Subtypes of Epstein-Barr Virus in Human Immunodeficiency Virus-Associated Non-Hodgkin Lymphoma

By M.J. Boyle, W.A. Sewell, T.B. Sculley, A. Apolloni, J.J. Turner, C.E. Swanson, R. Penny, and D.A. Cooper

Biopsy samples obtained from 20 patients with human immunodeficiency virus (HIV)-associated non-Hodgkin lymphoma (NHL) were assessed for evidence of Epstein-Barr virus (EBV) and HIV sequences. DNA was extracted from formalin-fixed, paraffin-embedded NHL tissue and specific viral gene sequences were sought using the polymerase chain reaction (PCR). EBV sequences were found in 10 NHL samples (50%), with five tumors showing A-type and five B-type sequences. By serologic testing, 18 of 19 patients had antibodies to EBV, with 14 patients having antibodies to A-type EBV and 11 to B-type EBV. Serology confirmed the high prevalence of type B EBV in HIV-infected patients, but was not a reliable indicator of the EBV subtype present in the lymphomas. HIV sequences were present in biopsy tissue but at a level consistent with an origin from bystander HIV-infected cells. All 20 patients were negative by enzyme-linked immunosorbent assay for antibodies to human T-cell leukemia virus-type I. The high prevalence of type B EBV in these tumors is similar to the findings in endemic Burkitt’s lymphoma, where 40% of the tumors have type B viral sequences. In normal populations, type B EBV is rarely found outside the nasopharynx. These studies support the hypothesis that EBV is an important cofactor in NHL in HIV-infected persons. The finding that B-type EBV is present in 25% of HIV-associated NHL suggests that this EBV subtype may be an important human pathogen with a wider geographic distribution than originally thought.

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diced, and sequentially washed three times in xylol, twice in 99% ethanol, and once in 95% ethanol before rehydration in water (30 minutes each). EBV and HIV control DNA was extracted from unfixed cells. Samples were incubated in DNA lysis buffer for 4 days (50 mmol/L NaCl; 10 mmol/L EDTA; 10 mmol/L TrisCl pH 8; 1% sodium dodecyl sulfate [SDS]; 100 μg/mL protease K) before phenol/chloroform extraction, RNase treatment for 3 hours at 37°C, and dialysis against 10 mmol/L TrisCl pH 8; 1 mmol/L EDTA. DNA concentration was determined by absorbance at 260 nm and 280 nm.

Amplification of HIV, Interleukin-5 (IL-5), β-Actin, and EBV Gene Sequences

Amplification was performed using the polymerase chain reaction (PCR). Each reaction was performed in a volume of 100 μL, which contained 0.5 μg of DNA sample, 10 mmol/L TrisCl pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.01% (wt/vol) gelatin, 0.5 μg of each amplification primer (see Table 1), 200 μmol/L of each dNTP, and 2 U Taq polymerase (Toyobo, Osaka, Japan). The reaction mixture was first heated for 3 minutes at 93°C and then incubated at three different temperatures using an intelligent heating block (Hybaid, Teddington, UK). To amplify HIV sequences, 36 cycles of 30 seconds at 93°C, 1 minute at 50°C, and 1 minute at 70°C were used. To amplify EBV sequences, 25 cycles of 1 minute at 93°C, 2 minutes at 55°C, and 1 minute at 72°C were used. To amplify IL-5 and β-actin sequences, 25 cycles of 1 minute each at 93°C, 50°C, and 70°C were used. The amplified products were electrophoresed in 1.2% agarose gels, transferred to Hybond N+ membranes (Amersham International, Amersham, UK) and sequences detected by DNA hybridization.

Preparation of Radiolabeled Probes

The radiolabeled EBV probes were produced by PCR amplification of each of the specific EBNA genes, using the primers shown in Table 1, with the substitution of 32P-dCTP (Amersham) for cold deoxycytidine 5'-triphosphate (dCTP) in the reaction mix. The EBNA-2A gene was obtained from the plasmid pM-BamH2, the EBNA-2B gene from the plasmid p-HKA7, and the EBNA-1 gene from the plasmid BamHI1K.

Radiolabeled probes for HIV, IL-5, and human β-actin were prepared by end-labeling synthetic DNA sequences using γ-ATP (Amersham) and polynucleotide kinase (Pharmacia, Uppsala, Sweden). These synthetic probes were designed to anneal to the PCR products at sites distinct from the primers. The sequences of the probes were for HIV: 5' GTTAAAAGCCAGGAGGAAGAAATAATA; for IL-5: 5' TGACAGTTTCTGTAATACC; and for β-actin: 5' CAGCCATGTACGTTGCCATAT.

