Epstein-Barr Virus in Benign Lymph Node Biopsies From Individuals Infected With the Human Immunodeficiency Virus Is Associated With Concurrent or Subsequent Development of Non-Hodgkin’s Lymphoma

By Darryl Shibata, Lawrence M. Weiss, Bharat N. Nathwani, Russell K. Brynes, and Alexandra M. Levine

Most adults have been infected by Epstein-Barr virus (EBV). The initial infection may be asymptomatic or may produce the clinical syndrome of infectious mononucleosis. During the acute infection, large numbers of EBV-infected lymphocytes are present in the peripheral blood (PB) and lymph nodes. Subsequently, the number of infected lymphocytes decreases as the host mounts an immune response. EBV then remains latent in B lymphocytes or nasopharyngeal epithelium, with periodic shedding of virus into saliva.

The mechanisms of EBV latency are complex and host dependent. In immunocompromised patients, EBV-related lymphoproliferative disorders have been well described. Furthermore, patients immunocompromised owing to infection by human immunodeficiency virus (HIV) have an increase in circulating EBV-infected lymphocytes and have also been shown to be at increased risk of developing high-grade B-cell lymphoma. Approximately one half of these lymphomas have been shown to contain EBV DNA sequences. The AIDS-related lymphomas behave aggressively and respond poorly to chemotherapy.

HIV-infected patients are also at increased risk for developing reactive lymphadenopathy, designated as persistent generalized lymphadenopathy (PGL), which is considered an AIDS-related condition (ARC). The histology of these enlarged PGL lymph nodes consists of a benign but florid follicular and interfollicular B-cell hyperplasia or follicular involution. The presence of monoclonal or oligoclonal immunoglobulin gene rearrangements in approximately 20% of these reactive lymph nodes suggests that this lymphoid hyperplasia may be a risk factor or prodrome for subsequent development of lymphoma, but because most HIV-infected individuals with PGL do not progress to lymphoma, other cofactors may be necessary. Because EBV may be associated with HIV-related lymphoma, the current study was performed to investigate the presence of EBV in PGL lymph node biopsies and to determine the possible relationship between EBV-related lymphoproliferations and the risk of malignant lymphoma.
SL1-3 detected the EBNA 1 gene from the Raji, Iijoye, EB1, EB2, EB3, and Daudi cell lines (American Type Culture Collection, Rockville, MD) and also detected EBV from more than 90% of all fixed biopsies of nasopharyngeal carcinoma (data not shown). The reaction volumes were 50 μL, and the primers were used at 40 μmol/L. Temperatures during the 42 amplification cycles were 95°C for 45 seconds, 50°C for 45 seconds, and 72°C for 60 seconds. As a positive amplification control, primers for a genomic sequence (low-density lipoprotein receptor) were also simultaneously present during the amplification. All EBV- and EBV+ samples with the exception of the B-5 fixed biopsies demonstrated amplification of this normal genomic sequence. Fixation with B-5 greatly reduces PCR amplification (D. Shibata, unpublished observations), and only EBV was detected from the B-5-fixed tissues. The remainder of the PGL biopsies and all biopsies from the non-HIV-infected patients were fixed in formalin, and dilution studies demonstrated that most of the DNA was suitable for amplification (described below). Positive controls consisting of Raji DNA and negative controls consisting of human genomic DNA as well as an assay with no added sample were performed with each experiment.

The sensitivity of the EBV EBNA PCR reaction on fixed tissue was determined by amplifying formalin-fixed, paraffin-embedded mixtures of Raji cells (50 EBV copies per cell) and a non-EBV-infected T-cell line (Molt 3). Deparaffinized slices containing approximately 50,000 cells were boiled and directly amplified as above. The assay can detect at least 0.01% infected cells or an average of 0.005 EBV copies per cell (data not shown).

Amplifications were performed in a blinded fashion without knowledge of the HIV or lymphoma status. The EBNA PCR assay was performed at least twice for each biopsy. In some cases, a sample was positive on one run but negative on the second. For these samples, five PCR assays were performed. A sample was considered positive for EBV if most of the amplifications were positive. If two of the five amplifications were positive, the sample was considered weakly positive (+), as indicated in Table 1. EBV DNA was not detected from any of the negative biopsies except for one specimen (case 15), which was considered negative because only one of the five assays was positive. The relative amounts of EBV DNA were further characterized by subjecting serial dilutions of the DNA extracted from the formalin-fixed specimens to 50 PCR cycles.

