ABSTRACT

While Epstein-Barr viral (EBV) DNA is nearly always detectable in African Burkitt’s lymphoma (BL), the relatively low frequency of BL in EBV-positive African children and the frequent finding of EBV in American BL suggest that other cofactors may contribute to the malignant transformation. Among these possible cofactors are type C oncornaviruses. To evaluate this possibility, we screened the cellular DNA from 16 lymphomas (2 African, 14 American) and the DNA from 20 lymphoma-derived cell lines (4 African, 16 American) with a radiolabeled viral DNA probe from EBV and two oncornaviral probes (murine amphotropic 1504A virus and simian sarcoma virus). The radiolabeled EBV DNA probe hybridized with 18 of 36 tumor or cell line DNA’s. Only 2 of 11 American BL tumors contained detectable EBV sequences. However, the cell lines derived from three EBV-negative tumors converted in vitro to EBV positivity, suggesting that some of the tumor cells could be infected with EBV. In contrast, none of the tumors or the cell lines derived therefrom hybridized with either the 1504A or the simian sarcoma virus probes, decreasing the likelihood that type C viruses are cofactors with EBV.

INTRODUCTION

Since its discovery in the cultured tumor cells from an African patient with BL, the EBV has become a leading candidate for a human tumor virus (17, 18). This is based on several observations. EBV DNA can be found in 97% of African Burkitt’s tumors (55, 70), and 100% of these patients have significantly elevated serum antibodies to the viral capsid antigen and early antigen of EBV (28, 30). Furthermore, in vitro infection with EBV results in the transformation of human umbilical cord lymphocytes into continuous cell lines (24), and the inoculation of EBV into owl monkeys and cotton-topped marmosets can result in a fatal lymphoproliferative disorder (13, 19, 66). While EBV at an early age eventually develops Burkitt’s lymphoma (15). Hence, the possibility that other factors operate in addition to EBV to produce malignant transformation has been raised. Among the possible cofactors which have been proposed are genetic defects [e.g., the 8; 14 translocation (32, 51) and the X-linked lymphoproliferative syndrome (61)] or the immunosuppression from holoendemic malaria which may permit uncontrolled B-cell proliferation (16, 56). The possibility that specific strains of EBV may be oncogenic or that other tumor viruses may serve as cofactors with EBV has also been suggested (25–34, 36).

Type C oncornaviruses have been described in a variety of subhuman species and tumors (20, 22). Moreover, some of these oncornaviruses (MuLV, SiSV, gibbon ape leukemia virus, and baboon endogenous virus) have been associated with human lymphoid cancers. This includes the finding of immature intracytoplasmic type C particles with a sedimentation coefficient of 550S, containing 70S RNA with homology to SiSV, MuLV, and baboon endogenous virus by cDNA hybridization; the presence of SiSV and MuLV antibody-inhibitable reverse transcriptase; the electron microscopic demonstration of virus-like particles, and, less frequently, the presence of proviral DNA sequences (21, 22). In Burkitt’s tumor tissue, Kufe et al. (41–43) described 70S particles containing RNA-dependent DNA polymerase, and Kotler et al. (40) observed the release of type C RNA virus particles from arginine-deprived human lymphoblastoid cell lines which also contain EBV.

To further examine the possible association of oncornaviruses with human lymphomas, their derivative cell lines, and EBV, we have studied a variety of African and American lymphomas and their cell lines for the presence of EBV and/or oncornavirus proviral sequences. Two oncornavirus probes were used. The amphotropic 1504A virus was selected as a representative MuLV since at least 60% of its sequences are homologous with the ecotropic and xenotropic viruses of laboratory mice and 10% of its specific sequences (which are not contained in the 60% of ecotropic and xenotropic sequences) hybridize with the Friend, Rauscher, and Moloney leukemia viruses (7, 8, 10, 26, 44, 47, 63). We also used a simian sarcoma viral probe, since this virus has been associated previously with human leukemias and lymphomas (20–22). Together, these probes provide a broad screen for detecting oncornavirus sequences.