Hybridization Conditions

EBV filters were prehybridized in 900 mmol/L NaCl, 90 mmol/L Na citrate pH 7, 5X Denhardt's solution, 0.1% SDS, 5 mmol/L EDTA pH 8, 0.1 mg/mL salmon sperm DNA, 2 mmol/L phosphate buffer pH 6.5 for 2 hours at 50°C and then hybridized overnight in the same solution with the addition of the probe. The final wash was for 30 minutes in 150 mmol/L NaCl, 15 mmol/L Na citrate pH 7, 0.1% SDS at 65°C. HIV, IL-5, and β-actin filters were prehybridized in 7% SDS, 0.25 mol/L NaPi pH 7.2, 1 mmol/L EDTA for 2 hours at 50°C and then hybridized overnight at 50°C in the same solution to which 10 mmol of radiolabeled probe had been added. The final wash was for 10 minutes at 50°C in 150 mmol/L NaCl, 15 mmol/L Na citrate pH 7, 0.1% SDS. Autoradiography was performed at ~70°C using Cronex x-ray film (DuPont, Boston, MA).

All samples were amplified and autoradiography performed for EBV genes on at least three occasions and in two separate laboratories to confirm the results and ensure reliability. However, each figure represents results obtained from a single experiment, with all samples amplified in the same PCR.

Ig gene rearrangement studies. Ig gene rearrangement studies were performed using a recently published method, which exploits primers directed toward conserved sequences of the V and J regions in a PCR. Briefly, each reaction was performed in a volume of 25 μL, which contained between 0.3 and 0.9 μg of DNA sample, 67 mmol/L TrisCl pH 8.8, 1 mmol/L 2-mercaptoethanol, 170 μg/mL bovine serum albumin, 16.6 mmol/L (NH₄)₂SO₄, 1.5 mmol/L MgCl₂, 0.2% (wt/vol) gelatin, 6.7 μmol/L EDTA, 0.25 μg of each amplification primer, 100 μmol/L of each dNTP, and 1 U Taq polymerase (Toyobo). The reaction mixture was first heated for 3 minutes at 95°C and then incubated for 1 minute at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C through 40 cycles using a Gene Machine (Innovonics, Melbourne, Australia). The amplified products were electrophoresed in polyacrylamide gels, stained with ethidium bromide, and the presence of monoclonal bands (100 to 120 bp long) determined under UV illumination. Polyclonal B-cell populations are seen as a broad band of staining in this region. Assessment by restriction enzyme digestion and Southern blotting was not possible due to both the small quantities and the degraded nature of the DNA available.

Serology. Serum samples obtained from the 20 patients were tested in two ELISAs for antibodies to HTLV-I (Genetic Systems, Seattle, WA; Abbott, North Chicago, IL). Antibodies to the EBNA-1, 2A, and 2B nuclear antigens were detected using a recently published method, which exploits primers directed toward conserved sequences of the V and J regions in a PCR. Briefly, each reaction was performed in a volume of 25 μL, which contained between 0.3 and 0.9 μg of DNA sample, 67 mmol/L TrisCl pH 8.8, 1 mmol/L 2-mercaptoethanol, 170 μg/mL bovine serum albumin, 16.6 mmol/L (NH₄)₂SO₄, 1.5 mmol/L MgCl₂, 0.2% (wt/vol) gelatin, 6.7 μmol/L EDTA, 0.25 μg of each amplification primer, 100 μmol/L of each dNTP, and 1 U Taq polymerase (Toyobo). The reaction mixture was first heated for 3 minutes at 95°C and then incubated for 1 minute at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C through 40 cycles using a Gene Machine (Innovonics, Melbourne, Australia). The amplified products were electrophoresed in polyacrylamide gels, stained with ethidium bromide, and the presence of monoclonal bands (100 to 120 bp long) determined under UV illumination. Polyclonal B-cell populations are seen as a broad band of staining in this region. Assessment by restriction enzyme digestion and Southern blotting was not possible due to both the small quantities and the degraded nature of the DNA available.

Statistics. Statistical analyses were performed using the BMDP statistical package. Proportional-hazards model analyses were computed using program P2L of the BMDP statistical package.

