ABSTRACT

Epstein-Barr viral (EBV) DNA is nearly always detectable in African Burkitt’s lymphoma (BL) but is infrequently found in the histologically indistinguishable American BL. We have derived a tumor cell line from a patient with American BL which produces EBV, and we have compared this virus isolate [JLP(c)] with African BL EBV. The American JLP(c) virus immortalizes human umbilical cord lymphocytes in vitro, and its DNA is indistinguishable from African BL EBV DNA by nucleic acid hybridization and preliminary restriction endonuclease cleavage analysis.

INTRODUCTION

An environmental or infectious etiology was first postulated by Denis Burkitt to explain the unique cases of childhood lymphoma which were clustered in East Africa (6, 7). In 1964, the EBV2 was discovered in the cultured cells from a patient with this lymphoma, now referred to as BL (10). Subsequent studies have shown that African patients with histologically demonstrable BL have significantly elevated antibody titers to BL have significantly elevated antibody titers to EBV2 were discovered in the cultured cells from a patient with this lymphoma which were clustered in East Africa (6, 7). In 1964, the EBV2 was discovered in the cultured cells from a patient with this lymphoma, now referred to as BL (10). Subsequent studies have shown that African patients with histologically demonstrable BL have significantly elevated antibody titers to EBV DNA by nucleic acid hybridization and preliminary restriction endonuclease cleavage analysis.

MATERIALS AND METHODS

Derivation of the Cell Line. The JLP cell line was derived from the pleural fluid of an 11-year-old Caucasian American male with histologically confirmed BL. The patient’s initial presentation included extensive abdominal tumor with malignant ascites and pleural effusions. Malignant cells obtained from a pleural fluid sample (7.2 x 10^6 cells/ml with 93% tumor cells) and bone marrow from which mature polymorphonuclear leukocytes and RBC had been removed by Ficoll-Hypaque separation were diluted with fresh Roswell Park Memorial Institute Medium 1640 plus 20% heat-inactivated (56°, 30 min) fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.) to an initial cell concentration of 1 x 10^6 cells/ml in 75-sq cm plastic flasks and incubated at 37° in an atmosphere containing 5% CO2. Outgrowth of cell lines occurred without a lag phase, and the permanent lines were subcultured every 3 to 4 days, using the same culture medium.

Fresh tumor cells were also pelleted, washed with phosphate-buffered saline (made with 0.0067 M concentrations of Na2HPO4, KH2PO4, and 0.85% NaCl, pH 7.2), and stored at -70°. Cells were also harvested and pelleted from the established cell lines after different culture passages and were similarly pelleted, washed with phosphate-buffered saline, and stored at -70°. Cells were also examined at intervals for the presence of EBNA.

Virus and Viral DNA Isolation. During serial culture, the JLP cell line converted from 0.5 to 1% EBNA positive at the time of its initiation to 88% 8 weeks later. The EBNA-positive cell line [designated JLP(c)] was examined for the cytoplasmic viral capsid antigen and was found to be approximately 8% positive at 37°. Unconcentrated supernatant was assayed in a transformation assay using Ficoll-Hypaque-purified human umbilical cord leukocytes by a previously described method (22).

JLP(c) cells were grown to obtain sufficient virus for biochemical study in 150-sq cm plastic flasks at 32° (15 liters) and were labeled with [3H]thymidine (Amersham/Searle; Arlington Heights, Ill.; 45 Ci/mmol) at 5 μCi/ml on the eighth day of culture. After 14 days of continuous culture, the supernatant was clarified by low-speed centrifugation, passed through a Millipore filter (RATF 14200; Millipore Corp., Bedford, Mass.), and concentrated using the Millipore Pellicon cassette system with a molecular weight cutoff of 1 x 10^6 to 3 x 10^6. This concentrated supernatant was then pelleted at 113,000 x g for 1 hr at 4° and resuspended in approximately 3 ml of buffer (0.01 M Tris·HCl pH 7.8-0.001 M EDTA-0.1 M NaCl). The subsequent schema for virus purification has been described previously (21) and includes an initial dextran velocity gradient (ρ 1.04 to 1.09 g/ml) followed by isopyknic sedimentation in sucrose (30-60%, w/v). The radiolabeled virus-containing peaks were pooled, diluted with 2 volumes of 0.1 M Tris, pH 7.6-0.001 M EDTA buffer, pelleted, and dialyzed. To obtain

