Epstein-Barr Virus Latent Membrane Protein-1 Oncogene Deletions: Correlations With Malignancy in Epstein-Barr Virus–Associated Lymphoproliferative Disorders and Malignant Lymphomas

By D.W. Kingma, W.B. Weiss, E.S. Jaffe, S. Kumar, K. Frekko, and M. Raffeld

LMP-1, an Epstein-Barr viral (EBV) latency protein, is considered a viral oncogene because of its ability to transform rodent fibroblasts in vivo and render them tumorigenic in nude mice. In human B cells, EBV LMP-1 induces DNA synthesis and abrogates apoptosis. LMP-1 is expressed in EBV-transformed lymphoblastoid cell lines, nasopharyngeal carcinoma (NPC), a subset of Hodgkin’s disease (HD), and in EBV-associated lymphoproliferative disorders (EBV-LPDs).

Recently, focused deletions near the 3’ end of the LMP-1 gene (del-LMP-1, amino acids 346-355), in a region functionally related to the half-life to the LMP-1 protein, have been reported frequently in human immunodeficiency virus (HIV)-associated HD (100%) and EBV* Malaysian and Danish peripheral T-cell lymphomas (100%, 61% respectively), but less frequently in cases of HD not associated with HIV (28%, 33%) and infectious mononucleosis (33%). To further investigate the potential relationship of del-LMP-1 to EBV-LPDs associated with immunosuppression or immunodeficiency, we studied 39 EBV-associated lymphoproliferations (10 benign, 29 malignant) from four distinct clinical settings: posttransplant (4 malignant, 1 reactive); HIV* (18 malignant, 2 reactive); nonimmunodeficiency malignant lymphoma (ML) (7 cases); and sporadic EBV infection with lymphoid hyperplasia (7 cases). The presence of EBV within lymphoid cells was confirmed by EBV EBER1 RNA in situ hybridization or by polymerase chain reaction (PCR) analysis. EBV strain type and LMP-1 deletion status were determined by PCR. EBV strain types segregated into two distinct distributions: HIV* (9 A; 11 B) and non-HIV (19 A, 0 B), consistent with previous reports. Overall, del-LMP-1 were found in 6 of 5 (20%) Burkitt lymphomas (BL); 17 of 24 (71%) aggressive non-Hodgkin’s lymphoma (agg-NHL), and 2 of 10 (20%) reactive lymphoid proliferations. Of the agg-NHLs, del-LMP-1 were present in 4 of 4 PT-ML (100%); 10 of 15 HIV* ML (67%); and 3 of 5 nonimmunodeficiency malignant lymphoma (ML, 60%). A total of 2 of 7 (28%) sporadic EBV-associated lymphoid hyperplasias contained a del-LMP-1. All del-LMP-1 were identical by DNA sequence analysis. No correlation was identified between the presence of del-LMP-1 and the EBV strain type observed. The high incidence of del-LMP-1 observed in agg-NHLs (71%), in contrast to the relatively low incidence observed in reactive lymphoid proliferations (28%), suggests that the deleted form may be preferentially selected in lymphomatous processes. All posttransplant agg-NHLs contained a del-LMP-1, and a similar frequency of del-LMP-1 was observed in both HIV-associated ML (66%) and nonimmunodeficiency ML (60%), suggesting that impairment of immune function alone is not a requirement for the expansion of malignant cells infected by EBV strains containing the deleted LMP-1 gene.

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partial deletions clustered about the 3' end of the LMP-1 gene and located within the carboxy-terminal domain of the LMP-1 protein, have been reported to be more tumorigenic when inoculated into severe combined immunodeficient (SCID) mice. Moreover, these partial deletions have been identified in some lymphomas. The characterized 30-bp deletions have involved an identical DNA segment corresponding to amino acids 346-355.

EBV-LPDs developing in the setting of immunosuppression and immunodeficiency are reported to display a Lat-III phenotype, thereby expressing the LMP-1 protein. A wide spectrum of histologic transformation, ranging from a reactive polymorphic B-cell hyperplasia to unequivocal malignant lymphoma, is typical of these lesions. We studied 39 EBV-associated lymphoproliferations (10 benign, 29 malignant) from four distinct clinical settings: postransplant (4 malignant, 1 reactive); HIV+ (18 malignant, 2 reactive); nonimmunodeficiency ML (7 cases); and sporadic lymphoid hyperplasia, EBV+ (7 cases) to further investigate the potential influence of del-LMP-1 on tumorigenicity.