MATERIALS AND METHODS

Tumors and Cell Lines. Fresh tumor material was divided into portions which were stored at −70°C or which were finely minced, filtered through sterile gauze, and cultured in RPMI 1640 plus 20% FCS (Grand Island Biological Co., Grand Island,
cultures at 37° in 75-sq cm plastic flasks and subcultured
Waring Blendor and tissue grinder. DNA extraction was then
from both tumor tissue and cell pellets. Tumor tissue was
cell lines, approximately 5 liters of each cell line were cultured
lyzed recently (45, 46). For the preparation of DMA from these
properties of many of these derived cell lines have been ana
P. A. Pizzo et al.
to 1.95.
(129,000 x g for 60 hr at 20°). The single
hybridization reactions were each sheared twice by passage through a French
isolation of cDNA, and its characterization have been described
elsewhere (64, 65).
Synthesis of Single-stranded, Virus-specific [3H]DNA. Single-stranded virus-specific [3H]DNA was synthesized in an
endoogenous reverse transcriptase reaction using detergent-lysed purified virions in the presence of actinomycin D. The synthesized
probe was purified by deproteinization with chloroform: isoamyl alcohol (24:1, v/v) and phenol, and unincorporated radioactivity was removed by Sephadex G-50 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) chromatography. RNA was removed by alkali treatment followed by neutralization and
dialysis. The 3H-viral probe synthesized by this method had a specific activity of 2 x 10^7 cpm/µg and was 98% single-
stranded and about 200 to 400 nucleotides long. More than 90% of the probe sequences are present in the viral RNA (11).
Hybridization Reactions. The unlabeled DNA extracted from tumors and cell packs and the 3H-labeled EBV DNA probes
were each sheared twice by passage through a French pressure
cell (American Instrument Co., Silver Spring, Md.) with a
pressure drop of 40,000 psi. Sheared DNA's were each passed through a Metrical GA-6 filter (cellulose acetate; Gelman
Instrument Co., Ann Arbor, Mich.), extracted twice with chloro-
form:isoamyl alcohol, and precipitated with ethanol.
Hybridization reactions with the radiolabeled EBV DNA probe consisted of sheared unlabeled tumor or cell line DNA (5.6 mg/ml) mixed with 7 x 10^{-7} µg of 3H-AG876 EBV DNA in a 1-
mL Reactivial (Pierce Chemical Co., Rockford, Ill.). The mixture was denatured by heating to 105° for 5 min in 0.12 m PB which
was then adjusted to 0.48 m PB. The mixture was incubated at 65°, and aliquots were removed at different time points and
diluted to 2 mL with a final concentration of 0.14 m PB plus 0.4% SDS. The percentage of hybridization was determined by
hydroxyapatite chromatography (Bio-Gel HTP; Bio-Rad Labo-
ratories, Richmond, Calif.), unhybridized molecules being eluted from the columns at 60° with 8 mL of 0.14 m PB plus
0.4% SDS and hybridized molecules being eluted with 8 mL of
0.32 m PB. Twelve mL of Instagel (Packard Instrument Co.,
Downtowns Grove, Ill.) were added to each for liquid scintillation
counting. A minimum of 12 to 15 samples were assayed for
each reaction mixture during a 72-hr incubation, and the per-
RD cells which were chronically infected with 1504A virus were
maintained and propagated in McCoy's Medium 5A (Grand
Island Biological Co., Grand Island, N. Y.) with 10% heat-
inactivated FCS. Cells were seeded in 150-sq cm plastic flasks
(Corning Products, Corning, N. Y.); when confluent, the super-
natant (20 mL/flask) was collected at 12-hr intervals, centri-
fuged at 9150 x g for 10 min at 4°, decanted into a sterile 20-
liter carboy, and stored at 4°. The virus was concentrated by
in a 20 to 50% sucrose density gradient in a Model K ultracentrifuge
(Electronucleonics Corp.). The virus-containing fractions (mea-
sured by absorbance at A_{280}) were banded a second time in a
20 to 50% sucrose density gradient in a CF-32 rotor (Beckman
Instrument Co., Palo Alto, Calif.). The virus-containing fractions
were then pooled, pelleted at 99,000 x g, resuspended in 0.1
m NaCl:0.01 m Tris (pH 7.4):0.001 m EDTA, and stored at
-70°.
SiSV-1 virus was prepared together with woolly monkey
leukemia virus in a manner similar to that for the NC-37 cell
line. The 3H-SiSV-1-cDNA probe used in this study was pro-
duced from Drs. D. Lowy and E. M. Scolnick (National Cancer
Institute, Bethesda, Md.). The propagation of the virus, synthesis
of [3H]cDNA, and its characterization have been described
elsewhere (64, 65).
Preparation of Radiolabeled Viral DNA Probes. EBV was
purified from the AG876 cell line according to a previously
described method (58). In brief, AG876 cells were cultured in
RPMI 1640 plus 10% FCS at 32°-33° and were labeled on the
eighth day of culture with [3H]thymidine (Amersham/Searle,
Arlington Heights, Ill.; 45 Ci/mmol) at a concentration of 5 ¿Ci/
ml. After 14 days of continuous culture, cellular debris was
pelleted by low-speed centrifugation, and the supernatant was
filtered through a 1-/im Millipore filter (RATF 14200; Millipore
Co., Bedford, Mass.), concentrate was pelleted by ultracentrifugation (113,000 x g
60%, w/v); and the radioactive peak was pelleted and dialyzed
against TE buffer. The purified virions were then lysed with
against TE buffer. The viral DNA peak was pooled, dialyzed against TE buffer, and
radioactive peak at a density of 1.720 g/ml was pooled,
in cesium chloride (129,000 x g for 60 hr at 20°). The single
amphototropic 1504 virus (1504A) used in this study was
grown in a human rhabdomyosarcoma (RD114) cell lines (47).
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percentage of hybridization of viral DNA was analyzed as a function of $C_0$,t (the product of the viral DNA concentration in the reaction mixture and the duration of the hybridization reaction, calculated as nucleic acid absorbance at 260 nm times the duration of incubation in hr divided by 2).

