Epstein-Barr Virus-Associated Non-Hodgkin’s Lymphoma in Patients Infected With the Human Immunodeficiency Virus

By Darryl Shibata, Lawrence M. Weiss, Antonio M. Hernandez, Bharat N. Nathwani, Leslie Bernstein, and Alexandra M. Levine

Lymphoproliferations associated with Epstein-Barr virus (EBV) commonly arise in settings of immune dysfunction, including human immunodeficiency virus (HIV) infection. In this study, EBV was associated with 39 of 59 (66%) HIV-associated systemic lymphomas. Unlike the lymphoproliferations in the setting of transplantation, the HIV-related lymphomas were monoclonal, as evaluated by Ig heavy chain rearrangements and EBV termini analysis, and associated (40%) with c-MYC rearrangements. Furthermore, analysis of multiple lymphoma tissues from one autopsy showed evidence that a single lymphoma clone was responsible for dissemination. The latent EBV nuclear antigen (EBNA-1) transcripts detected in the HIV-related lymphomas were characteristic of the pattern found in Burkitt lymphoma (g1 EBNA1) and not in transplant-related lymphoproliferations. However, unlike Burkitt lymphoma, EBV latent membrane-associated protein (LMP) transcripts were also detected, thereby constituting an EBV expression pattern (g1 EBNA1+, LMP+) not previously observed in B-cell lymphomas. These findings demonstrate a high frequency of EBV-associated lymphomas in the setting of HIV infection that are distinct from the lymphoproliferations that arise during iatrogenic transplant-associated immunosuppression or in the general population. However, it is also apparent that HIV-related lymphomas are biologically heterogeneous, which may reflect the multiple mechanisms or steps necessary for eventual malignant transformation.
EBV-ASSOCIATED HIV-RELATED LYMPHOMA

RESULTS

EBV was detected in specimens from 39 of the 59 (66%) HIV-related lymphoma cases by PCR, along with ISH or Southern blot assays for EBV termini (Tables 1 and 2). In contrast, EBV was detected in tissues from only 2 of the 37 (5%) non–HIV-related lymphoma cases (P < .00001). A specimen was considered EBV associated only if EBV was detected by at least two methods; thus, four HIV-related cases in which EBV was detected by PCR but could not be localized to the lymphoma cells by ISH were considered EBV negative. Large numbers of EBV sequences were estimated to be present in the EBV-positive cases, based on serial dilutions followed by PCR (not shown). In the EBV-positive specimens, EBER-1 RNA was localized to the nucleus as previously reported26 (Fig 1). The large number of EBER-1 RNA molecules expressed per infected cell (up to 10^5) facilitated detection by ISH.

The EBV-positive, HIV-related cases did not have distinctive histologic features and included 6 of 11 (55%) cases of diffuse large cell, 16 of 28 (57%) cases of small noncleaved, and 17 of 20 (85%) cases of immunoblastic lymphoma (Table 3). None of the HIV-related small noncleaved lymphomas had the classic histology of Burkitt lymphoma (BL). In this study, which excluded primary CNS lymphoma, the majority of the HIV-related systemic lymphomas were extranodal (59%). Approximately equal proportions of the lymphomas arising in nodal sites (67%) and extranodal sites (66%) were EBV associated.

EBV may be subclassified into two groups, type A and type B, based on sequence polymorphisms in the EBNA-2 gene.13,21 The EBV type in the 39 positive HIV-related cases was determined to be type A in 25 (64%) cases and type B in 10 (26%) cases. No mixed infections were detected. The EBV type could not be determined in four cases. The two

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Abbreviations: IBL, immunoblastic lymphoma; SNC, small noncleaved lymphoma; LC, large cell lymphoma.

