Epstein-Barr Virus Infection Precedes Clonal Expansion in Burkitt’s and Acquired Immunodeficiency Syndrome-Associated Lymphoma

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The Epstein-Barr virus (EBV) is associated with distinct forms of human lymphoid malignancies. EBV sequences are found in malignant cells in virtually 100% of cases of endemic Burkitt’s lymphoma (eBL) and in 10% to 20% of cases of sporadic BL (sBL).1 EBV is also associated with B-cell lymphoma developing in the context of a variety of congenital and acquired immunodeficiency states,2,3 including undifferentiated B-cell lymphoma associated with the acquired immunodeficiency syndrome (AIDS) where EBV sequences have been found in 40% of the cases.4 The EBV genome has been also detected in biopsies of some cases of central nervous system lymphoma,5 lymphoma in patients with ataxia telangiectasia,6 immunoblastic lymphoproliferative disorder,7 and Hodgkin disease.8

Despite these associations, the role of EBV in B-cell lymphomagenesis remains controversial. In support of a pathogenetic role are the epidemiologic observations, which are particularly convincing in the case of eBL, and the ability of the virus to efficiently immortalize human B lymphoblasts in vitro.9 On the other hand, the inconsistent presence of EBV in malignancies such as sBL and AIDS-associated non-Hodgkin lymphoma (AIDS-NHL) and the distinct B-cell tropism of EBV could also be interpreted as indicating a noncausative role in at least some types of malignancy.

To assess the possible etiologic contribution of EBV to malignant transformation we sought to determine whether EBV infection has preceded and, thus, possibly contributed to clonal expansion, or whether infection has occurred after clonal expansion and thus is unlikely to contribute to pathogenesis. Toward this end we analyzed the structure of the heterogeneous genomic termini of EBV as markers of clonal infection.10,11 The termini of the linear intermediate form of EBV DNA contain a variable number of direct tandem repeats (about 500 bp in length), which are joined intracellularly to form covalently closed episomal DNA on viral infection of host cells.12 Because of this termini heterogeneity, independently amplified EBV termini, ie, independent infection events, can be identified as differently sized fragments on Southern blot analysis. Therefore, in most situations characterized by a low multiplicity of infection, a single form of fused EBV termini will be detectable in the clonally expanded progeny of a single infected cell. Conversely, heterogeneous EBV termini will be detected in the case of infection of either an already-expanded clonal population or a polyclonal population.12 This assay has been used to determine that EBV-associated nasopharyngeal carcinomas are monoclonal,13 whereas both clonal and polyclonal forms of EBV have been found in a heterogeneous collection of EBV-associated lymphoproliferative diseases.14

Using this assay, we show that clonal forms of EBV termini are uniformly detectable in sBL, eBL, and AIDS-NHL, strongly suggesting that EBV infection has preceded and, thus, possibly contributed to clonal expansion in these malignancies.

MATERIALS AND METHODS

Pathologic samples and diagnostic criteria. Representative samples of lymph nodes and/or involved bone marrow were collected during the course of standard diagnostic procedures and stored at −80°C as intact tissue blocks (eBL) or mononuclear cell suspensions (sBL and AIDS-NHL). All the eBL samples analyzed in this study originated from Ghana and were obtained through the National Cancer Institute’s Burkitt Tumor Project. sBL samples originated from North America and Europe. AIDS-NHL samples were collected at the New York University Medical Center. Histopathologic subclassification was performed according to the working formulation of NHL. The diagnosis was further confirmed by immunophenotypic analysis of expression of surface Ig (sIg), sheep erythrocyte rosettes, terminal deoxynucleotidyl transferase, and the B-cell–associated antigens HLA-DR, CALLA, B1, and B2. All of these samples were previously analyzed (see Table 1) for Ig heavy- and light-chain gene rearrangements, for rearrangements of the c-myc locus, for the presence of EBV DNA sequences

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and expression of EBV proteins by immunofluorescence analysis as previously described.

**DNA extraction and Southern blot analysis.** DNA was prepared by cell lysis, digestion with proteinase-K, extraction with phenol/chloroform, and precipitation with ethanol. For Southern blot analysis, 10 μg of DNA was digested with the appropriate restriction endonuclease, electrophoresed in 0.7% agarose, denatured, neutralized, and transferred to nitrocellulose membranes. Hybridization to the EBV probe (see below) was performed in 3X standard saline citrate (SSC), 50% formamide at 37°C for 16 hours. Filters were then washed in 0.2X SSC/0.5% sodium dodecyl sulfate for 2 hours at 60°C and then autoradiographed using intensifying screens (Quanta II; Dupont, Wilmington, DE).

