of cells formed in these wells, and one was selected at random from each well and isolated. Each newly isolated cluster of cells was allowed to expand and was designated as a subline.

Preparation of Cellular DNA. Cells were lysed and incubated (65°C, overnight) in a solution of Tris (10 mM, pH 7.5) containing EDTA (5 mM), NaCl (100 mM), proteinase K (0.2 mg/ml; Sigma, St. Louis, MO), and 1% sodium dodecyl sulfate. Genomic DNA was extracted with phenol followed by chloroform as described elsewhere (13) and precipitated with 2-propanol. DNA was dialyzed against Tris (10 mM) containing EDTA (1 mM), pH 8.0, and quantified by spectrophotometry at 260 nm.

Southern Blots. Purified DNA preparations from biopsy tissue of patients with B-cell lymphoma and from their respective EBV-transformed cell lines were digested with four different restriction endonucleases (HindIII, BamHI, XbaI, and EcoRI; all from Bethesda Research Laboratories, Bethesda, MD) and electrophoresed in 0.7% agarose gels. Electrophoresed restriction fragments were transferred from agarose gels to nylon membrane (Zetabind; AMF Cuno, Meriden, CT) according to the method first described by Southern (14). Immunoglobulin gene rearrangements were identified by autoradiography using 32P-radiolabeled DNA probes. Heavy chain gene rearrangements were detected using an 800-base pair BgIII-HindIII restriction fragment from the second exon of C\(\alpha\). All probes originated as clones received as gifts from Philip Leder (Harvard University) (15). HindIII restriction fragments from \(\lambda\)-phage DNA were used in Southern blots as markers to determine lengths of immunoglobulin gene fragments. Germline (nonrearranged) immunoglobulin gene fragments were identified as previously described (16) by comparison to similarly digested DNA extracted from nonlymphoid human cell lines.

RESULTS

Biopsy samples from five patients with B-cell lymphoma were used in this study (Table 1). All of the patients were diagnosed on the basis of clinical, histological, immunophenotypic, and cell kinetic information as having low grade, B-cell malignant lymphoma.

We derived cell lines from biopsy cells of each of the five patients by transformation of B-cells with EBV. Individual cultures were grown until their cell number approached 200 \(\times 10^6\) (usually 12 to 16 wk) and were then harvested for analysis. Initial cell cultures were slow to expand after transformation, and these early cultures were not very stable in that the cell number would sometimes expand and contract. Thus, estimates of doubling times of these early cultures were not made. After 3 or 4 wk of culture, the cells grew at a more uniform and predictable rate with doubling times of 4 to 5 days.

DNA from the transformed cells and from the respective primary tumor cells was analyzed by Southern blot for immunoglobulin gene rearrangements. Genomic DNA preparations were digested with four different endonucleases and probed for heavy chain and light chain gene rearrangements. Electrophoretic patterns of bands representative of immunoglobulin gene rearrangements from the primary tumor cells were compared with those of the respective EBV-transformed cells. Data from this type of analysis demonstrate that two of the transformed cell lines have neoplastic origins.

In the first example, Case 840, the tumor cells express IgM with \(\kappa\)-type light chains, as determined by surface immunophenotyping. Genomic DNA isolated from Case 840 biopsy cells was digested with HindIII restriction endonuclease and a Southern blot prepared. The autoradiograph of the Southern blot after hybridization with a 32P-radiolabeled probe which reveals heavy chain gene rearrangement is shown in Fig. 1a. The autoradiograph shows a fragment representative of heavy chain genes in the germline configuration (contributed by non-tumor cells present in the biopsy) and two fragments representative of rearranged heavy chain genes. HindIII-digested DNA from EBV-transformed cells from Case 840 has the same rearranged heavy chain gene fragments as the DNA from the primary biopsy cells (Fig. 1a). The EBV-transformed cells lack heavy chain genes in the germline configuration, which is consistent with the presence of two rearranged heavy chain gene alleles.