MATERIALS AND METHODS

Case Selection

Cases of EBV+ benign and malignant lymphoproliferations selected for this study comprised four clinically distinct groups: (1) human immunodeficiency virus (HIV)-infection, (2) postransplantation, (3) EBV+ lymphomas arising in immunocompetent patients, and (4) sporadic reactive lymphoproliferations consistent with EBV infection. Cases were retrieved from the files of the Hematopathology Section, Laboratory of Pathology, National Cancer Institute, National Institutes of Health (NIH) and the Department of Pathology, University of Texas Medical Branch, Galveston, TX. The criteria for inclusion into this study were (1) a benign or malignant lymphoproliferative process; (2) EBV positivity shown by in situ hybridization or polymerase chain reaction (PCR), and (3) availability of frozen tissue or paraffin blocks from which DNA could be extracted for molecular studies. ML were classified according to the Working Formulation. Posttransplant lymphoproliferative disorders were classified according to the criteria of Frizzera et al. Lymphomas classified according to the Working Formulation as either large cell lymphoma, large cell immunoblastic lymphoma, or small noncleaved non-Burkitt's lymphoma, were evaluated as a group and reported as aggressive non-Hodgkin's lymphomas (agg-NHLs). The cell lineage of the malignant lymphomas were determined by immunohistochemistry and/or Southern blot analysis. Tumors were classified as B-cell origin if the malignant cells stained positively for L26 (CD20), clonally expressed either kappa or lambda light chain, or if they were shown to contain a clonal Ig gene rearrangement (JH, J-Kappa) by Southern blot analysis. Tumors were reported as T-cell phenotype if tumor cells stained with T-cell associated antibodies (anti-CD45RO, anti-CD3) or if they were shown to contain a clonal T-cell receptor (TCRβ) by Southern blot analysis. Any case exhibiting both T-cell and B-cell antigen receptor gene rearrangements was reported as of indeterminate phenotype.

Southern Blot Analysis for c-myc Rearrangement

From 15 cases (12 HIV-, 1 posttransplant, 2 aggressive NHL), high molecular weight DNA was isolated directly from involved frozen tissue samples of cell suspensions as described previously. Following restriction enzyme digestion with HindIII, EcoRI, and

<table>
<thead>
<tr>
<th>Table 1. DNA Sequences for PCR Primers: LMP1 and EBNA2</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMP-1</td>
</tr>
<tr>
<td>EBNA-2</td>
</tr>
</tbody>
</table>

EBV In Situ Hybridization

The RNA in situ hybridization technique has been described previously. Briefly, 5-μm sections of paraffin-embedded tissue were prepared on silanated slides. Tissue sections were deparaffinized, rehydrated, permeabilized with triton X-100, and digested with proteinase K (10 μg/mL). Riboprobe were applied in a 50% formamide hybridization buffer, and the slides were hybridized overnight. Following posthybridization washes, an antidigoxigenin alkaline phosphatase antibody-conjugate was applied to each slide. The slides were then washed and placed into a color developing solution consisting of nitrobluetetrazolium (NBT) and X-phosphate. The reaction was stopped by briefly washing the slides in an appropriate buffer. The slides were counterstained with eosin and coverslips were applied.

The integrity of the RNA in each tissue section was evaluated with a digoxigenin-labeled riboprobe directed at an abundant cellular RNA polymerase III transcript, U6 (gift from Dr Richard Ambinder, Johns Hopkins Oncology Center, Baltimore, MD). Sections showing hybridization signal with the U6 probe were determined adequate for analysis with the EBER1 probe. The EBV EBER1 riboprobe was prepared as previously described. A control slide, prepared from a paraffin-embedded tissue block containing metastatic nasopharyngeal carcinoma to lymph node, accompanied each hybridization run.

