Epstein-Barr Virus–Related Oral T-Cell Lymphoma Associated With Human Immunodeficiency Virus Immunosuppression

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Epstein-Barr virus (EBV) is generally held to infect B cells and epithelial cells, although there are now reports of EBV infection in normal T cells and neoplastic T-cell diseases. In patients with human immunodeficiency virus (HIV) infection, EBV is associated with the benign epithelial lesion, hairy leukoplakia, and has been reported in up to 80% of acquired immunodeficiency syndrome (AIDS)-related B-cell lymphoma. This study shows the presence of EBV in malignant oral T-cell lymphoma in three AIDS patients, two of whom had concurrent manifestation of hairy leukoplakia. The T-cell lineage of the tumor cells was determined by positive immunophenotyping for T-cell markers and lack of B-cell or nonhematopoietic (cytokeratin) determinants. All tumors contained monoclonal T-cell populations shown by polymerase chain reaction, which showed amplification of T-cell receptor γ chain DNA without evidence of Ig heavy chain gene rearrangement. Furthermore, these lesions showed the presence of EBV DNA and expression of EBV latent gene products in the tumor cells. EBV involvement in AIDS-related T-cell lymphoma has not been widely reported and may represent a further manifestation of opportunistic EBV infection arising in the HIV-immunocompromised host.

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EPSTEIN-BARR VIRUS (EBV) is a human herpes virus that infects B cells and certain squamous epithelia. Both cell types are thought to contribute to the long-term carriage of EBV in vivo. Full replication with virus production takes place in oropharyngeal epithelium followed by a latent/nonproductive virus infection in circulating B cells. In immunocompetent individuals, this persistent cellular infection by EBV is under strict immunologic control and rarely causes severe disease in normal seropositive individuals. In circumstances of severe immune suppression, it is now well recognized that EBV is closely associated with a range of diseases that histogenically reflect the host cell targets of infection. These diseases include rapidly progressive B-lymphoproliferative disorders associated with congenital immunodeficiency or with iatrogenically induced immunosuppression in bone marrow and organ transplant recipients, undifferentiated B-cell lymphoma in patients with acquired immunodeficiency syndrome (AIDS) in which 40% to 77% of tumors are associated with EBV, and the benign AIDS-related oral lesion, hairy leukoplakia (HL), which is a unique clinical manifestation of productive EBV infection in squamous epithelium.

The restricted target-cell specificity for EBV has lately been challenged by evidence that normal T cells can support stable EBV infection in vitro, and by reports of EBV DNA and/or viral gene products in various T-cell disorders ranging from relatively benign T-cell hyperplasia to aggressive T-cell lymphoma (TCL). Although evidence of abnormal EBV serologic patterns in a small number of cases has been suggested to reflect some degree of immune impairment, and may contribute to the development of some of these lesions, hitherto close association between clinically overt immunosuppression and EBV-related TCL has not been widely reported.

In this study, we have investigated oral TCLs arising in three AIDS patients for the presence of EBV-DNA using in situ hybridization, DNA amplification by polymerase chain reaction (PCR), and immunophenotyping techniques. Demonstration of EBV genome and virus latent gene products in each tumor sample has important implications on the role of EBV in the pathogenesis of these lesions, and the possible effect(s) of human immunodeficiency virus (HIV)-induced immunosuppression in widening the range of target cells susceptible to EBV infection in vivo.

MATERIALS AND METHODS

Patients: All patients were homosexual men with no history of intravenous (IV) drug use.

Case 1, aged 40 years, presented in May 1991 with a 2-week history of painful ulceration of the right hard palate and clinically suggestive HL on the lateral tongue margin. The patient was found to be HIV seropositive, but at this time had no overt clinical symptoms of AIDS. Hematologic investigation showed evidence of anemia (hemoglobin [HB], 9.5 g/dL; red blood cell count [RBC], 4×10^12/L; mean corpuscular volume [MCV], 79 fL; mean corpuscular hemoglobin [MCH], 23.6 pg; MCH concentration [MCHC], 29.8%), elevated platelet count (503×10^9/L), and white blood cell (WBC) values within the normal range (WBC, 6.9×10^9/L; mean corpuscular hemoglobin [MCH], 23.6 pg; MCH concentration [MCHC], 29.8%), elevated platelet count (503×10^9/L), and white blood cell (WBC) values within the normal range (WBC, 6.9×10^9/L; lymphocytes, 1×10^9/L). EBV serology showed a normal seropositive profile; the serologic status for human T-lymphotropic virus (HTLV)-I was not determined. The 2-cm diameter indurated palatal ulcer showed radiographic evidence of interdental bone loss. Biopsies of the oral lesions showed histologic appearances consistent with a T-cell lymphoma in the palatal ulcer, and typical HL changes in the tongue lesion. The patient was started on zidovudine (AZT) and acyclovir, and referred for management of his lymphoma. Four weeks after presentation, his condition had deteriorated rapidly with symptoms of progressive anemia, ascites, and hepatosplenomegaly. He had evidence of marked immunosuppression with low CD4 count, and liver dysfunction with raised alkaline phosphatase. A computer tomography (CT) scan showed multiple hepatic nodules. The patient was started on PACE-BOM cytotoxic...
AIDS-RELATED EBV+ T-CELL LYMPHOMA