EBV was detected using HindIII and BamHI/HindIII digestions with a 5.4-kb HindIII-BamHI JH probe (Oncogene Science, Uniondale, NY). T-P chain receptor rearrangements were analyzed using BamHI or EcoRI digestions with a 0.4-kb constant region probe (Oncogene Science). c-MYC rearrangements were detected using EcoRI and HindIII digestions with a third exon 1.4-kb Cla I-EcoRI probe (Oncor). EBV termini structure was analyzed using BamHI digestion and a 1.9-kb Xho I fragment located near the right EBV terminus (generously provided by Dr N. Raab-Traub).5 The DNA probes were labeled with 32P-dATP using the random hexamer method.15

Detection of EBV mRNA. Reverse PCR was performed to detect the group 1 (g1) or group 3 (g3) EBNA-1 RNA as previously described.17 A single frozen section was placed in a sterile 1.5 mL microfuge tube. Poly A+ RNA was isolated using oligo(dT)-cellulose columns with a commercial kit (QuickPrep; Pharmacia, Piscataway, NJ). First-strand cDNA synthesis was performed on approximately 15% of the RNA from each section using Moloney murine leukemia virus reverse transcriptase (BRL) in standard PCR buffer at 37°C for 3 minutes.19 The "K" primer15 was used to prime the first-strand synthesis. The cDNA was split into two different aliquots and then subjected to PCR with either the "Q" and "K" primers to detect the g1 or the "Y3" and "K" primers to detect g3 EBNA-1 mRNA. A total of 50 PCR cycles was performed, with denaturation at 95°C for 60 seconds, annealing at 54°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension at 72°C for 7 minutes. The PCR products were separated on a 2.5% Nuseive (FMC, Rockland, ME), 0.5% agarose gel and Southern blotted to a nylon filter (Genetran 45; Plasco, Woburn, MA). The filter was hybridized to a 32P-labeled fluorescent "U" exon oligomer probe7 and washed in 2× SSPE at room temperature. Autoradiography was performed for 3 to 72 hours. A positive control for poly A+ RNA isolated from the EBV-positive Raji cell line demonstrated high g3 EBNA-1 mRNA levels and very low g1 EBNA-1 mRNA levels.

The EBV mRNAs for latent membrane-associated protein 1 (LMP1), LMP2A, and LMP2B were detected by reverse PCR as above, using the same aliquots of poly A+ RNA with primers described by Brooks et al. The primers for LMP1 were CCCTGAGAGAGACCTCTCTGT (upstream) and ATACCATGACAGAAGTACGCT (downstream and used for cDNA synthesis). The oligomer ACAATGCCTGTCCGTGCAA was used as an LMP1 probe. LMP2 mRNA was detected using a common downstream primer for cDNA synthesis and then a different upstream primer specific for either LMP2A or LMP2B. An oligonucleotide common to both LMP2A and LMP2B was used as a labeled probe.10 The PCR was performed as above with an annealing temperature of 50°C and 50 cycles.

Criteria for EBV-associated lymphoma. A lymphoma was considered EBV associated only if EBV could be detected by at least two methods including PCR, demonstration of labeling of the tumor cells by ISH, or clonal infection using the EBV termini probe.

Table 1. EBV Analysis of HIV-Positive and HIV-Negative Lymphoma

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EBV-associated lymphomas from the non–HIV-infected patients contained type A in one case and type B in the other.

The g1 pattern of EBNA-1 mRNA expression was detected in four EBV-associated, HIV-related lymphomas (Fig 2). There was no evidence of the g3 expression pattern. In contrast, mRNA isolated from the Raji cell line demonstrated a predominance of the g3 EBNA-1 message with scant g1 expression. Using reverse PCR, the mRNAs for LMP1 or LMP2A were also detected. No LMP2B transcripts were detected. Attempts to demonstrate LMP by immunohistochemistry were technically unsatisfactory (not shown).