RESULTS

Patients

The 20 patients were all homosexual or bisexual men with no other risk factors for HIV infection. Seven patients had high-grade, malignant lymphoma, small, noncleaved cell

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Product Size (bp)</th>
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<tbody>
<tr>
<td>EBNA1-5'</td>
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<tr>
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<tr>
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Gene and pseudogene products are found with β-actin. The β-actin genes are on separate exons and the pseudogenes lack introns. Thus, a shorter sequence is amplified.
type, similar to Burkitt’s lymphoma (SNCC). Twelve patients had high-grade malignant lymphoma, large cell immunoblastic type (LCI). One patient (patient 14) had high-grade NHL by cell cytology criteria but was unable to be classified further with the available tissue. All tumors successfully phenotyped were of B-cell origin (N = 16). Four patients (nos. 8, 13, 14, and 16) were unable to be phenotyped. Patients 13, 14, and 16 had no further material available for phenotyping after DNA extraction. Patient 8 had a tumor that was positive for leukocyte common antigen, but did not mark for CD20, MB2, IgG, M, κ, λ, CD3, and UCHT1. Seventy percent of tumors had monoclonal bands evident using the PCR technique to assess clonality (data not shown). This rate of monoclonality is similar to that found by the original investigators of this technique, who suggested that the failure to show monoclonality in all patients was due to aberrant rearrangements, such as inversions of the heavy chain locus.24-26 Using both immunohistology and gene rearrangement criteria, only patient 14 was not shown to have a B-cell tumor. Nine patients had limited nodal disease at presentation (5 = LCI; 4 = SNCC), whereas 11 had disseminated or extranodal disease at presentation (3 = SNCC; 7 = LCI). The details are given in Table 2.

Detection of EBV Sequences in NHL Biopsy Samples

To identify the presence of EBV sequences in the biopsy samples, primers complementary to regions within the EBNA-1 gene were used in the PCR. This region was chosen because every strain of EBV must contain this gene to be stably expressed in transformed cells. As a control, DNA extracted from the EBV-transformed cell line QIMR-JS/B95-8 (in which all cells contain EBV sequences) was included in each set of PCR amplifications. Amplified DNA was visualized by hybridization with a 32P-labeled probe and the intensity of this reaction in samples was compared to the reactions obtained with the control DNA (Fig 1). Significant amounts of EBV DNA were detected in 10 of the biopsy samples (patients 1 through 7, 12, 17, 18) and low levels in biopsies from patients 10, 14, and 19 (detected after prolonged exposure of the autoradiograph).

Sample quality, as assessed by ethidium bromide staining of gels, showed significant and variable degradation of the DNA derived from formalin-fixed tissues. Thus, it was possible that the low level of EBV detected in the biopsy material from patients 10, 14, and 19 was due to extensive degradation in these samples. Therefore, control studies were necessary to assess and normalize each of the samples for amounts of amplifiable DNA. This was accomplished by using primers directed against the IL-5 and β-actin genes in PCR reactions. The results, shown in Fig 1, demonstrate that there was significant variability between the samples in regard to the amount of amplifiable DNA, even though 0.5 μg of DNA (as assessed by absorbance at 260 nm) was used in each PCR. Amplification of both the IL-5 and β-actin genes yielded similar results, with the biopsies of patients 5, 6, 10, and 14 showing the greatest amounts of amplifiable DNA. The discrepancy between the amounts of total DNA and PCR-amplifiable DNA reflects the degree of degradation of the DNA in these samples. Even after normalization of the samples for excessive degradation of the DNA, it was apparent that only patients 1 through 7, 12, 17, and 18 contained significant amounts of EBNA-1 gene sequence. This conclusion was supported by titration of biopsy sam-

### Table 2. Patient Details and Serology

<table>
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<tr>
<th>Patient</th>
<th>Tumor Type</th>
<th>Histology</th>
<th>Presentation</th>
<th>CDC Group at Diagnosis</th>
<th>CDA Count at Diagnosis</th>
<th>Survival (d)</th>
<th>EBNA-1 Serology</th>
<th>EBNA-2A Serology</th>
<th>EBNA-2B Serology</th>
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</table>

*The patient number refers to the same patient as in other figures. Serology results are recorded as + when antibody was detected and 0 where undetected. Dashes indicate a result was not available.

Abbreviation: EN, extranodal disease.

*The patient is still alive, and the survival was calculated to September 30, 1990.
EBV SUBTYPES IN HIV-ASSOCIATED NHL

There was also a trend for EBV tumor incorporation in the less immunodeficient patients. Eight of the 14 patients with CDC group II or III disease at diagnosis had EBV genome in their tumor DNA (57%), whereas only two of the six patients with a prior acquired immunodeficiency syndrome (AIDS) diagnosis had EBV in their tumors (33%; \( P = .62 \), two-tailed Fisher’s exact test). This trend was also evident when CD4 cell count was used to assess immunodeficiency, as 5 of the 8 patients with T4 counts over 200 \( \times 10^6 \) cells/L at diagnosis had tumor EBV, whereas 4 of the 10 patients with counts less than 200 \( \times 10^6 \) cells/L at diagnosis had EBV detectable in their tumors (\( N = 10; 40%; P = .64 \), two-tailed Fisher’s exact test).