Fig 1. Tongue biopsy in case 3 showing highly pleomorphic tumor cells with frequent mitoses. (Hematoxylin and eosin; original magnification ×630).

chemotherapy (prednisolone, diaminopyrimidine, cyclophosphamide, etoposide alternating with bleomycin, vincristine, and melphalan for 12 cycles). Treatment was completed in November 1991 and had produced complete regression of the oral tumor. To date, the patient is well but has evidence of persistent liver disease.

Case 2, aged 57 years, was diagnosed as HIV seropositive and began AZT therapy in September 1990, after he presented with a 3-month history of night sweats, fever, and weight loss. Symptoms resolved with treatment but returned in May 1991, at which time he had developed skin Kaposi sarcoma, oral symptoms of candidiasis, acute necrotizing ulcerative gingivitis, and a large midline interdental swelling of the upper jaw. He also had a hyperkeratotic lesion, suggestive of HL, on the lateral tongue margin. Serologically, the patient was toxoplasma positive and HTLV-1 positive; EBV serology was not determined. After biopsy of the gingival swelling and tongue lesion, the patient was treated with acyclovir, radiotherapy, and chemotherapy. The tumor showed a rapid response to treatment with complete regression. However, the patient continues to show evidence of progressive AIDS.

Case 3, aged 49 years, had a long history of AIDS treated with AZT and in May 1991 developed a rapidly growing midline tongue ulcer, 3 cm in diameter, that was biopsied. The patient was HTLV-I seronegative and EBV serology was not investigated. At this time, the patient was undergoing terminal care and treatment of the oral tumor was directed at alleviating symptoms only. The patient died 3 months later.

**Biopsy analysis.** Cell marker profiles were examined in tissue sections from conventionally processed biopsies of the oral tumor and HL from case 1 and snap-frozen tumor biopsies from cases 2 and 3. The HL biopsy from case 2 was not available for analysis. Samples were examined with monoclonal antibodies (MoAbs) to formalin-resistant determinants of common leukocyte (CD 45R; Dako, Ltd, High Wycombe, UK), T-cell (MT1; Bionuclear, Reading, UK; CD3; Dako, Ltd), B-cell (L26/CD20; Dako, Ltd), and epithelial pan-cytokeratin (PKK1; Dako, Ltd) antigens. Cryostat sections of tumor biopsies from cases 2 and 3 were analyzed with an additional panel of MoAbs to (1) T-cell-related antigens, βF1 reacting with the β subunit of the αβ T-cell receptor (TCR) molecule (Lab Impex Ltd, Teddington, UK), CD2 (Dako, Ltd), CD4 (Coulterclone, Luton, UK), CD8 (Coulterclone and UCH T4 from the ICRF Human Tumour Immunology Laboratory, London, UK), mucosal lymphocyte antigen (MLA-BerAct 8; Dako, Ltd), CD30 (Ki-1; Dako, Ltd; (2) CD21/EBV-C3d binding receptor antigen (HB5; Becton Dickinson, Oxford, UK; OKT7; Orthomune, Ortho Diagnostics, High Wycombe, UK; B2; Coulterclone); class I (W6/32) and class II (1B5) major histocompatibility (MHC) antigens (MoAbs from the ICRF Tissue Antigen Laboratory); and (4) cell adhesion molecules, CD54 (leukocyte function antigen [LFA]-3) and CD58 (intercellular adhesion molecule-1 [ICAM-1]), which, respectively, were detected with MoAbs TS2/9 and RR1/11 (supplied by Prof T. Springer, Laboratory of Immunocytochemistry, Dana Farber Institute, Boston, MA). Phenotypic evidence of EBV latent infection was investigated with human sera containing high activity to EB nuclear antigens (EBNAs) 1 through 4 and 6, using the anti-complement immunoperoxidase (ACP) method, and MoAbs to EBNA species 2 (PE-2; supplied by Dr L. Young, Cancer Research Campaign Laboratories, University of Birmingham, Birmingham, UK), and the EBV latency-associated membrane protein (LMP) (CSI-4; Dako, Ltd). EBV lytic cycle antigen expression was investigated with MoAbs to virus early antigens (EA) (1108-I; Biogenesis, Bournemouth, UK), viral capsid antigens (VCA) (F323; Seralab, Sussex, UK), and the membrane antigen (MA) complex (H140; Seralab). Evidence of HIV infection was investigated with MoAb to p24 HIV core protein (KL1; Dako Ltd). MoAbs were detected by the peroxidase antiperoxidase (PAP) method.