In hybridization reactions with the radiolabeled oncornaviral cDNA, sheared DNA (10 mg/ml) from tumor or tissue culture cells was mixed with $1 \times 10^{-3} \mu$g of the amphotropic 1504A MuLV cDNA probe or with $1 \times 10^{-3} \mu$g of the SISV-1 cDNA probe in 0.3-ml Reactivials. The mixtures were denatured in 0.14 M PB by heating to 100° for 4 min, adjusting to 0.48 M PB, and incubating at 65°. A minimum of 8 samples were removed from each reaction mixture during the subsequent 72 hr of incubation. Hybridization was again determined by hydroxyapatite chromatography, with unhybridized molecules being eluted from the column with 8 ml of 0.14 M PB plus 0.4% SDS at 60°. Hybridized molecules were eluted from the column at 99° with 8 ml of 0.14 M PB plus 0.4% SDS. The percentage of hybridization of the unlabeled cell DNA was measured in each sample by the spectrophotometric absorbance at 260 nm. Following this, 12 ml of Instagel were added to each sample, and the percentage of hybridization of the labeled viral DNA probe was measured by liquid scintillation counting.

RESULTS

Thirty-six cellular DNA’s from lymphoma patients were studied, 16 tumors (2 African, 14 American) and 20 cell lines (4 African, 16 American). In 11 cases, both the original lymphoma tissue and its derivative cell line were available for study. All 36 cellular DNA’s were studied with the radiolabeled EBV probe. Thirty-three of the 36 cell DNA’s were also studied with the amphotropic MuLV and SiSV-1 viral cDNA probes; insufficient quantities of 3 tumor DNA’s were available for testing with the amphotropic MuLV and SiSV-1 probes. In addition, cellular DNA’s from human placental tissue, from a human rhabdomyosarcoma tumor, and from RD114 cells infected with amphotropic MuLV served as controls.

Hybridization with $^3$H-EBV DNA. Eighteen of the 36 unlabeled DNA’s from tumor tissue culture cell lines contained detectable EBV DNA sequences. Three patterns of hybridization with $^3$H-AG876 EBV DNA were observed. The first was complete hybridization (i.e., >90%) and was demonstrated in 6 of the 18 DNA’s (Chart 1A). Four of these DNA’s were from EBV producer cell lines (P3HRi, B95-8, JLP, and AG876). A second pattern of hybridization, in which the radiolabeled EBV probe hybridized from 81 to 89%, was observed with 5 of 18 cell DNA’s (Chart 1B). Hybridization with EBV was incomplete (62 to 80%) with 7 cellular DNA’s (Chart 1C), one of these cell lines being a virus producer. The remaining 18 DNA’s showed no discernible hybridization with EBV [3H]DNA (Chart 1A).