PCR Analysis for LMP-1 Deletions and EBNA-2 Strain Typing

Primers flanking the clustered LMP-1 deletion segment were selected using the Oligo Primer Analysis Software (National Biosciences, Plymouth, MN) and are listed in Table 1. Control template DNA for LMP-1 PCR included DNA extracted from Raji (EBV+, wildtype-LMP-1), B95-8 (EBV+, wildtype-LMP-1), and MOLT4 (EBV-) cell lines. A positive and negative control accompanied each PCR reaction. LMP-1 PCR products were analyzed on a 1.5% agarose gel, and product lengths for clinical samples were compared with those obtained with RAJI. Those cases exhibiting equivalent PCR product lengths were compared with those obtained with RAJI. Samples exhibiting shorter PCR product length than those obtained with RAJI were interpreted as containing a deletion (del-LMP-1) and were further subjected to DNA sequence analysis for confirmation. The EBV EBNA2 PCR primer sequences for EBV strain typing have been previously reported and are listed in Table 1.
<table>
<thead>
<tr>
<th>Case No.</th>
<th>Sex/Age</th>
<th>Biopsy Site</th>
<th>Biopsy Diagnosis</th>
<th>Phenotype</th>
<th>Molecular</th>
<th>EBV RISH</th>
<th>EBV LMP: PCR</th>
<th>EBV Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M/2</td>
<td>LN, R axilla</td>
<td>Atypical lymphoid hyperplasia with plasmacytosis and an IBL proliferation</td>
<td>JH+ J-kappa+ TCR- EBV clonal c-myc-</td>
<td>&gt;100/hpf</td>
<td>W</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>M/10m</td>
<td>LN, R cervical</td>
<td>Atypical lymphoid hyperplasia with polyclonal plasmacytosis</td>
<td>JH+ J-kappa- TCR- EBV clonal c-myc-</td>
<td>&gt;100/hpf</td>
<td>W</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>M/26</td>
<td>Bowel</td>
<td>Plasmacytoma</td>
<td>B</td>
<td>ND</td>
<td>D</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>M/37</td>
<td>Pleural fluid</td>
<td>ML, plasmacytoid differentiation</td>
<td>B, lambda</td>
<td>ND</td>
<td>D</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>M/3</td>
<td>LN, L cervical</td>
<td>ML, Diffuse, aggressive histologic grade, plasmacytoid features</td>
<td>B, kappa</td>
<td>pos</td>
<td>D</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>M/43</td>
<td>Ascites, abdomen</td>
<td>Anaplastic plasma cell tumor</td>
<td>B</td>
<td>ND</td>
<td>D</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>M/30</td>
<td>ST, rectal</td>
<td>Anaplastic plasma cell tumor</td>
<td>B</td>
<td>pos</td>
<td>W</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>M/29</td>
<td>Pleural fluid</td>
<td>ML, NOS</td>
<td>B</td>
<td>ND</td>
<td>D</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>M/14</td>
<td>Lung</td>
<td>ML, DLC</td>
<td>B, IgG, kappa</td>
<td>pos</td>
<td>W</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>M/47</td>
<td>Brain</td>
<td>ML, DLC</td>
<td>B</td>
<td>ND</td>
<td>pos</td>
<td>D</td>
<td>B</td>
</tr>
<tr>
<td>11</td>
<td>M/29</td>
<td>Lung</td>
<td>ML, DLC</td>
<td>B</td>
<td>pos</td>
<td>D</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>M/4</td>
<td>LN, I inguinal</td>
<td>ML, LC, IBL</td>
<td>B</td>
<td>pos</td>
<td>D</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>M/32</td>
<td>ST, thigh</td>
<td>ML, LC, IBL</td>
<td>B, kappa</td>
<td>ND</td>
<td>pos</td>
<td>D</td>
<td>B</td>
</tr>
<tr>
<td>14</td>
<td>M/30</td>
<td>Buccal mucosa</td>
<td>ML, LC, IBL</td>
<td>B</td>
<td>ND</td>
<td>pos</td>
<td>D</td>
<td>B</td>
</tr>
<tr>
<td>15</td>
<td>M/33</td>
<td>Buccal mucosa</td>
<td>ML, LC, IBL</td>
<td>B</td>
<td>pos</td>
<td>W</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>M/26</td>
<td>Bone &amp; ST, forehead</td>
<td>ML, SNC, NOS</td>
<td>B, lambda</td>
<td>ND</td>
<td>pos</td>
<td>D</td>
<td>A</td>
</tr>
<tr>
<td>17</td>
<td>M/40</td>
<td>Kidney</td>
<td>ML, SNC, NOS</td>
<td>B</td>
<td>EBV+ c-myc-</td>
<td>ND</td>
<td>W</td>
<td>B</td>
</tr>
<tr>
<td>18</td>
<td>F/37</td>
<td>Tonsil</td>
<td>ML, SNC, Burkitt's</td>
<td>B, IgG, kappa</td>
<td>pos</td>
<td>D</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>M/23</td>
<td>ST, rectal</td>
<td>ML, SNC, Burkitt's</td>
<td>B</td>
<td>EBV+ c-myc-</td>
<td>ND</td>
<td>W</td>
<td>B</td>
</tr>
<tr>
<td>20</td>
<td>M/30</td>
<td>LN, inguinal</td>
<td>ML, SNC, Burkitt's</td>
<td>B</td>
<td>EBV+ c-myc-</td>
<td>ND</td>
<td>W</td>
<td>B</td>
</tr>
</tbody>
</table>

Abbreviations: LN, lymph node; R, right; L, left; ST, soft tissue; ML, malignant lymphoma; IBL, immunoblastic, NOS, not otherwise subclassified; DLC, diffuse large cell; LC, large cell; SNC, small noncleaved; TCR, T-cell receptor; JH, immunoglobulin heavy chain, joining region; ND, not done; pos, positive; hpf, microscopic high power field; D, deleted; W, wildtype EBV LMP-1; A, EBV strain type A; B, EBV strain type B.
template DNA for EBNA2 PCR included DNA extracted from Raji (strain A) and Jijoye (strain B) cell lines. EBNA2 PCR products from cases studied were analyzed on a 2% agarose gel and compared with PCR products obtained with Raji and Jijoye. Cases exhibiting PCR product lengths equivalent to that obtained with Raji were interpreted as containing EBV, strain A. Cases with PCR product lengths equivalent to Jijoye were interpreted as containing EBV, strain B.

