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

Individuals infected with the human immunodeficiency virus (HIV) have an increased incidence of high-grade B-cell lymphoma. In many instances, these lymphomas contain Epstein-Barr viral (EBV) genomes. To investigate the role of EBV in development of HIV-related lymphoma, benign fixed lymph node biopsies from normal individuals and HIV-infected individuals with persistent generalized lymphadenopathy (PGL) were analyzed for EBV sequences by polymerase chain reaction and in situ DNA hybridization techniques. EBV DNA was not detected in any of 16 benign lymph node biopsies from normal individuals, but could be detected from 13 of 35 PGL biopsies. The EBV-infected cells were present in both follicular and interfollicular areas and in both small and large lymphoid cells. The presence of detectable amounts of EBV DNA in the 13 PGL biopsies was associated with an increased incidence of concurrent lymphoma at another site (n = 3) or development of lymphoma in time (n = 2). In contrast, only 1 of 22 individuals with EBV-negative PGL biopsies developed lymphoma in time (P < .05). EBV was detected in all five lymphomas in which tissue was available for subsequent analysis, including the lymphoma that developed in the individual without EBV in his previous PGL biopsy. These findings support the hypothesis that EBV plays a role in development of some HIV-related lymphomas. Detectable EBV lymphoproliferations occur in a few PGL biopsies and are associated with a significant risk of EBV DNA-positive non-Hodgkin’s lymphoma.

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MATERIALS AND METHODS

The slides and paraffin-embedded benign lymph node biopsies from 35 HIV-infected and 16 non–HIV-infected patients from the Los Angeles County-University of Southern California (LAC-USC) Medical Center were analyzed. None of the 35 HIV-infected patients had histories of opportunistic infection or Kaposi’s sarcoma, but three HIV-infected patients subsequently developed lymphoma and three HIV-infected patients had lymphoma (“concurrent lymphoma”) at another site at the time the initial benign lymph node biopsy was performed. Morphologic lymphoma classification, performed by three of the authors (D.S., R.K.B., B.N.N.), was based on the Working Formulation. The benign lymph node biopsies were classified either as normal or as reactive with follicular hyperplasia, follicular involution, or dermatopathic lymphadenitis.

DNA was extracted from single 10-μm slices of the fixed tissues into a 50-μL extraction solution. Approximately 0.5 to 2.0 μL extraction solution was used for each reaction.

The polymerase chain reaction (PCR) was performed with primers (SL1 5’ GGACCTCAAAGAAGAGGGGG and SL3 GC-TCTGGTGTCCTCGGC) and a probe (SL2 GGACGAGGACGCGGAGAGG) specific for the EBNA 1 gene of EBV. The primers amplify and detect an 80-base pair (bp) region of the EBNA 1 gene (starting at position 8037′). As expected, the EBNA 1 gene (position 8037′) was detected in all five EBV-positive PGL biopsies. These EBV-positive PGL lymphomas were associated with a significant risk of EBV DNA-positive non-Hodgkin’s lymphoma.
SL1-3 detected the EBNA 1 gene from the Raji, Ijioye, 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 (1+), 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.25–26 The primers (SL18, GGGCAGCCCTGGAGGTTGTCC; and SL19, TTCCAGCAGATGCTAGT) 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) agarose gel, using a 32P-labeled probe (SL20, TGACAAATGCCCCACAG-GACCCT) homologous to the tandem repeats.

In situ DNA hybridization was performed using an 32P-labeled BamHI-W fragment of the EBV genome.2 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 SK 38 and SK 39.27 The HIV gag gene was amplified using the primers SK 38 and SK 39.27 The HIV PCR results for seven of the non–HIV-infected and 16 of the HIV-infected patients were reported previously.17 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>Case</th>
<th>Type</th>
<th>HIV PCR</th>
<th>PCR</th>
<th>EBV</th>
<th>Type</th>
<th>Months From</th>
<th>PCR</th>
<th>EBV</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PGL BX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>FH</td>
<td>+</td>
<td>–</td>
<td>Negative</td>
<td>SNC</td>
<td>1</td>
<td>5+</td>
<td>SNC cells +</td>
</tr>
<tr>
<td>2</td>
<td>FH</td>
<td>+</td>
<td>1+</td>
<td>Rare +</td>
<td>SNC</td>
<td>22</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td>3</td>
<td>Fl+</td>
<td>–</td>
<td>1+</td>
<td>Atypical cells +</td>
<td>IBL</td>
<td>5</td>
<td>1+</td>
<td>IBL cells +</td>
</tr>
<tr>
<td>4</td>
<td>FH</td>
<td>+</td>
<td>3+</td>
<td>Atypical cells +</td>
<td>IBL</td>
<td>0</td>
<td>1+</td>
<td>IBL cells +</td>
</tr>
<tr>
<td>5</td>
<td>FH</td>
<td>+</td>
<td>4+</td>
<td>ND</td>
<td>SNC</td>
<td>0</td>
<td>5+</td>
<td>SNC cells +</td>
</tr>
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<td>+</td>
<td>3+</td>
<td>Rare +</td>
<td>SNCT</td>
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<td>–</td>
<td>SNC cells +</td>
</tr>
<tr>
<td>7</td>
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<td>NA</td>
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<td>NA</td>
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<td>Rare +</td>
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<td>NA</td>
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<td>NA</td>
</tr>
<tr>
<td>11</td>
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<td>2+</td>
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<td>NA</td>
</tr>
<tr>
<td>13</td>
<td>Fl</td>
<td>+</td>
<td>2+</td>
<td>Rare +</td>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>14</td>
<td>Fl</td>
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<td>NA</td>
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<td>NA</td>
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<td>NA</td>
</tr>
<tr>
<td>18-32</td>
<td>FH</td>
<td>+</td>
<td>–</td>
<td>Not done</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>33-34</td>
<td>Fl</td>
<td>+</td>
<td>–</td>
<td>Not done</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>35</td>
<td>DM</td>
<td>+</td>
<td>–</td>
<td>Not done</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

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; Fl, 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.

1B5-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, $P < .05$).

To characterize further the relative amounts of EBV DNA present, we amplified serial dilutions of 14 EBV PCR$^+$ formalin-fixed samples (Table 1 and Fig 3). The EBV PCR$^+$ formalin-fixed lymphomas and the Raji cell line could be diluted at least 10,000-fold, with persistence of PCR positivity for both EBV and the genomic sequence. In contrast, the benign biopsies became negative for EBV at lesser dilutions.

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

<table>
<thead>
<tr>
<th>HIV Status</th>
<th>EBV PCR</th>
<th>EBV$^+$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>13</td>
<td>22</td>
</tr>
<tr>
<td>−</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>pos/neg</td>
<td>(P &lt; .01)</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations as in Table 1.
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>
</thead>
<tbody>
<tr>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>+</td>
<td>5</td>
</tr>
<tr>
<td>-</td>
<td>1</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 HIV PCR 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 PCR 18,19 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. 7 Despite this increase in the numbers of EBV-infected peripheral blood mononuclear cells, detection of EBV sequences within PGL tissues has been extremely difficult. 34,35 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. 23,36 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, 6,10 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,5,6,10,31,35 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.37,38

Although the number of HIV-infected cells in PGL lymph node 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 proliferate41 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),32,43 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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