The clonality of EBV infection and structure of the EBV genome was then analyzed by determining the number and size of fused terminal fragments.16 This analysis was performed on 12 EBV-positive cases from which sufficient frozen tissue was available, using an EBV-terminal probe and a BamHI digestion (Fig 3). A single band greater than 8 kb, indicating one species of EBV episome, was detected in each case. There was no evidence of linear EBV genomes except for very faint smears between the 4- and 6-kb regions. These results are consistent with a monoclonal expansion of the EBV-infected lymphoma cells. Further, active viral replication, if present at all, comprised only the minority of the EBV genomes.

The 15 HIV-related and 22 non–HIV-related lymphoma specimens with frozen tissue available were further studied by Southern blot analysis. In all of these specimens, clonal Ig heavy chain rearrangements were detected. Rearrangement of the T-β receptor was not detected in the 22 cases examined. Using a third exon probe, c-MYC rearrangements were de-
tected in 6 (40%) HIV-related lymphomas. The HIV lymphomas with rearranged c-MYC genes were all EBV positive. In contrast, c-MYC rearrangements were detected from only 3 (14%) of the non–HIV-related lymphomas and only 1 of these was EBV positive.

In one autopsy case (case 11), lymphoma tissues from multiple sites (lymph nodes, rectal mass, and vesical mass) were available for Ig heavy chain and EBV termini clonal analysis. In every involved site, the identical monoclonal Ig heavy chain and EBV termini rearrangements were detected (Fig 4). An additional, very faint higher molecular weight EBV-terminal fragment was also detected at one lymphoma tissue site (hilar node, not visible in Fig 4). These data suggest that dissemination of lymphoma in this patient occurred primarily through the clonal expansion of a single transformed EBV-infected B-lymphoid cell.

**DISCUSSION**

The majority (66%) of HIV-related systemic lymphomas in this study were EBV associated. This frequency is somewhat higher than that noted in the majority of prior studies, in which 38% to 50% of cases were found to be EBV associated. Higher frequencies of EBV involvement, similar to our own, have also been noted. The explanation for the differences between these studies is unclear, but may involve differences in patient population, histologic types of lymphoma, or anatomical sites of disease. For example, the vast majority of HIV-related primary CNS lymphomas are EBV associated. However, no case of primary CNS lymphoma was examined in the current study and we found no difference in the frequency of EBV-positive lymphomas arising from extranodal or nodal sites. The frequency (59%) of systemic extranodal lymphoma observed in this study is similar to that found in other reports.

Similar to the lack of uniformity in the setting of HIV-related lymphoma, different frequencies of EBV association have also been found in non–HIV-related lymphoma occurring throughout the world. Thus, EBV is present in nearly all examples of BL from Africa, but is much less frequent (10% to 20%) in cases from the United States. Studies of similar lymphomas from other geographic regions such as South America have demonstrated an intermediate (50%) frequency of EBV association. The different frequencies of EBV involvement observed in BL from diverse geographic areas may be a reflection of diverse mechanisms of lymphomagenesis, which may or may not require preceding EBV infection. The relatively low frequency of c-MYC rearrangements (40%) detected in this study of HIV-related lymphoproliferations, compared with prior studies, may also reflect this diversity. More detailed analysis of the specific c-MYC and Ig heavy chain breakpoints in these lymphomas may allow better comparisons between groups. In our population from Los Angeles, it is clear that lymphomas that occur in the setting of underlying HIV infection are more likely to be associated with EBV than similar lymphomas that occur in the absence of HIV infection.