Similar results were obtained from a Southern blot which contained DNA from Case 840 digested with \(\lambda\)baI endonuclease and which was hybridized with a probe specific for \(\kappa\)-chain gene rearrangement (Fig. 1b). The biopsy specimen contains cells which have \(\kappa\)-chain genes in the germline configuration and tumor cells which have two rearranged \(\kappa\)-chain genes. The EBV-transformed cells have no \(\kappa\)-chain genes in the germline configuration and have the same two \(\kappa\)-chain gene rearrangements as the primary biopsy cells.

Restriction fragments generated from DNA digested with HindIII, \(\lambda\)baI, BamHI, and EcoRI endonucleases were shared between the primary biopsy cells of Case 840 and the EBV-transformed cells when probed for either heavy chain or \(\kappa\)-chain gene rearrangements. These results are summarized in Table 2 where the lengths of the restriction fragments are given. It is evident that a clonal population of tumor origin is present in the transformed cell population of Case 840.

The \(\lambda\)-chain genes in the primary biopsy cells of Case 840 and in the cells which were EBV transformed were found to be in the germline configuration regardless of the enzyme used to digest the DNA (data not shown). This result is as expected for B-cells which express \(\kappa\)-type light chains. In addition, it is further evidence that a clonal population of nontumor origin is not present.

In the second example, Case 901, the tumor cells expressed immunoglobulin with \(\kappa\)-type light chains. An autoradiograph from Southern blot analysis of Case 901 DNA digested with HindIII endonuclease and probed for heavy chain gene rearrangement is shown in Fig. 2a. DNA from the primary biopsy cells of Case 901 has a fragment representing heavy chain genes
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EBV T

EBV T

EBV T

EBV T

Fig. 1. Autoradiographs from Southern blots containing DNA from EBV-transformed cells (EBV) and DNA from the primary tumor cells (T) of Case 840. Bands due to genes in the germline configuration are indicated by dashes with estimated kilobase lengths given, and clonal rearrangements by arrows. In a, the DNA was digested with HindIII and hybridized with a probe specific for the JH region. Use of this probe on HindIII-digested DNA results in an additional 3.5-kilobase fragment (and/or a 3.2-kilobase fragment due to polymorphism) that appears regardless of heavy chain gene rearrangement and has been observed by others (17, 18). The origin of this band is not determined. In b, the DNA was digested with XbaI and hybridized with a probe specific for the J region. The EBV-transformed cells have heavy chain and \( \kappa \) chain gene rearrangements which are identical to the rearrangements of the primary tumor cells but, as might be expected, the EBV-transformed cells have none of these genes in the germline configuration.

Table 2 Immunoglobulin heavy chain and \( \kappa \) chain gene fragments for Case 840

<table>
<thead>
<tr>
<th>Probe</th>
<th>Enzyme</th>
<th>Primary tumor cells</th>
<th>EBV-transformed cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>( J_w )</td>
<td>HindIII</td>
<td>(9.7)/10.5/23</td>
<td>10.5/23</td>
</tr>
<tr>
<td>( XbaI )</td>
<td>6.1/4.9</td>
<td>4.9/9.1</td>
<td></td>
</tr>
<tr>
<td>EcoRI</td>
<td>6.4/9.3</td>
<td>6.4/9.3</td>
<td></td>
</tr>
<tr>
<td>BamHI</td>
<td>15/11.5/12.0</td>
<td>11.5/12.0</td>
<td></td>
</tr>
<tr>
<td>( J_\kappa )</td>
<td>HindIII</td>
<td>(5.4)/1.7/3.1</td>
<td>1.7/3.1/3.5</td>
</tr>
<tr>
<td>( XbaI )</td>
<td>14.0/7.6/9.0</td>
<td>7.6/9.0</td>
<td></td>
</tr>
<tr>
<td>EcoRI</td>
<td>8.2/5.8/6.3</td>
<td>5.8/6.3</td>
<td></td>
</tr>
<tr>
<td>BamHI</td>
<td>8.4/7.1/8.0</td>
<td>7.1/8.0</td>
<td></td>
</tr>
</tbody>
</table>

Numbers in parentheses, nonrearranged immunoglobulin genes.

Rearranged immunoglobulin genes.