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2 The abbreviations used are: EBV, Epstein-Barr virus; BL, Burkitt’s lymphoma; EBNA, Epstein-Barr virus nuclear antigen; PB, phosphate buffer.
viral DNA, the purified virions were lysed with sodium dodecyl sulfate and sedimented through sucrose (10 to 30%). The 55S viral DNA peak was pooled, dialyzed, extracted twice with buffer-saturated redistilled phenol and twice with chloroform-isoamyl alcohol, precipitated with ice cold ethanol, resuspended in 0.1 M Tris, pH 7.6-0.001 M EDTA buffer, and centrifuged to equilibrium in cesium chloride. The single radioactive peak at a density of 1.7180 g/ml was pooled, dialyzed, and shown to have a \( A_{260}/A_{280} \) spectrophotometric ratio of 1.90. This highly purified viral DNA was compared with 3 other EBV DNA’s by molecular hybridization and restriction endonuclease analysis.

**RESULTS**

**Cell Lines.** The original tumor cell line contained approximately 1% EBNA (16). After 6 weeks of culture, the number of EBNA-positive cells had increased to 14% and then to 67% at Week 7 and 88% by Week 8. We therefore examined the original tumor cells which had been frozen, as well as cells which had been harvested from the established cell lines after 1 month (passage 8) and after 7 months of tissue culture (passage 56) for the presence of the EBV genome. Chromosomal examination of the JLP(c) cell line showed it to have an aneuploid karyotype and an 8;14 translocation consistent with the original tumor. EBV DNA sequences were detectable in the original tumor cells which had been frozen, as well as cells which had been harvested from the established cell lines after 1 month (Chart 1A). Similarly, cells from the bone marrow cell lines (JL-BM) which had been harvested 1 month after their establishment in tissue culture also contained detectable sequences. These EBV DNA sequences were increased 13-fold in the pleural and bone marrow cell lines which were harvested after 7 months in tissue culture. It is of note that the patient’s anti-viral capsid antigen titer was only weakly positive (1:10) at the time of diagnosis, while the anti-early antigen was <1:10 and the anti-EBNA was 2.

**Virus Characterization.** The JLP(c) supernatant virus contained 10^4 of the 50% transforming units per ml. However, the same JLP(c) supernatant virus failed to induce early antigen in the EBV genome-positive RAJI cells. As shown in Chart 1B, the 3H-JLP(c) EBV DNA hybridized to 92% with both AG876 and P3HR1 cell DNA’s and to 82% with the unlabeled B95-8 cellular DNA. This pattern of hybridization is very similar to that observed with P3HR1 and AG876 (14, 22), in spite of the known biological differences between these viral strains (i.e., P3HR1 has generally lost its capacity to transform umbilical cord lymphocytes into EBNA-positive continuous cell lines, while both the African BL-derived AG876 and the JLP(c) virus strains have this transformation capacity, as do all other naturally occurring EBV isolates). The extent of hybridization of the 3H-JLP(c) EBV DNA with unlabeled B95-8 cell DNA is also similar to that observed with the radiolabeled AG876 and P3HR1 viral probes, again showing the deletion of EBV sequences in B95-8. Hybridization of the 3H-AG876 or the 3H-P3HR1 viral DNA’s with the unlabeled JLP(c) cell line cellular DNA’s is shown in Chart 1A. The AG876 and P3HR1 viral DNA probes hybridize to at least 75% with both the JLP(c) and JL-BM cellular DNA’s (passage 56). The final extent to which this hybridization is complete cannot be concluded from this analysis.

**Restriction Enzyme Cleavage.** Restriction endonuclease cleavage patterns of various purified EBV DNA were studied using approximately 3 x 10^5 cpm of 3H-labeled EBV DNA (specific activity, 1 to 1.5 x 10^4 cpm/µg) which were digested with either Eco RI in 100 mM Tris-HCl (pH 7-2)-50 mM NaCl-5 mM MgCl_2-2 mM 2-mercaptoethanol or Sal I in 10 mM Tris-HCl (pH 7.6)-150 mM NaCl-5 mM MgCl_2, in the presence of 1 µg carrier phage λ DNA. The digests were analyzed by electrophoresis in 0.6% agarose slab gels containing 40 mM Tris base-30 mM NaH_2PO_4 (pH 8.2)-1 mM EDTA. The completeness of each reaction was confirmed by the total cleavage of carrier λ DNA as visualized by staining with ethidium bromide (0.5 µg/ml). 3H-labeled EBV DNA fragments were detected by fluorography (4). Exposures were for 2 days at -70°.