Each of the 6 DNA’s of African origin (2 tumors, 4 cell lines) hybridized to between 89 and 94% with the $^3$H-AG876 EBV probe. Of the 14 lymphoma tumor samples of American origin (9 Burkitt’s, 3 undifferentiated, and 2 lymphoblastic lymphomas), 2 (both Burkitt’s) hybridized with $^3$H-EBV (one to 62% and the second to 86%). Of the 16 cell lines derived from American lymphoma tissue, 9 were EBV genome positive (62 to 93% hybridization) while 7 contained no detectable EBV sequences (Table 1).

The original tumor and its derivative cell line were available for hybridization with EBV in 11 cases. One of these tumors
Table 1

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<th>Cellular DNA</th>
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<td>Human placenta</td>
<td>Normal tissue</td>
<td>22</td>
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</table>

* CL, cell line; T, tumor, NA, cytogenetics not available; other, chromosome markers other than No. 8 or No. 14 present.

was of African origin (AG876), and the $^3$H-EBV DNA probe hybridized to $>90\%$ with the DNA from both the tumor tissue and its derived cell line (Table 1). The other 10 tumor cell line pairs were of American origin. With one pair (JL), the $^3$H-EBV DNA probe hybridized with both the tumor and its derived cell lines. The remaining tumors contained no detectable EBV sequences, and in 6 cases the derived cell line was similarly negative for EBV. In 3 cases, however, the original tumor was EBV negative, but the derived cell line contained detectable EBV sequences. In one of these cases (JA), the derived cell lines appeared to be diploid and probably represented the overgrowth of a normal EBV lymphoblastoid cell line. In the other 2 cases (DW and KK), however, the cell lines were aneuploid and had karyotypes consistent with that of the original tumor (including an 8;14 translocation for the DW and DW6) and hence were considered to be tumor derived. One of these, cell line KK124, was established from tumor ascites in a patient with American BL who had relapsed shortly after a serologically documented episode of infectious mononucleosis. It is notable that the tumor samples obtained from this patient at the time of initial diagnosis and after infectious mononucleosis were EBV genome negative. It is possible that some of the tumor cells (too few for detection by hybridization) obtained at the time of relapse had been converted in vivo to EBV genome positivity.

### Hybridization with C-Type Probes

Reaction of the $^3$H-amphotropic 1504A-MuLV cDNA probe with the unlabeled DNA extracted from the amphotropic virus-infected RD114 cells hybridized to 90% (Chart 1D). However, both the amphotropic and SiSV-1 cDNA probe failed to hybridize with any of the 33 tumor or derived cell line DNA's or with human placental DNA or rhabdomyosarcoma DNA. The kinetic patterns of these reactions were virtually identical for all tumor and cell line DNA's. Representative patterns are shown in Chart 1D for 6 such hybridization reactions. In each of these reactions, spectrophotometric analysis confirmed that the cellular DNA self-hybridization had progressed to completion.

### DISCUSSION

Serological and biochemical evidence for the association of EBV with African BL can be found in approximately 97% of the tumors and their derivative cell lines (17, 18, 28, 30, 55, 70). In contrast, a similar association with EBV is available in only 9 to 17% of the histologically indistinguishable American BL (3, 57, 69). Whether there are 2 types of BL (one associated with EBV and the other not) remains unresolved. In addition, whether additional factors contribute to or enhance the onco-
genic potential of EBV is not known. The known association of EBV with a nonmalignant disease (infectious mononucleosis) (29, 53) further confounds an understanding of the oncogenicity of EBV.

The evidence to date also suggests that there is no apparent difference in the linear integrated or high-molecular-weight circular episomal forms of EBV in African or American BL (1, 2, 4, 6, 35, 36, 39). Furthermore, consistent differences have also not been demonstrated in the circular DNA’s found in nontumorous EBV-infected lymphoblastoid cell lines in comparison with African BL. Whether different strains of EBV or different sites of integration are related to the oncogenic expression of this virus is also unknown but under study (23, 25, 27, 37, 48, 50, 58, 60).