There was no significant survival difference between those patients with EBV gene sequences in tumor tissue DNA and those without such sequences, nor was there a survival advantage for one EBV subtype over another.

Serology for EBV and HTLV-I

Nineteen patients were tested for antibodies to EBV nuclear antigens 1, 2A, and 2B (Table 2). The sera of all 10 patients with EBV in their tumor tissue contained antibodies to EBV antigens. Many of these sera showed dual reactivity to EBNA-2A and -2B antigens. EBV serology did not reliably predict the subtype of EBV in the tumor, nor was it helpful in predicting which patients had EBV-related tumors, as almost all subjects showed some reactivity to EBV antigens.

The HTLV-I serology was negative by both ELISAs in all 20 patients. Therefore, this retrovirus is unlikely to contribute to the pathogenesis of HIV-associated NHL.

Detection of HIV Sequences in NHL Biopsy Samples

HIV sequences could be found in nine of the patient samples after prolonged exposure of the autoradiograph (Fig 1). The patient samples were compared with dilutions of DNA from an HIV-infected cell line (Hut PW) amplified under the same conditions. Fifty picograms of cell line DNA was able to produce a signal equivalent to 500 nanograms of the strongest responding tumor-derived DNA (patient 12; Fig 1), a dilution of 1 in 10,000 (Fig 3). Again, the biopsies contained predominantly but not exclusively NHL tissue. Thus, the results of these titering experiments, though not strictly quantitative, suggest that the source of these sequences is HIV infection of surrounding, nonmalig-
Fig 2. PCR for the EBNA-1 gene. Samples 3 and 17 were amplified for EBNA-1 sequences starting with the following amounts of DNA (ng): tracks A, 500; tracks B, 50; tracks C, 5; tracks D, 0.5. Samples 2, 4, and 19 were amplified using 500 ng DNA. Molecular weight markers (bp) are shown on the left.

nant cells. The result suggests HIV does not play a direct role in these tumors.

DISCUSSION

Ten of the 20 NHL patients in this study had EBV DNA in their tumor samples. This is consistent with previously published work using both Southern analyses and in situ techniques to assess the incidence of EBV in NHL tumors. Five of the patients in the present study had type B EBV and five had type A virus. Evidence for the presence of HIV or HTLV-I in these tumors was not obtained, confirming previous reports.

We were constrained by two difficulties in assembling the present collection of patient samples. Firstly, to obtain a reasonable number of samples for analysis, it was necessary to use formalin-fixed specimens, rather than awaiting fresh material from new cases. Secondly, in a number of cases, the available biopsy was very small. We chose to use PCR to analyze these samples because it is effective on formalin-fixed tissue and it requires only small amounts of tissue for analysis. A significant limitation with the PCR is that, because of its high sensitivity, it is possible that the positive signals for EBV and HIV DNA reported in this work may have arisen from small numbers of nontumor cells present in the biopsy. However, this is unlikely on a number of grounds. The overall frequency of EBV-positive tumors in this series is within the range published by others using different techniques. Furthermore, 90% of the patients had positive EBV serology (Table 2) whereas only 50% of the tumors were positive for EBV sequences (Fig 1). Therefore, we found 40% of the tumors did not contain EBV sequences, although they arose in patients with positive serology for EBV and in whom nontumor cells containing EBV DNA might have caused us to classify the tumors as positive for EBV. Indeed, three samples showed weak but consistently detectable EBNA-1 sequences (patients 10, 14, and 19; Fig 1). These sequences were only weakly present compared with the positive controls (Fig 2), a finding consistent with the notion that they were present in a minority of cells. Further evidence that the EBV signals arose from tumor cells was that in each EBV-positive tumor sample, only one EBNA-2 subtype was detected, although in 7 of these 10 cases both subtypes were detected serologically.

Initial studies of B-type EBV had suggested its distribution was limited to Central Africa and New Guinea. Later, B-type virus was found to be shed frequently from the nasopharynx of both HIV-infected and normal subjects, but peripheral blood B cells of normal subjects from North America who secreted both types of EBV showed only A-type virus incorporation. Work in progress suggests that B-type EBV is found in the peripheral blood of HIV-infected individuals (Kyaw MT, Harren L, Evans L, Moss DJ, Cooper DA, Benson E, Esmore D, Sculley TB: in
preparation). The studies reported here show that B-type EBV is present in nodal and extranodal sites in HIV-infected persons. Thus, it appears that B-type EBV has a much wider geographic distribution than originally thought and may be an important pathogen in HIV-infected subjects.