All PCR reactions were performed using the Perkin Elmer Cetus DNA thermal cycler (Norwalk, CT) and the PCR Core Kit (Boehringer Mannheim, Indianapolis, IN). The PCR parameters for both LMP-1 and EBNA2 were identical and consisted of an initial denaturation step of 5 minutes (94°C) followed by 35 cycles of (1) 94°C, 1 minute; (2) 56°C, 1 minute; and (3) 72°C, 1 minute, followed by a final extension step at 72°C for 10 minutes. DNA extracted from paraffin was subjected to two rounds of PCR using the hot start method, whereas DNA extracted from frozen tissue was subjected to a single round of PCR.

**DNA Sequencing of LMP-1 Deletions**

To confirm the specificity of the PCR reaction, deleted LMP-1 PCR products were sequenced using the Sequenase PCR product sequencing kit version 2.0 (US Biochemical, Cleveland, OH). The LMP-1 sequencing PCR primer (5’ CGG AAG AGG TGG AAA ACA AA 3’) produced a readable sequence of about 90 bp, which included the clustered deleted segment previously reported. All sequenced cases were compared with that obtained with B95-8, representing the wild-type LMP-1 gene (wt-LMP-1).

**RESULTS**

**HIV-Associated EBV+ Lymphoproliferations**

**Clinical.** The clinicopathologic and EBV molecular findings are listed in Table 2. There were 19 males and one female with a median age of 30 years (range, 10 months to 47 years) at diagnosis. The sites biopsied were nodal in six patients and extranodal in 14 patients. Two cases were diagnosed as atypical reactive lymphoid hyperplasia with plasmacytosis, 18 were diagnosed as malignant lymphoma. The two reactive cases (Table 2, cases 1 and 2) showed polymorphous cellular infiltrate exhibiting a spectrum of immunoblastic transformation, with marked plasmacytoid differentiation. Although polyclonal for kappa and lambda light chains by paraffin immunoperoxidase (case 2), the lymphoproliferation diffusely effaced the lymph node architecture, mimicking a malignant lymphoma. Although Southern blot analysis of case 2 did not show an antigen receptor gene rearrangement (JH, J-kappa-, TCRβ-), analysis of EBV terminal repeats showed a clonal episomal form in both cases. Southern blot analysis of case 1 showed a weak clonal band using a probe for J-kappa and a germline band using a probe for JH.

A histologic diagnosis of malignant non-Hodgkin’s lymphoma was made in 18 of 20 cases. These were further subclassified as diffuse, small noncleaved (5 cases; 3 Burkitt’s, 2 non-Burkitt’s); diffuse large cell (4); diffuse large cell, immunoblastic (4); anaplastic plasmacytoid tumor (2); plasmacytoma (1), malignant lymphoma with plasmacytoid features (1); and malignant lymphoma, not otherwise subclassified (1). All lymphomas were of B-cell lineage.

**EBV studies.** The presence of EBV was confirmed in all biopsy samples by EBV EBER1 RNA in situ hybridization (13 of 13) and/or EBNA2 PCR (20 of 20). In the two biopsies diagnosed as atypical lymphoid hyperplasia (Table 2, cases 1 and 2), EBV EBER1 RNA was identified in both small

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Note: The image contains a figure labeled as Fig 1, which is a gel electrophoresis image comparing different samples. The figure is not transcribed here as it contains visual data that is not easily represented in text form.
Table 3. Posttransplant EBV-LPD: Clinical, Histologic, and EBV Molecular Data

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Sex/Age</th>
<th>Transplant Site</th>
<th>Biopsy Site</th>
<th>Biopsy Diagnosis</th>
<th>Phenotype</th>
<th>Molecular</th>
<th>EBV RISH</th>
<th>EBV LMP-1 PCR</th>
<th>EBV Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F/8</td>
<td>Transplant kidney</td>
<td>kidney</td>
<td>Polymorphic B-cell hyperplasia</td>
<td>Indeterminate (B-cell IPOX)</td>
<td>JH+ J-kappa+ TCR-</td>
<td>pos</td>
<td>W</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>M/43</td>
<td>Liver</td>
<td>Tonsil</td>
<td>ML, aggressive histologic grade</td>
<td>B</td>
<td>JH+ J-kappa+ TCR-</td>
<td>pos</td>
<td>D</td>
<td>A</td>
</tr>
<tr>
<td>3</td>
<td>F/4</td>
<td>Heart</td>
<td>Bladder, mesenteric</td>
<td>ML, LC, IBL</td>
<td>B</td>
<td>ND</td>
<td>pos</td>
<td>D</td>
<td>A</td>
</tr>
<tr>
<td>4</td>
<td>M/50</td>
<td>Kidney</td>
<td>LN, axilla &amp; inguinal</td>
<td>ML, LC, IBL</td>
<td>B</td>
<td>JH+ TCR-clonal EBV c-myc-</td>
<td>ND</td>
<td>D</td>
<td>A</td>
</tr>
<tr>
<td>5</td>
<td>M/40</td>
<td>Kidney</td>
<td>LN, celiac</td>
<td>ML, LC, IBL, T-cell, c/w LCAL</td>
<td>T</td>
<td>JH+ TCR+</td>
<td>ND</td>
<td>D</td>
<td>A</td>
</tr>
</tbody>
</table>

Abbreviations: LN, lymph node; ML, malignant lymphoma; IBL, immunoblastic; LC, large cell; SNC, small noncleaved; TCR, T-cell receptor; JH, immunoglobulin heavy chain, joining region; ND, not done; pos, positive; D, deleted; W, wild-type EBV LMP-1; A, EBV strain type A; B, EBV strain type B.