**Chart 1.**

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<tr>
<th>COT</th>
<th>% Hybridization</th>
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<td>10^-4</td>
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<tr>
<td>10^-3</td>
<td>80</td>
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In A, the reaction mixtures consisted of the 3H-AG876 viral DNA probe with: CA46 (EBV genome-negative control) (O); P3HR1 (EBV genome-positive control) ( ); original tumor ( ); JLP cell line, passage 8 (A); JLP(c) cell line, passage 56 ( ); JL-BM cell line, passage 8 ( ); JL-BM cell line, passage 56 ( ). In B, the viral DNA probe used for these hybridization reactions was from the JLP(c) cell line. In these experiments, 3H-JLP(c) viral DNA was hybridized with: P3HR1 cell DNA ( ); AG876 cell DNA ( ); B95-8 cell DNA ( ); CA46 (EBV genome-negative) cell DNA ( ).
ysis because of the self-hybridization which occurred with the double-stranded DNA probe(s).

Restriction endonuclease cleavage fragments of purified $^3$H-labeled EBV DNA show no detectable difference between JLP(c) and AG876, using either Eco RI or Sal I, within limits of resolution of the gel (Fig. 1). Two separate preparations of the JLP(c) DNA and 3 of AG876 DNA have been examined and show to be identical each time. However, comparisons between JLP(c) and either B95-8 or P3HR1 DNA show that, while JLP(c) is highly related to these laboratory strains, there are detectable differences seen with both Eco RI and Sal I. These differences were seen reproducibly from preparation to preparation. These findings are clearly preliminary since larger cleavage fragments are under represented, and more detailed study is necessary to truly compare these DNA samples. Nonetheless, these findings fail to demonstrate any major difference in the smaller fragments between the EBV isolates obtained from either our African (AG876) or American [JLP(c)] BL-derived cell lines.

**DISCUSSION**

The JLP(c) virus is the first EBV isolate from an American BL which has been biochemically analyzed. The presence of the EBV genome in the original tumor material and in the early passages of the tumor-derived cell line, along with the low number of EBNA-positive cells (1%), suggests that the EBV genome was present in a small number of cells rather than distributed in low quantity in all cells. Furthermore, the derived cell line contained the same 8;14 translocation and have an HLA type consistent with that of the original tumor cells, suggesting that the conversion of the JLP cell line to EBNA positivity and ultimate virus productivity was due either to the selective survival of an EBV-containing subpopulation of tumor cells or to the in vitro infection of susceptible tumor cells by EBV released from genome-positive tumor cells. Alternatively, it is possible that the genome content of most of the tumor cells was initially below the level detectable by EBNA staining and that, as the number of genomes increased in vitro, so did the percentage of EBNA detectable in cells.

Although an etiological role for EBV in the genesis of this tumor cannot be excluded, the fact that the majority of the tumor cells were EBNA negative makes this unlikely. This is consistent with the observation that more than 80% of American BL’s do not contain evidence of the EBV genome (21). Although the reassocation kinetics and preliminary restriction enzyme analysis are not sensitive enough to exclude possible differences, the similarity of the EBV isolate from this patient with American BL with that recently isolated from an African patient with BL is noteworthy. It is of interest that Bornkamm et al. (5) recently described similar comparability in an analysis of EBV isolates from the lymphoid cell lines of patients with African BL, nasopharyngeal carcinoma, and infectious mononucleosis (5). Hence, the comparability of the viral isolate that we describe from a tumor with which EBV is only rarely associated with one in which it is commonly associated raises additional questions concerning the role of EBV in human oncogenesis.

**REFERENCES**

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Characterization of the Epstein-Barr Virus Isolated from a Cell Line Derived from a Patient with American Burkitt's Lymphoma

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