This additional band may be the result of a secondary change in the EBV-transformed cell line.

in the germline configuration and four other fragments presumably representing heavy chain gene rearrangements. The reason for more than the conventional two heavy chain gene rearrangements in this tumor population is not clear. Others have reported similar results in studies of B-cell lymphomas (17, 18). After EBV transformation of Case 901 biopsy cells, the cells growing in culture were polyclonal as determined by both surface immunoglobulin expression and immunoglobulin gene rearrangement (data not shown). Sublines were subsequently derived from the Case 901 EBV-transformed cells as described in "Materials and Methods," and DNA from two of three sublines analyzed showed restriction fragments with DNA from the primary tumor cell. One of these sublines was analyzed further. The transformed cells from the subline have two heavy chain gene rearrangements which are the same as two of the four rearrangements present in the primary biopsy cells (Fig. 2a). As is expected for a cell line with two rearrangements, these cells have no heavy chain genes in the germline configuration. The simplest interpretation of these results is that there were two concurrent populations of tumor cells in the biopsy sample, one of which we transformed with EBV.

When DNA preparations from Case 901 biopsy cells and from the EBV-transformed subline were probed to reveal \( \kappa \)-
chain gene rearrangements, a result very similar to that using the heavy chain gene probe was obtained (Fig. 2b). As was done for Case 840, DNA from Case 901 and from the EBV-transformed cells was digested with four restriction enzymes and analyzed by Southern blot for heavy chain and \(\kappa\)-chain gene rearrangements. These results are summarized in Table 3. Interestingly, when genomic DNA from Case 901 is digested with some endonucleases and probed for heavy chain or \(\kappa\)-chain gene fragments, multiple (more than two) rearrangements are not revealed. Regardless, the EBV-transformed cells have heavy chain and \(\kappa\)-chain gene rearrangements identical to gene rearrangements of the primary tumor cells. The \(\lambda\)-chain genes of the primary tumor cells and of the EBV-transformed cells both are in the germline configuration which is not unexpected (data not shown).

DNA preparations from the remaining three EBV-transformed cell lines were analyzed by Southern blot for heavy chain, \(\kappa\)-chain, and \(\lambda\)-chain gene rearrangements (data not shown). Results show that they have no restriction fragments in common with DNA preparations from their respective primary tumor biopsy cells. Thus, we conclude that the latter three cell lines originated from transformation of normal B-cells present in the biopsy samples.

The Southern blots that were hybridized with the immunoglobulin gene probes were subsequently hybridized with a BamHI restriction fragment specific for the W fragment of the EBV genome (19). Use of this probe demonstrated the presence of EBV DNA in all of our EBV-transformed cell lines (data not shown). In addition, a total of 16 primary B-cell tumors was represented on these blots, and only two hybridized with the EBV-specific probe: one was Case 901; and the other was unrelated to this study. Nonetheless, a parallel culture of Case 901 primary biopsy cells without the addition of exogenous EBV did not expand into a growing cell line.

DISCUSSION

EBV is a polyclonal stimulator and transformant of human B-cells. Because biopsy samples from patients with B-cell lymphoma contain both tumor cells and nonmalignant (normal) B-cells, transformation of normal and neoplastic B-cells would be expected following addition of EBV to a tumor biopsy sample. If this is the case, then our results suggest that normal B-cells may be preferentially transformed or adopt characteristics which give them a growth advantage in culture. Consequently, the cells which eventually dominate the culture most of the time are derived from normal B-cells rather than neoplastic B-cells as determined by surface marker and gene rearrangement analyses.

Table 3 Immunoglobulin heavy chain and \(\kappa\)-chain gene fragments for Case 901 DNA from EBV-transformed cells shares restriction fragments with DNA from the respective primary tumor.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Enzyme</th>
<th>Primary tumor cells</th>
<th>EBV-transformed cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jn</td>
<td>HindIII</td>
<td>(9.7)<em>/1.9</em>/6.8*/5.9/9.0</td>
<td>1.9/8.5</td>
</tr>
<tr>
<td>XbaI</td>
<td>(6.1)/6.4/7.7/8.8</td>
<td>(6.1)/7.7</td>
<td></td>
</tr>
<tr>
<td>EcoRl</td>
<td>(16)/13.0/20</td>
<td>13.0/20</td>
<td></td>
</tr>
<tr>
<td>BamHI</td>
<td>(15)/11.8/14.0</td>
<td>11.8/14.0</td>
<td></td>
</tr>
<tr>
<td>J</td>
<td>HindIII</td>
<td>(5.4)/5.1/10.0/13.0</td>
<td>5.1/10.0/13.0</td>
</tr>
<tr>
<td>EcoRl</td>
<td>(8.7)/5.2/10.5/12.0</td>
<td>7.0/10.5/12.0</td>
<td></td>
</tr>
<tr>
<td>BamHI</td>
<td>(8.4)/6.3/8.6/10.0</td>
<td>6.3/8.6/10.0</td>
<td></td>
</tr>
</tbody>
</table>