EBV-Associated Posttransplant LPD

Clinical. The clinicopathologic and EBV molecular findings are listed in Table 3. There were three males and two females with a median age of 40 years (range, 4 to 50 years) at diagnosis. The sites biopsied were nodal in three patients and extranodal in two patients (transplant kidney; mesenteric mass/bladder mass). The biopsy from case 1 histologically contained a polymorphous cellular infiltrate exhibiting a spectrum of immunoblastic transformation with marked plasmacytoid differentiation, consistent with polymorphic B-cell hyperplasia. Southern blot analysis showed the presence of both immunoglobulin (JH+, J-kappa+) and T-cell receptor (TCR-β) antigen receptor gene rearrangements. Moreover, EBV terminal repeat analysis demonstrated a single clonal episomal form.

Four cases were diagnosed as agg-NHL, further classified as diffuse large cell (1) and diffuse large cell, immunoblastic (3). Three cases exhibited a B-cell phenotype (Table 3, cases 2 to 4) whereas one case (Table 3, case 5) was of T-cell lineage, as demonstrated by immunophenotypic and genotypic studies.

EBV studies. The presence of EBV was confirmed in all biopsy samples by EBV EBER1 RNA in situ hybridization (4 of 5) or EBNA2 PCR (5 of 5). Case 1 (Table 3), a polymorphic B-cell hyperplasia, contained >100 EBV+ cells/HPF identified as both small lymphocytes and transformed immunoblasts. All malignant lymphomas studied by EBV EBER1 RNA in situ contained hybridization signal in >95% of tumor cells.

EBV strain type A was present in all 5 biopsies (100%).

c-MYC analysis. High molecular weight DNA was available in one case (Table 3, case 4; ML, LC, IBL). No rearrangement of c-MYC was identified.

EBV Malignant Lymphoma in Immunocompetent Patients

Clinical. The clinicopathologic and EBV molecular findings are listed in Table 4. There were 6 males and 1 female with a median age of 35 years (range, 3 to 75 years) at diagnosis. The sites biopsied were nodal in four patients and extranodal in three patients (breast, gastrointestinal, nasal septum). These were further classified as diffuse large lymphoid cells and immunoblasts. The number of infected cells was >100 per microscopic high power field (HPF). All malignant lymphomas studied by EBV EBER1 RNA in situ hybridization contained hybridization signal in >95% of tumor cells.

Shorter LMP-1 PCR products, indicating a del-LMP-1, were identified in 0 of 2 (0%) nonmalignant biopsies and 11 of 18 (61%) biopsies diagnosed as malignant lymphoma (Fig 1). DNA sequencing of the shorter PCR products showed an identical deleted segment spanning 30 bp in all cases studied (see Table 6). Del-LMP-1 were observed in 1 of 3 (33%) Burkitt’s lymphomas and 10 of 15 (66%) agg-NHLs. EBV strain type A (prototype RAJI) was present in 9 of 20 biopsies (45%), strain type B (prototype Jijoye) was present in 11 of 20 (55%) biopsies. LMP-1 deletions were observed more frequently in strain type B-infected lymphomas (73%) than in EBV strain A-infected lymphomas (43%).

c-MYC analysis. High molecular weight DNA was available for 12 cases (2 reactive, 7 agg-NHL, 3 BL). A c-MYC rearrangement was identified in 2 of 12 biopsies, both classified as Burkitt’s lymphoma. Interestingly, these two cases were wild type for EBV LMP-1. The one c-MYC negative Burkitt’s lymphoma contained a del-LMP-1.

EBV studies. The presence of EBV was confirmed in all biopsy samples by EBV EBER1 RNA in situ hybridization (4 of 5) or EBNA2 PCR (5 of 5). Case 1 (Table 3), a polymorphic B-cell hyperplasia, contained >100 EBV+ cells/HPF identified as both small lymphocytes and transformed immunoblasts. All malignant lymphomas studied by EBV EBER1 RNA in situ contained hybridization signal in >95% of tumor cells.

Shorter LMP-1 PCR products, indicating a del-LMP-1, were identified only in the cases diagnosed as malignant lymphoma (Fig 2). DNA sequencing of the shorter PCR products showed an identical deleted segment spanning 30 bp in all cases studied (see Table 6). EBV strain type A was present in all 5 biopsies (100%).

c-MYC analysis. High molecular weight DNA was available in one case (Table 3, case 4; ML, LC, IBL). No rearrangement of c-MYC was identified.
cell (2) or diffuse large cell, immunoblastic (2); and small noncleaved (3 cases: 2 Burkitt’s, 1 non-Burkitt’s). Each lymphoma exhibited a B-cell phenotype.