The EBNA+ formalin-fixed specimens were amplified with a second set of primers that immediately flank the portion of the EBV lymphocyte-determined membrane antigen (LYDMA) gene composed of variable numbers of tandem 33 bp repeats. The primers (SL18, GGCGCACCTGGAGGTGGTCC, and SL19, TT-TCCAGCAGTGCTGCTAGG) were used at 2.5 μmol/L with 40 amplification cycles at 95°C for 45 seconds, 50°C for 30 seconds, and 72°C for 2 minutes with a final extension for 10 minutes at 72°C. The PCR products were Southern-blotted after electrophoresis through a 3% NuSieve (FMC, Rockland, ME), 0.5% agarose gel, using a 32P-labeled probe (SL20, TGACAATGCCCCACAGGACCCTG) homologous to the tandem repeats.

In situ DNA hybridization was performed using an 32P-labeled BamHI-W fragment of the EBV genome. Negative controls consisting of normal lymph nodes from individuals not in this study were also analyzed.

HIV infection was determined by HIV enzyme-linked immunosorbent assay (ELISA) testing (Abbott Laboratories, Chicago, IL) and by PCR analysis of the lymph node biopsies. The HIV gag gene was amplified using the primers SK38 and SK39. The HIV gag gene was amplified using the primers SK38 and SK39. The HIV PCR results for seven of the non-HIV-infected and 16 of the HIV-infected patients were reported previously.

Clinical follow-up, subsequent to the time of initial PGL biopsy, was obtained by review of the medical records. Statistical analysis was performed with the chi-square test.

Table 1. Summary of Results of PGL and Lymphoma Biopsies

<table>
<thead>
<tr>
<th>Benign Lymph Node Biopsy</th>
<th>Lymphoma Biopsy</th>
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<tbody>
<tr>
<td>Case Type</td>
<td>HIV PCR</td>
</tr>
<tr>
<td>1</td>
<td>FH</td>
</tr>
<tr>
<td>2</td>
<td>FH</td>
</tr>
<tr>
<td>3</td>
<td>FI†</td>
</tr>
<tr>
<td>4</td>
<td>FH</td>
</tr>
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<td>5</td>
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</tr>
<tr>
<td>33-34</td>
<td>FI</td>
</tr>
<tr>
<td>35</td>
<td>DM</td>
</tr>
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Negative (-); positive (+): weak amplification (1+)(described in the Results section and legend to Fig 2); amplification to the 10-fold dilution (2+); amplification to the 100-fold dilution (3+); amplification to the 1,000-fold dilution (4+); and amplification to the 10,000-fold dilution (5+).

Abbreviations: PGL, persistent generalized lymphadenopathy; EBV, Epstein-Barr virus; HIV, human immunodeficiency virus; FH, follicular hyperplasia; FI, follicular involution; DM, dermatopathic lymphadenitis; SNC, small non-cleaved lymphoma; IBL, immunoblastic lymphoma; NA, not applicable.

*Lymphoma cells were obtained by fine-needle aspiration; insufficient material was available for EBV studies.

†BS-Fixed tissue; amplification of genomic sequences was not possible (described in the Results section).
RESULTS

The EBV EBNA PCR was performed on histologically benign lymph node biopsies from 16 non–HIV-infected patients and 35 HIV-infected patients. The results are shown in Fig 1 and Tables 1 and 2. EBV could be detected in biopsies from 13 of the 35 HIV-infected patients and from none of those from the 16 non–HIV-infected patients ($P < .01$). The lymph node biopsies from the non–HIV-infected patients were classified either as normal (n = 1) or as reactive with follicular hyperplasia (n = 9) or dermatopathic lymphadenitis (n = 6).

The EBNA PCR+ formalin-fixed specimens were also amplified for the portion of the EBV LYDMA gene composed of variable numbers of tandem 33 bp repeats. The size of this heterogeneous region is characteristic for a given EBV isolate and can vary between EBV isolates. Each EBNA PCR+ formalin-fixed specimen was also LYDMA PCR+, and the LYDMA PCR products were variable in size (Fig 2). An exception was the lymphoma of case 1, which was EBNA PCR+ but LYDMA PCR−. In situ hybridization (described below) confirmed the presence of EBV DNA in this lymphoma. Under the conditions used, a single major PCR product band was produced from each EBV-infected cell line, although multiple minor bands could be detected after prolonged exposure. Mixtures of the EBV-infected cell lines produced multiple predominant bands. A single predominant LYDMA PCR band was produced from the specimens in this study except for cases 12 and 14, which produced multiple predominant bands, indicating the presence of at least two EBV species. In case 5, the only instance in which formalin-fixed material was available for both the lymphoma and the PGL biopsy, a single identical LYDMA band was produced.