In the present series, we were able to demonstrate detectable EBV in only 2 of the 11 (18%) American BL tumors examined. Two additional non-Burkitt’s American lymphoma tissues were examined and were found to lack EBV sequences. Ten American lymphoma tumors gave rise to lymphoid cell lines. In 6 cases, both the original tumor and derived cell line were negative, while in one case the tumor and cell line were positive. However, EBNA staining showed that only a small fraction of this cell line, when first derived, contained EBV. This rapidly increased to 100%. The remaining 3 original tumors were EBV negative, while the derived cell line was positive. All of these cell lines have an aneuploid karyotype consistent with the original and 2 had 8;14 translocation, which suggests that they are tumor-derived cell lines. In one of these (DW), the conversion to EBV positivity occurred in vitro and is unexplained. In the case of KK, the original tumor was EBV negative. However, several months following the initial diagnosis, the patient developed serologically documented infectious mononucleosis and then relapsed. A tumor cell line established from the ascitic fluid at the time of relapse was EBV positive. These findings suggest that, while EBV was not detected in the original tumor, at least some of the tumor cells could have been infected by EBV. While these observations seem to further minimize the role of EBV in American BL, our data do not permit us to exclude the possibility that small portions or fragments of the EBV genome were present (but currently undetectable) in the apparently genome negative DNA’s. In this regard, the partial hybridization of the EBV probe with some of the cell DNA’s is of interest and may reflect that only a portion of the EBV genome is present (59).

It is notable that the human rhabdomyosarcoma tumor selected as a control contained detectable EBV sequences. While the most likely explanation is that this was due to infiltration of the sarcomatous tissue by EBV-positive lymphocytes (31), it remains possible that the rhabdomyoblasts contained the viral sequences. Since it is known that the epithelial cells found in nasopharyngeal carcinoma can contain EBV, a precedent for nonlymphoid EBV infection is available.

We were surprised by the absence of any detectable homology with the 2 oncornavirus probes used in this study. Since previous workers have described oncornavirus-like particles in at least 2 of the cell lines that we examined (RAJI and P3HR1), our negative findings are in direct conflict. Several possibilities might explain the lack of detectable proviral DNA sequences in this study. First, the probe(s) that we selected might have simply missed or failed to detect resident oncornaviral sequences. While this cannot be excluded, we chose the SiSV-1 probe because of its previous association with human leukemia, although proviral sequences have been generally less detectable. The murine amphotropic viral probe was selected since it shares 80% homology with the Rauscher and Moloney leukemia viruses which have also been detected in human lymphoid tumors. Consequently, we hypothesized that the amphotropic cDNA would permit an initial screening which could be more specifically explored with selected oncornaviruses. The lack of proviral sequences in this study does not exclude the possibility that 7OS RNA or RNA-dependent DNA polymerase might be present, nor can we exclude the possibility that a C-type virus had previously entered the “tumor” cell, contributed to its transformation, and was then excised and rendered undetectable (20–22). Our hybridization technique would also fail to detect the presence of small fragments of oncornavirus.

It is of additional interest that the tumor-derived cell lines also failed to reveal the presence of proviral sequences. It is well established that prolonged tissue culture may permit the expression of virus activity not demonstrated in the original tumor (33, 34, 68). On the other hand, while tissue culture may enhance type C virus replication, it may also permit the laboratory contamination of cell lines by oncornaviruses. Finally, it should be noted that, in addition to reports finding oncornaviral material in human leukemias and lymphomas, negative studies have also been reported (9, 38, 52).

Thus, the majority of the American lymphomas that we examined lack both EBV and type C viral sequences. The lack of association of these tumors with EBV and the observation that some of the negative tumor cells could be infected with EBV suggest that it is unlikely that this virus is a causative factor in American BL. Furthermore, it might be concluded either that the histologically identical EBV-positive African BL is truly a separate disease in which EBV plays an important etiological role or that EBV is simply resident in a cell which undergoes transformation by some other process. The contribution of oncornaviruses in this transformation process is not substantiated by this study.

REFERENCES


EBV and C-Type Proviral Sequences in Lymphomas


Examination of Epstein-Barr Virus and C-Type Proviral Sequences in American and African Lymphomas and Derivative Cell Lines


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