**EBV studies.** The presence of EBV was confirmed in all biopsy samples by EBV EBER1 RNA in situ hybridization (3 of 7) or EBNA2 PCR (7 of 7). All malignant lymphomas studied by EBV EBER1 RNA in situ hybridization contained hybridization signal in >95% of tumor cells.

Shorter LMP-1 PCR products, indicating a del-LMP-1, were identified in 3 of 5 (60%) cases (1 large cell; 1 large cell, immunoblastic, 1 small noncleaved, non-Burkitt’s). DNA sequencing of the shorter PCR products showed an identical deleted segment spanning 30 bp in all cases studied (see Table 6). A total of 0 of 2 BL contained a del-LMP-1. EBV strain type A was identified in all biopsies.

**c-MYC analysis.** High molecular weight DNA was available for two cases (Table 4, cases 6 and 7). Both cases were Burkitt’s lymphomas, contained a c-MYC rearrangement, and were wild type for EBV LMP-1.

**Sporadic EBV Infection With Lymphoid Hyperplasia**

**Clinical.** The clinicopathologic and EBV molecular findings are listed in Table 5. There were four males and three females with a median age of 18 years (range, 6 to 52 years) at diagnosis. The sites biopsied were nodal in all patients. Histologically, each demonstrated features of reactive hyperplasia and contained a polyclonal proliferation of lymphoid cells exhibiting a spectrum of immunoblastic transformation and plasma cell differentiation. Histologic features of malignancy were not identified in any of the biopsies.

**EBV studies.** The presence of EBV was confirmed in all biopsy samples by EBV EBER1 RNA in situ hybridization. Hybridization signal was identified in >50 lymphoid cells/HPF and localized to the interfollicular regions.

Shorter LMP-1 PCR products, indicating a del-LMP-1, were identified in 2 of 7 (28%) cases (Table 5, cases 1 and 2; Fig 3). DNA sequencing of the shorter PCR products showed an identical deleted segment spanning 30 bp (Table 6). EBV strain type A was identified in all biopsies.

**DISCUSSION**

Previous studies have suggested that del-LMP-1 may play a role in the oncogenic potential of EBV. We wished to study EBV+ LPDs and EBV+ ML to determine if LMP-1 deletions (del-LMP-1) play a role in the evolution to malignant lymphoma. EBV-LPDs are a useful model to study this question because (1) EBV-LPDs show a marked histologic, phenotypic and genotypic spectrum of transformation and (2) EBV-LPDs frequently express the EBV LMP-1 protein.

Del-LMP-1 were found in 18 of 29 (62%) biopsies diagnosed as unequivocal malignant lymphoma, but were found in only 2 of 10 (20%) biopsies containing an EBV reactive lymphoid proliferation. Specifically, del-LMP-1 were identified in 4 of 4 (100%) posttransplant malignant lymphomas (ML), 11 of 18 (63%) ML developing in HIV-infected patients, and 3 of 7 (43%) ML arising in nonimmunodeficient patients. When analyzed according to the histologic type of the malignant process, del-LMP-1 were found in 17 of 24 (71%) agg-NHLs, but in only 1 of 5 (20%) EBV+ Burkitt’s lymphomas (BL). Our results indicate that in EBV-associated LPD and agg-NHL known to express the LMP-1 protein, del-LMP-1 may have a more potent tumor promoting activity than EBV containing the full size LMP-1 gene. Moreover, impairment of immune function alone does not
Table 4. EBV+ Malignant Lymphomas Arising in Immunocompetent Patients: Clinical, Histologic, and EBV Molecular Data

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Sex/Age</th>
<th>Biopsy Site</th>
<th>Biopsy Diagnosis</th>
<th>Phenotype</th>
<th>Molecular</th>
<th>EBV RISH</th>
<th>EBV LMP-1: PCR</th>
<th>EBV Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M/73</td>
<td>Nasal septum</td>
<td>ML, DLC</td>
<td>B</td>
<td>ND</td>
<td>pos</td>
<td>D</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>M/31</td>
<td>LN, supracervical</td>
<td>ML, DLC</td>
<td>B, IgM, kappa</td>
<td>ND</td>
<td>ND</td>
<td>W</td>
<td>A</td>
</tr>
<tr>
<td>3</td>
<td>M/46</td>
<td>LN, cervical</td>
<td>ML, LC, IBL</td>
<td>B</td>
<td>JH-</td>
<td>ND</td>
<td>D</td>
<td>A</td>
</tr>
<tr>
<td>4</td>
<td>M/75</td>
<td>LN, cervical</td>
<td>ML, LC, IBL</td>
<td>B</td>
<td>J-kappa+</td>
<td>ND</td>
<td>ND</td>
<td>WD</td>
</tr>
<tr>
<td>5</td>
<td>M/35</td>
<td>LN, axillary</td>
<td>ML, SNC, non-Burkitt's</td>
<td>B</td>
<td>ND</td>
<td>pos</td>
<td>D</td>
<td>A</td>
</tr>
<tr>
<td>6</td>
<td>M/33</td>
<td>Ileum, appendix</td>
<td>ML, SNC, Burkitt's</td>
<td>B</td>
<td>EBV+</td>
<td>ND</td>
<td>W</td>
<td>A</td>
</tr>
<tr>
<td>7</td>
<td>F/32</td>
<td>ST, breast</td>
<td>ML, SNC, Burkitt's</td>
<td>B</td>
<td>EBV+</td>
<td>c-myc+</td>
<td>ND</td>
<td>W</td>
</tr>
</tbody>
</table>