* Results are of one subline of EBV-transformed cells from Case 901.

** Numbers in parentheses, nonrearranged immunoglobulin genes.

A rapidly growing \textit{in vitro} tumor cell line can result from transformation by EBV of tumor cells in certain instances as we were able to transform the tumor cells in two of five cases. In one, Case 840, the tumor cells represented the vast majority of B-cells present (95%). Thus, a clonal population of EBV-transformed tumor cells dominated the culture probably because of a limited population of competing normal B-cells. In the other, Case 901, the tumor cells were not the majority of cells present, and presumably some normal B-cells were transformed by the EBV. However, we were able to isolate the EBV-transformed tumor cells before transformed cells of normal B-cell origin could dominate the culture.

Because of the small number of lymphoma samples we transformed, it is not possible to predict at what frequency transformation of the tumor cell will occur for low grade B-cell lymphomas. In the two cases which resulted in a transformed tumor cell culture, the tumor cell was present in the transformed cell population at the time the culture was harvested for Southern blot analysis. Even in Case 901, in which the culture required further manipulation to isolate the transformed tumor cell, immunoglobulin gene rearrangements of the transformed tumor cell could be identified in the unselected cell culture. In the other three cases, there was no indication that the tumor cell was present in the transformed cell population at the time of our assay. These results suggest that low grade B-cell lymphomas are not all equally susceptible to EBV transformation and/or adaptation to growth in cell culture.

The presence of multiple immunoglobulin gene fragments in Case 901 DNA digested with HindIII and XbaI is intriguing. The presence of more than the conventional two heavy chain gene rearrangements in this tumor population may put this tumor into the category of "biclonal lymphomas" as described previously by others (17, 18). Alternatively, the multiple fragments may reflect secondary changes in a single primary tumor. Mechanisms such as somatic mutation or secondary IgH gene rearrangements could give rise to subpopulations of tumor cells which differ in their immunoglobulin gene rearrangements or sequences.

Our interpretation of the data obtained for Case 901 is complicated by the fact that varying numbers of immunoglobulin gene fragments were observed depending on the enzyme used to digest the DNA. Most reports of immunoglobulin gene rearrangement studies describe the use of only one or two restriction enzymes, although a study reporting the use of four different enzymes on DNA from non-T-acute lymphoblastic leukemia showed no examples of discrepant results (20). However, a recently published abstract (21) describes the phenomenon of differing numbers of immunoglobulin gene fragments as a result of digestion with different enzymes even though all of the enzymes used should reveal immunoglobulin gene rearrangement. Other cases in our laboratory display this phenomenon, and studies are in progress to help us further understand the significance of these results.

Transformation of tumor cells by EBV offers the potential for creating \textit{in vitro} models for human low grade B-cell lymphomas. The question still remains as to how stable these lines will be in continuous, long-term culture and what impact EBV transformation may have on their tumor phenotype. Our cell lines were harvested and analyzed after 3 or 4 mo of continuous culture. EBV-transformed cell lines of normal origin become aneuploid following continuous \textit{in vitro} passage and adopt growth characteristics not present initially after transformation (6, 22). EBV-transformed cell lines of tumor origin should be...
useful for comparison to EBV-transformed cell lines of normal origin from the same patients.

ACKNOWLEDGMENTS

We thank Dr. L. Hutt-Fletcher at the University of Florida for supplying plasmid DNA containing the W fragment of the EBV genome.

REFERENCES

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