**DNA probes.** The DNA probe specific for the EBV genomic termini is the 5.2-kb *BamHI-EcoRI* isolated from the fused *BamHI* terminal fragment NJ-het.8 For use as probes, DNA fragments were labeled (specific activity 1 to 2 X 10^9/μg) with ^32P by the random priming method.15

**RESULTS**

We have analyzed 26 EBV-positive tumor biopsies, including 11 cBL, 9 sBL, and 11 AIDS-NHL, which were previously classified as positive for EBV sequences and EBV protein expression (see Materials and Methods and Table 1). As summarized in Table 1, five AIDS-NHL cases were representative of both Burkitt-type and non-Burkitt-type NHL. All of these tumor biopsies were previously analyzed for Ig heavy-chain gene rearrangements and shown to display a predominant clonal pattern.18 Rare cases of AIDS-NHL displaying polymorphic EBV forms18 may also correspond to those cases displaying multiple B-cell clonal expansions accompanying the malignant clone.19 Taken together, these data indicate that EBV infection precedes and may have directly contributed to clonal expansion in most cases of EBV-associated lymphoproliferative disorders. The absence of EBV sequences in a significant fraction of sBL and AIDS-NHL cases suggests, however, that even in these malignancies EBV may not be strictly necessary for clonal expansion and that alternative pathogenetic mechanisms may be involved.

**DISCUSSION**

In the present study we have attempted to determine whether EBV enters the malignant cell in BL and AIDS-NHL before or after clonal expansion. Our results are consistent with a monomorphic pattern of episomal EBV DNA in all of the tumor biopsies analyzed. Because all of the biopsies studied contained a prominent monoclonal B-cell population uniformly expressing EBV proteins, the detection of a clonal form of EBV DNA indicates that this clonal expansion derived from a progenitor B cell that was already infected by EBV. The presence of clonal circular EBV plasmids and the apparent absence of linear EBV genomes in all of the samples tested also confirm the latent as opposed to lytic status of the virus in these malignancies.

**Table 1. Summary of Relevant Genotypic Features of the Pathologic Samples Studied**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>IgH Gene Clonality*</th>
<th>c-myc Oncogene Activation†</th>
<th>EBV Sequences‡</th>
<th>EBV Terminus Clonality</th>
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<td>sBL</td>
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<tr>
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<td>5/5</td>
</tr>
<tr>
<td>Non-Burkitt-type</td>
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<td>3/5</td>
<td>5/5</td>
<td>5/5</td>
</tr>
</tbody>
</table>

*Positive for clonal IgH gene rearrangements.6,16,19†c-myc gene rearrangements (1 eBL; 7/8 sBL; 8/9 AIDS-NHL) or mutations within first exon sequences (10 eBL, 2/9 sBL, and 1 AIDS-NHL) consistent with the presence of chromosomal translocations (see references 21 and 24).‡Positive for EBV DNA sequences (see Materials and Methods).§Two cases diagnosed as large-cell, immunoblastic-plasmacytoid cell lymphoma and three cases as large-noncleaved B-cell lymphoma.
Fig 1. Analysis of EBV termini heterogeneity in BL and AIDS-NHL. DNAs were digested with BamHI restriction endonuclease and subjected to Southern blot hybridization analysis using a DNA probe specific for the fused termini of the EBV genome. Normal human placenta DNA is used as a negative control (lane a). A lymphoblastoid cell line derived by EBV infection of normal polyclonal B cells (lane b) and the EBV-positive clonal B-lymphoma line Daudi (lane c) were used as controls for polymorphic and monomorphic EBV termini, respectively. Representative results for eBL (lanes 1 through 4) and AIDS-NHL (lanes 5 through 8) biopsies are shown.

gene activation, which is detectable in virtually all cases of BL and in at least 70% of AIDS-NHL. This hypothesis is supported by the detection of multiple clonal B-cell expansions in the lymph nodes and peripheral blood of patients with AIDS or AIDS-related complex, which could represent targets for c-myc activation. In vitro studies have also shown that the introduction of activated c-myc oncogenes into EBV-infected B lymphoblasts from AIDS patients causes their tumorigenic conversion. Our results are consistent with this model by showing that EBV is in fact present before clonal expansion. However, these results are also consistent with the model, suggesting that EBV infection and the subsequent clonal expansion may occur in a cell that already carries an activated c-myc oncogene.

REFERENCES

forms of Epstein-Barr virus DNA in lymphomas and lymphoproliferative diseases. J Infect Dis 160:589, 1989


Epstein-Barr virus infection precedes clonal expansion in Burkitt's and acquired immunodeficiency syndrome-associated lymphoma [see comments]

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