Abbreviations: LN, lymph node; ML, malignant lymphoma; IBL, immunoblastic; DLC, diffuse large cell; LC, large cell; SNC, small noncleaved; TCR, T-cell receptor; JH, immunoglobulin heavy chain, joining region; ND, not done; pos, positive; D, deleted; W, wild-type EBV LMP-1; A, EBV strain type A; B, EBV strain type B.

appear to be a requirement for the expansion of malignant cells infected by EBV strains carrying the deleted LMP-1 gene, as the frequency of del-LMP in HIV-associated agg-NHLs (66%) and nonimmunodeficient agg-NHLs (60%) was similar.

In vitro studies of C-terminal deletion mutants have shown that deletions involving the LMP-1 gene segment analyzed in this study (amino acids 346-355) are associated with prolongation of the LMP-1 half-life by 30% to 70%. Martin and Sugden49 postulated that the sequence between amino acid positions 322 and 364 is required for rapid LMP-I protein turnover. Because LMP-1 expression within human B cells induces DNA synthesis, abrogates apoptosis, and likely plays an important role in the initiation and/or maintenance of the immortalized state, it is possible that the effects of LMP-1 may be accentuated through loss of a genomic segment required for its rapid turnover. The resulting prolonged biological effects of LMP-1 might also increase the likelihood of infected cells carrying the deletion to undergo additional molecular alterations. Alternatively, cells carrying the LMP-1 deletion may express an altered LMP-1 protein product with decreased immunogenicity for EBV-specific cytotoxic T cells. These EBV-infected cells may escape immunosurveillance, thereby acquiring a survival advantage, and the possibility for further mutagenic events.

It is likely that del-LMP-1 is only one in a series of molecular events leading to malignant transformation in EBV-associated LPDs and ML. For example, Knowles et al48 have recently shown the presence of one or more oncogene or tumor suppressor gene alterations in unequivocal cases of monomorphic lymphoma developing in posttransplant patients, whereas polymorphic lymphoproliferations lacked such alterations. This sequence is manifested by a range in histologic transformation. EBV-LPDs may regress if the underlying defect is reversed; otherwise, the course is progressive and terminates in overt lymphoma. In immuno-

Table 5. Acute Reactive EBV Infection: Clinical, Histologic, and EBV Molecular Data

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Sex/Age</th>
<th>Biopsy Site</th>
<th>Biopsy Diagnosis</th>
<th>EBV RISH</th>
<th>EBV LMP-1 PCR</th>
<th>EBV Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M/19</td>
<td>Tonsils</td>
<td>Atypical paracortical hyperplasia with focal necrosis</td>
<td>&gt;50/hpf</td>
<td>D</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>M/6</td>
<td>LN, NOS</td>
<td>Atypical IBL proliferation c/w infectious mononucleosis (IM)</td>
<td>&gt;50/hpf</td>
<td>D</td>
<td>A</td>
</tr>
<tr>
<td>3</td>
<td>F/13</td>
<td>LN, cervical</td>
<td>Florid atypical paracortical hyperplasia</td>
<td>&gt;50/hpf</td>
<td>W</td>
<td>A</td>
</tr>
<tr>
<td>4</td>
<td>M/23</td>
<td>LN, cervical</td>
<td>Reactive follicular hyperplasia with atypical IBL proliferation</td>
<td>&gt;50/hpf</td>
<td>W</td>
<td>A</td>
</tr>
<tr>
<td>5</td>
<td>M/18</td>
<td>LN, axillary</td>
<td>Reactive follicular and paracortical hyperplasia c/w IM</td>
<td>&gt;50/hpf</td>
<td>W</td>
<td>A</td>
</tr>
<tr>
<td>6</td>
<td>F/52</td>
<td>LN, cervical</td>
<td>Atypical LPD with marked IBL proliferation</td>
<td>&gt;50/hpf</td>
<td>W</td>
<td>A</td>
</tr>
<tr>
<td>7</td>
<td>F/6</td>
<td>LN, cervical</td>
<td>Atypical paracortical hyperplasia c/w IM</td>
<td>&gt;50/hpf</td>
<td>W</td>
<td>A</td>
</tr>
</tbody>
</table>

Abbreviations: LN, lymph node; hpf, microscopic high power field; ND, not done; pos, positive; D, deleted; W, wild-type EBV LMP-1; A, EBV strain type A; B, EBV strain type B.
EBV LMP-1 ONCOGENE DELETIONS IN EBV-LPDs

LI3 acd > UF+

Fig 3. The EBV LMP-1 gene contained a 30-bp deletion in 2 of 7 acute reactive EBV-infected biopsies (lanes 3 and 4; 131-bp PCR product). A wild-type EBV LMP-1 161-bp PCR product was identified in the remaining five biopsies (lanes 5 and 6). (Lane 1, molecular weight markers, 6x174 Hae III digest; lane 2, Raji LMP-1 wild-type control; lane 7, negative control.)