The HIV-related lymphomas in this study were all diffuse lymphomas. Similar to other studies, the majority were high-grade small noncleaved or immunoblastic lymphomas. Of note, the presence of EBV was not restricted to a specific histologic type of lymphoma. Thus, EBV sequences were present in more than half of the diffuse large cell, small noncleaved, and immunoblastic lymphomas. As observed by others, the majority (85%) of the immunoblastic lymphoma cases in the current study were EBV associated. However,
Fig 2. (A) PCR products representing the group 1 (gpl) EBNA-1 mRNA (239 bp) were detected from four EBV-associated, HIV-related lymphomas (A, case 1; B, case 2; C, case 3; F, case 4). The group 3 (gp3) EBNA-1 mRNA (268 bp) was detected from the Raji cell line control but not from the specimens. The negative control is poly A RNA extracted from an uninfected T-cell line (CEM). Specimen D is an EBV-negative, HIV-related lymphoma (case 12). No EBNA-1 signal was detected from specimen E (EBV-associated, HIV-related lymphoma, case 5), but its RNA appeared degraded (not shown). (B) PCR products from the same specimens demonstrating positive LMP1 signals (153 bp) in B, C, and F, and positive LMP2A signals (260 bp) in A, B, C, and F. No LMP2B signals (342 bp) were detected. The 231-bp signal in the LMP1 assay is derived from EBV DNA isolated with the RNA because it persists if the reverse transcription step is omitted (with loss of the 153-bp RNA signal).

it is clear that the presence or absence of EBV genomes is not confined to one morphologic type of lymphoproliferation in the setting of HIV infection.

The high frequency of EBV association in the setting of HIV-related lymphoma is not surprising, because EBV genomes have been found within lymphoma tissue in diverse types of underlying immune dysregulation. In the setting of transplantation, almost 100% of the diffuse B-cell lymphoproliferations are EBV related. Of interest, transplant-related lymphoproliferations may have relatively limited malignant potential, because they often regress after reduction or withdrawal of iatrogenic immunosuppression. In contrast to the lymphomas arising in the general population, the transplant-associated lymphoproliferative disorders may be oligoclonal, multiclonal, or polyclonal, and chromosomal translocations or c-MYC rearrangements are characteristically absent.

Of interest, the EBV-related lymphomas in HIV-infected patients appear to be biologically distinct from the transplant-related lymphoproliferations (Table 4). Similar to other studies, the HIV-related lymphomas in the current study were predominantly monoclonal proliferations, as analyzed by Ig heavy chain rearrangements and EBV termini. The detection of only a single episomal form of EBV DNA suggests that EBV infection preceded transformation. In addition, replicative EBV genomes, observed in transplantation-associated lymphoproliferations, were not significantly detected in the current HIV-related lymphomas. Furthermore, unlike the multiple, distinct, EBV-associated clones that arise simultaneously at different anatomic sites in the setting of transplantation, the one autopsy case analyzed herein demonstrated an identical clone at all involved sites. Thus, although multiple small oligoclonal expansions have been observed in the reactive biopsies and lymphoma tissues from some HIV-infected patients, it appears that a single transformed clone can be responsible for dissemination of lymphomatous disease. The additional minor oligoclonal Ig heavy chain rearrangements noted by Pelicci et al were not detected in our lymphoma biopsies, although our assay may be less sensitive because similar minor clonal rearrangements are typically not detected in the benign hyperplastic lymph node biopsies from our population of HIV-infected patients (data not shown). These findings, along with the detection of c-MYC rearrangements in 40% of our studied cases, indicate pathophysiologic mechanisms in HIV-related lymphomas that are distinct from those found in the setting of iatrogenic immunosuppression and organ transplantation.
EBV-associated HIV-related lymphoma

EBV-related lymphoproliferative disorders can also be compared in terms of their patterns of latent viral expression. Thus, multiple EBV nuclear and latent membrane-associated antigens (EBNAs 1, 2, 3, and LMPs) are expressed in transplant-related lymphoproliferations, in lymphoblastoid cell lines, and in the EBV-associated lymphoproliferations that occur in severe combined immunodeficient mice. This EBV expression pattern has been designated “group 3” (g3). A second pattern of EBV expression, present in BL, is characterized by the expression of only the EBNA-1 nuclear antigen and is designated “group 1” (g1). The EBNA-1 mRNA transcripts are different in the two patterns because although they all include EBNA-1 sequences, the g1 EBNA-1 mRNA arises from a different EBV promoter and is differentially spliced compared with the g3 EBNA-1 mRNA. A PCR assay, using a common downstream primer for cDNA synthesis, can detect and compare the relative abundance of these two mRNAs. The predominant expression of EBNA-1 mRNA in the current HIV-related lymphomas appears restricted to the g1 pattern, similar to that found in endemic and sporadic BL. By analogy, expression of g1 EBNA-1 mRNA is characteristically associated with the absence of other g3 nuclear antigens (EBNA-2 and EBNA-3) because these g3 mRNAs all arise from the same promoters used for g3 EBNA-1 transcription. However, EBV expression may be heterogeneous in patients with HIV-related lymphoma, because EBNA-2 has been detected in the lymphoma cells of some cases with immunohistochemical methods.