Of the 13 HIV-infected patients with EBV PCR+ PGL biopsies, 3 had lymphoma diagnosed by biopsy at another site (concurrent lymphoma) and 2 subsequently developed lymphoma. In contrast, only 1 of 22 HIV-infected patients (median follow-up 12 months) with EBV− PGL biopsies developed lymphoma (Table 3, $P < .05$).

<table>
<thead>
<tr>
<th>HIV Status</th>
<th>EBV PCR+ (%)</th>
</tr>
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<tbody>
<tr>
<td>+</td>
<td>13</td>
</tr>
<tr>
<td>−</td>
<td>0</td>
</tr>
<tr>
<td>pos/neg</td>
<td>16</td>
</tr>
</tbody>
</table>

Abbreviations as in Table 1.

Table 2. EBV in Benign Lymph Node Biopsies From HIV-Infected and Non–HIV-Infected Patients

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Table 3. EBV as a Risk Factor for Lymphoma in HIV-Infected Patients

<table>
<thead>
<tr>
<th>EBV PCR in PGL Biopsy</th>
<th>Lymphoma*</th>
</tr>
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<tr>
<td>Present</td>
<td>5</td>
</tr>
<tr>
<td>Absent</td>
<td>21</td>
</tr>
</tbody>
</table>

*Patients with concurrent lymphoma or patients subsequently developed lymphoma.

Abbreviations as in Table 1.

To identify the cells infected with EBV, in situ DNA hybridization was performed (Fig 4). EBV DNA was identified in the lymphoma cells of all five cases examined. The diagnosis of lymphoma was made by fine-needle aspiration in case 2, and lymphoma tissue was not available for further analysis. Cells containing EBV DNA could also be identified in most of the EBV PCR* PGL biopsies, although the PCR was more sensitive. The in situ DNA hybridization technique, however, could analyze the B-5-fixed tissues unsuitable for the PCR. In PGL cases 3 and 4, small clusters of large atypical cells present in interfollicular and pericapsular adipose tissue were positive. In the remaining positive PGL cases, scattered large to small cells constituting less than 1% of the cells were positive in focal follicular as well as interfollicular areas. Presence of EBV DNA was limited to the cells in several germinal centers alone in cases 7 and 11.

HIV DNA was detected from 34 of the 35 benign lymph nodes of the HIV-infected patients (Table 1). Unlike some of the EBV amplifications, the HIV PCR assays were consistently positive. EBV DNA was detected from the one PGL biopsy. HIV sequences were not detected from any of the 16 lymph node biopsies from uninfected patients.

DISCUSSION

After an acute EBV infection, a life-long persistent infection is established, reflecting a balance between viral replication and host immunity.2 EBV can be cultured from the pharynx of most asymptomatic seropositive individuals.28 EBV can also be cultured from a very small proportion (< 1 in 1,000,000) of PB mononuclear cells.1 Direct detection of EBV-infected cells by nucleic acid hybridization techniques during latent infection has not been possible, however.29 Therefore, we used the PCR18,19 to enhance the sensitivity of EBV detection. Despite this increased sensitivity (at least an average of 0.005 EBV genomes per cell), EBV could not be detected in the lymph node biopsies from 16 normal individuals. Similarly, EBV has not been detected in benign lymph node biopsies from normal individuals by other investigators using the PCR.20,31 These results, analogous to the values obtained by culture assays for EBV, indicate that the numbers of EBV-infected lymph node cells is normally extremely small in latent infection.

In contrast, individuals infected with HIV may be immunocompromised, with resultant increased numbers of EBV-infected cells. Serologic data have demonstrated that more than 90% of HIV-infected individuals are also EBV infected and that reactivation of the EBV infection may be a common occurrence:32,33 eg, EBV can be cultured from a higher but still small proportion (~ 1 in 100,000) of PB B cells.3 Supersensitivity in the numbers of EBV-infected peripheral blood mononuclear cells, detection of EBV sequences within PGL tissues has been extremely difficult.34,36 In the current study, by using the PCR technique, EBV DNA was successfully detected in 37% of benign PGL biopsies.