Table 6. DNA Sequence Data for EBV LMP-1 Wild Type (wt-LMP1) and del-LMP-1

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>358</th>
<th>355</th>
<th>346</th>
<th>343</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (B95-8) EBV LMP-1</td>
<td>CAC ACC TAG ATA CGG CAG TAC CGG CTT TAG TCA TGC TCA CGG CGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deleted LMP-1 (del-LMP-1)</td>
<td>CAC ACC TAG ----------------------------------------------- TGG CGG CGG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All biopsies showing a shorter PCR product than that observed with the LMP wild-type controls (B95-8, RAJI) contained an identical 30-bp deletion, which did not correlate with EBV strain type. The highlighted sequence (italics) corresponds to the deleted DNA sequence observed in all biopsies exhibiting a del-LMP-1.

deficiency, polyclonal EBV-infected B cells expand, eventually leading to clonal selection of an EBV-infected cell. However, the progression to unequivocal malignant lymphoma probably requires additional molecular events, which immortalize and transform the cells irreversibly.

As noted previously, the importance of a del-LMP-1 in malignant transformation has been suggested by other studies involving both lymphoid and nonlymphoid malignancies.\(^2\)\(^3\) Cases of EBV+ HD carrying the del-LMP-1 were reported to be associated with histologic signs of particular aggressivity, including the presence of giant (anaplastic) HD cells, numerous Reed-Sternberg cells, and necrosis.\(^2\) LMP-1 genes isolated from NPC and carrying partial deletions of LMP-1 were reported to be more tumorigenic when inoculated into SCID or nude mice.\(^2\)\(^3\)

Although the number of cases represented in each clinical group is relatively small, the EBV strain distribution in our series was consistent with previous reports.\(^19\)\(^24\)\(^25\) EBV strain type B was identified in 11 of 18 (61%) HIV-associated malignant lymphomas, whereas EBV strain type B was not identified in any of the non-HIV associated lymphomas (0 of 4 posttransplant ML, 0 of 7 sporadic EBV+ ML). Moreover, EBV strain type B was not identified in any of the nonmalignant biopsies (0 of 2 HIV; 0 of 1 posttransplant, 0 of 7 acute reactive EBV infections).

Each of the biopsies showing a LMP-1 deletion by PCR was found to have an identical 30-bp deletion, regardless of clinical setting, leading us to question whether a correlation might exist between the presence of a del-LMP-1 and EBV strain type. EBV strain type B is more often identified in lymphomas from equatorial Africa (50%) and HIV-infected patients (46%), both settings associated with immunosuppression, than in sporadic EBV+ lymphomas from North America (7%) or South America (14%).\(^4\)\(^4\) There was no correlation between EBV strain type and the presence or absence of a deletion. Nevertheless, when taking the clinical setting into account, HIV-associated lymphomas containing a del-LMP-1 were more likely to contain EBV strain type B (8 of 11, 73%) than strain type A (3 of 7, 43%).

Del-LMP-1 were identified more frequently in agg-NHLs (17 of 24, 71%) than in those classified as BL (1 of 5; 20%). The fact that del-LMP-1 were more frequently identified in agg-NHL is not unexpected. LMP-1 expression is frequently present in EBV-positive agg-NHLs arising in the setting of immunosuppression,\(^12\)\(^36\) but is usually absent in BL.\(^4\)\(^2\)\(^1\)

Although most instances of Burkitt’s lymphoma have a c-myc arrangement, the case of BL containing a del-LMP gene was negative for translocations involving the c-myc oncogene by Southern blot analysis. In BL, constitutive over-
expression of c-myc is thought to lead to malignant transformation, and the LMP gene may not be as critical to oncogenesis. Thus this case, while morphologically resembling BL, had a molecular and antigenic profile of an agNHL, not BL.

REFERENCES
32. Knecht H, Bachmann E, Brouss P, Sandvej K, Nadal D, Bachmann F, Odermaat B, Delsol G, Pallesen G: Deletions within the LMP1 oncogene of Epstein-Barr virus are clustered in Hodgkin's
disease and identical to those observed in nasopharyngeal carcinoma. Blood 82:2937, 1993
Epstein-Barr virus latent membrane protein-1 oncogene deletions: correlations with malignancy in Epstein-Barr virus--associated lymphoproliferative disorders and malignant lymphomas

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