The serologic findings reported show that dual infection with both EBV subtypes is common in HIV-infected persons, as 11 of the 20 patients (55%) had specific antibodies to B-type virus and 10 (50%) had specific antibodies to both type A and B virus. Recent studies by another group showed dual secretion of EBV subtypes in throat washings from 50% of EBV secreting HIV-infected patients, again suggesting that many HIV-infected patients harbor both viral types.**1** HIV-infected subjects are known to be defective in their ability to regulate EBV-infected B cells,**11** and the HIV-induced deficiency in cell-mediated immunity in these patients may cause type B EBV spread beyond the nasopharynx. The equal frequency of A- and B-type EBV incorporation in lymphoid tumors from HIV-infected patients may be a reflection of the similar levels of infection of these two virus types in these subjects.

The high prevalence of type B EBV found in HIV-associated NHL is similar to the findings in endemic Burkitt's lymphoma, in which 40% of the tumors have type B viral sequences.**13** This contrasts with the pattern of the c-myc translocation, found in 75% of HIV-associated NHL, that is typical of sporadic rather than endemic Burkitt's lymphoma.**17,32,33** Three cases of phenotypically indeterminate HIV-associated NHL, which on molecular analysis proved to be EBV-positive, monoclonal B-cell tumors with germline c-myc on restriction enzyme analysis, have also been described.**33,34** Thus, HIV-associated NHL has both similarities and differences with endemic Burkitt's lymphoma. The pattern of EBV subtyping and the relatively high frequency of EBV incorporation in tumor DNA is reminiscent of endemic Burkitt's lymphoma, but the c-myc changes are more typical of the sporadic form. Also, c-myc rearrangement and EBV incorporation in tumor DNA is found in all HIV-associated lymphoma histologies, not limited to the Burkitt-like, small noncleaved cell tumors.**17,34-36**

There are also distinctions between the lymphoproliferations of HIV-infected patients and those found in patients with other forms of immunodeficiency. Small, noncleaved cell histology is not a regular feature of posttransplant or chemotherapy-induced NHL.**6** We and others have also found, using various techniques, that EBV is not associated with the lymphadenopathy syndrome of HIV infection**17,39** (Boyle MJ, Sculley TB, Cooper DA, Turner JJ, Penny R, Sewell WA; in preparation), though it is a common finding in the benign and malignant lymphoproliferations of transplant recipients, in whom antiviral therapy is sometimes effective in controlling disease.**30,41** The nature of the immunodeficiency is different in the various disease states and this will have an impact on the regulation of EBV and other infective agents, as well as a potential impact on the translocation of c-myc and any subsequent NHL. Indeed, endemic Burkitt's lymphoma is thought to arise in the setting of an immune deficiency consequent to malaria.
infection. It may be that the explanation for these variations in c-myc translocations and in tumor histologies lies in the subtle differences in immune deficit in different patient groups, rather than being linked to the presence or absence of EBV in the tumor. Further studies will be needed to address this question.

EBV sequences tended to be more prevalent in the less immunodeficient patients, as assessed both by CDC group and CD4 cell count before the diagnosis of NHL, though the result did not reach statistical significance in the small number of patients studied. B-cell proliferation and tumor development may be more dependent on EBV-driven mechanisms in less immunodeficient patients. As immunodeficiency progresses, other mechanisms of B-cell proliferation and tumor induction may become more potent. Such alternative mechanisms may involve other viral cofactors, chronic antigenic stimulation, or drug-induced changes to the cells, as a number of the drugs used in patients with HIV infection have tumor-inducing potential. Further studies will be needed to address these questions.

These studies suggest that EBV is an important cofactor in the development of NHL in HIV-infected persons and confirm previous reports that show no direct involvement of HIV or HTLV-I in these tumors. B-type EBV has previously been reported in oral hairy leukoplakia, and the finding that this virus is present in 25% of HIV-associated NHL supports the thesis that this type of EBV is an important human pathogen with a worldwide distribution.

ACKNOWLEDGMENT

The primers and methodology for amplification of HIV were a generous gift from Brian Nightingale (Rachel Forster Hospital, Sydney, Australia). We also thank Dr A. Morley and M. Brisco for their help with the studies of clonality using the PCR.

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Subtypes of Epstein-Barr virus in human immunodeficiency virus-associated non-Hodgkin lymphoma

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