The EBV-infected cells present in the PGL biopsies were identified using very sensitive in situ DNA hybridization techniques. Only a small proportion (<1%) of small and large lymphoid cells located in focal follicular and interfollicular regions were positive. In cases 7 and 11, infected cells were identified predominantly within isolated germinal centers. The patterns of EBV infection in the PGL lymph node biopsies are markedly different from the patterns present in infectious mononucleosis, in which greater numbers of EBV-infected cells are located almost exclusively in interfollicular areas and are predominantly immunoblasts.26 EBV-infected cells are otherwise not detectable in reactive lymph node biopsies from normal individuals.

Because approximately half of all HIV-related lymphomas have been shown to contain EBV sequences,9-10 the presence of detectable EBV-infected cells in the current PGL biopsies was studied for possible correlations with the presence of lymphoma. EBV positivity was significantly associated with the concurrent presence of EBV* non-Hodgkin's lymphoma at another site or subsequent development of EBV* lymphoma. These findings demonstrate that
existence of increased numbers of EBV-infected cells commonly precedes or is associated with development of lymphoma in HIV-infected individuals and supports the hypothesis that EBV may play a significant role in its genesis. None of the 37 HIV-infected individuals with PGL developed lymphomas negative for EBV. HIV-related lymphomas appear to be a heterogeneous group, and patients with PGL and detectable EBV sequences apparently have a greater risk for EBV⁺ but not EBV⁻ lymphoma, although the small numbers of patients in this study precludes definitive analysis.

This association between EBV in the PGL biopsies and EBV⁺ lymphoma is not unexpected. As hypothesized by other investigators,⁵,⁶,¹⁰,³³,³⁵ EBV-related lymphoproliferations that occur secondary to immunodeficiency may allow development of genetic aberrations such as the chromosomal translocation 8;14, activation of c-myc oncogene, and eventual termination in lymphoma. This study provides definitive evidence that the hypothesized EBV-related lymphoproliferations do occur in benign PGL lymph node biopsies. Amplification of the polymorphic LYDMA gene sequence can provide some information about the clonal nature of the EBV-related lymphoproliferation. Although polyclonal and clonal proliferation cannot be distinguished if an individual is infected with EBV species homogeneous for the polymorphic region, multiple bands were detected in two individuals in this study, indicating that these individuals were infected with at least two different EBV species. Unless the same cell was infected by the different EBV species, these EBV-related lymphoproliferations were probably oligoclonal or polyclonal.

Increased numbers of EBV-infected cells, however, are neither necessary nor sufficient for development of lymphoma. In case 1, EBV was not detected in the PGL biopsy performed 1 month before development of an EBV⁺ lymphoma. In this case, EBV-related lymphoproliferation may have been extremely focal and not present in the lymph node biopsied. In other currently reported PGL cases, EBV lymphoproliferations were not associated with lymphoma; whether the EBV reactivation represented a transient phenomenon in these patients or whether lymphoma will eventually develop during longer follow-up is unknown.
HIV-related lymphomas are characterized by the presence of widely disseminated disease. Therefore, in some instances, detection of EBV in the morphologically benign PGL biopsies may represent occult involvement with EBV-containing lymphoma cells. This may have occurred in cases 3 and 4 because the small number of EBV-infected cells identified by in situ hybridization appeared to have the abnormal morphology of lymphoma cells. Similar examples of histologically occult lymphoma detected by molecular techniques have been well described previously.6,7,40

Although the number of HIV-infected cells in PGL lymph nodes is small,17,39,40 HIV provirus, in contrast to EBV, was detected in virtually all benign lymph node biopsies from HIV-seropositive individuals. This difference may be related to the progressive nature of HIV infection as compared with the stable latent infection typically established by EBV. HIV can stimulate B cells to proliferate11 and may promote follicular hyperplasia of the EBV+ benign biopsies. Certain growth factors produced by HIV-infected monocytes and T lymphocytes, such as interleukin-6 (IL-6),9,15 may be operative in this regard.

In summary, the detectable presence of EBV DNA in benign PGL biopsies was a significant risk factor for EBV DNA+ lymphoma and suggests the need for close surveillance of these individuals. The presence of EBV in the PGL lymph node biopsies from AIDS patients with lymphoma may reflect widely disseminated occult lymphoma or increased immunosuppression leading to EBV reactivation. The exact biologic mechanisms responsible for the association between EBV reactivation and B-cell lymphoma are currently unknown.

ACKNOWLEDGMENT

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REFERENCES


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