Although similar to BL, the patterns of EBV expression in the current HIV-related lymphomas were not identical to the g1 model, because LMP transcripts were also detected. This pattern of latent EBV expression (g1 EBNA1+, LMP+) has been called “latency II” and can be induced in vitro by stimulating EBV-infected g1 B-lymphoblastoid cell lines. This phenotype of latent EBV expression has not previously been observed in NHL, because BL does not express LMP. Interestingly, this EBV expression pattern has been observed in Hodgkin’s disease and nasopharyngeal carcinoma. LMP has transforming properties and its observed expression in the HIV-associated lymphomas may potentially contribute to the process of lymphomagenesis. Further, LMP is an immunogenic cytotoxic target, which may prevent its expression except in the setting of immunodeficiency.

The pattern of EBV expression observed in the HIV-related lymphomas may explain the detection of EBV types A and B within tumor cells. Similar to previous studies, and analogous to endemic BL, both EBV types A and B were present in the HIV-related lymphomas. In contrast, sporadic (American) BL is characterized by a predominance of type A. EBV types A and B differ at their EBNA-2 sequences, and lymphocyte transformation is more difficult with type B virus. However, this difference observed in vitro may not be pertinent in HIV-related lymphomagenesis because the EBNA-2 gene does not appear to be strongly expressed.

The contribution of EBV to the etiology and pathogenesis of HIV-related lymphoma is currently unknown, but may be similar to that in BL because the pattern of EBV EBNA-1 transcription, timing of infection with respect to transfor-

EBV-ASSOCIATED HIV-RELATED LYMPHOMA

Fig 4. Southern blot analysis of lymphoma tissue from multiple autopsy sites from case 11. (A) Ig heavy chain analysis (HindIII digestion) demonstrates a single and similar molecular weight rearranged band at all lymphoma sites except for the uninvolved liver tissue, which only demonstrates the germline 11-kb band. (B) EBN termini analysis (BamHI digestion) demonstrates a single and similar molecular weight band at all sites except the uninvolved liver. There was no evidence of smaller linear EBV fragments. A faint, slightly higher molecular weight band was evident in the lymphoma tissue from the hilar lymph node with a longer exposure. The controls demonstrated the expected monoclonal episomal pattern (MONO, single band present on shorter exposure) and a polyclonal pattern (POLY) with linear viral fragments.

Table 4. Comparison Between Different Types of EBV-Related Lymphoproliferative Disorders

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Abbreviations: BL, Burkitt lymphoma; g1, group 1 EBV EBNA expression pattern; g3, group III EBV EBNA1 expression pattern.
...dysregulation of the immune system distinct from known examples of NHL because they coexpress in the setting of chronic malarial infestation in those regions of Africa endemic for BL. The evolution of lymphoma in the setting of HIV infection most likely involves multiple steps over a relatively short period of time, which may include EBV infection, c-MYC mutations, and other genetic alterations such as p53 inactivation. Of interest, the molecular genetic aberrations observed in this study and others are not uniform, but rather heterogeneous, and most likely reflect diverse pathogenic mechanisms in the setting of chronic exposure to multiple antigenic stimuli, characteristic of patients with underlying HIV infection.

ACKNOWLEDGMENTS

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REFERENCES

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Epstein-Barr virus-associated non-Hodgkin's lymphoma in patients infected with the human immunodeficiency virus [see comments]

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