**DNA studies.** EBV DNA was analyzed in all samples by nonisotopic in situ hybridization (ISH), as previously described, and by PCR. For ISH, the 3.1-kb BamHI W restriction fragment of the EBV genome cloned in pBR322 was biotinylated by nick translation (Amersham) and detected immunocytochemically with goat antibody (G) antibiotin and biotinylated rabbit anti-Ig antibodies and avidin-biotin-peroxidase complex (Dako Ltd). Biotin-labeled plasmid served as negative DNA probe and the system was controlled on EBV-positive (Raji, P3HR1) and -negative (Ramos) Burkitt lymphoma cell lines. For PCR amplification of DNA, three 5-mm paraffin sections from the tumor biopsies were suspended in 40 mL of a sterile solution of NaCl (200 mmol/L) and EDTA (100 mmol/L) to which 5 mL of proteinase K (10 mg/mL) was added, and the mixture was incubated at 37°C for 48 hours to release tissue DNA. The mixture was then boiled for 10 minutes to inactivate the proteinase K. Positive and negative control DNA was extracted from an EBV-positive B-cell line and an EBV-negative B-cell tumor line. Extracted DNA was amplified as previously described. Oligonucleotide primers were synthesized to a predicted 286-bp fragment of the EBV BZLF1 gene. Primer sequences were (1) 5'ACAGTAGAATTGTGCCTCCAGG3' and (2) 5'GACCAAGCTACCAGAGTC-
Table 1. Molecular and Phenotypic Profiles of EBV+ Oral TCL in Three AIDS Patients

<table>
<thead>
<tr>
<th>Case/Age/Sex</th>
<th>Phenotype</th>
<th>Genotype</th>
<th>EBV/DNA*</th>
<th>EBV Profile</th>
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<tr>
<td>1/40/M</td>
<td>CD45R+, MT1+, CD3+, CD20+</td>
<td>TCR γR, IgH G</td>
<td>+</td>
<td>NT</td>
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<tr>
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<td>TCR γR, IgH G</td>
<td>+</td>
<td>EBNA 1-4, 6+, 2+, LMP+, EA-, VCA, MA-</td>
</tr>
<tr>
<td>3/49/M</td>
<td>CD45R+, MT1+, CD3+, CD2+, CD8+, CD30+, CD20+, CD21+, MHC I+, II+ CD54+, CD58+</td>
<td>TCR γR, IgH G</td>
<td>+</td>
<td>EBNA 1-4, 6+, 2+, LMP+, EA-, VCA, MA-</td>
</tr>
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Abbreviations: R, rearranged; G, germline; NT, not tested.
* EBV-DNA detected by PCR and ISH.

RESULTS

Histology. The tumors in each case showed similar histologic features with extensive subepithelial lymphocytic infiltrates of large atypical malignant cells exhibiting marked cellular pleomorphism and frequent mitoses (Fig 1). Overlying epithelium in cases 1 and 2 showed no evidence of tumor cell infiltration, although minimal epidermotropic small lymphocytes were observed. In case 3, the large tumor extended deeply into muscle and was covered by ulcer slough. The tongue biopsy from case 1 showed many typical histologic features of HL, including patchy acanthosis, koilocytic vacuolation of the prickle cell layer with nuclear disintegration, and parakeratosis of overlying epithelium, which contained candida hyphae.6 However, superficial epidermal hyperparakeratosis, which contributes to the typical “hairy” appearance of these lesions, was not a feature.

Immunophenotyping studies (Table 1). Morphologically malignant cells in all tumor samples were CD45R+. In case 3, the majority of cells showed strong staining for βF1, CD2, CD8, as well as CD30 antigens, and weak activity for CD21/EBV-binding receptor antigen. In case 2, the weak CD3+ tumor cells showed no activity for these markers. In both samples, small reactive CD3+, CD2+, CD4+, or CD8+ lymphocytes were identified among the tumor cells, and in case 2, low numbers of MLA-BerAct 8–positive cells were shown in subepithelial and intraepithelial areas of overlying mucosa. Tumor cells in both cases were MHC class I+, CD58+, and, in case 3, the cells were also MHC class II+, CD54+. Neither tumor showed positive staining for HIV p24 core protein.

Virus studies (Table 1). Nuclear EBV DNA hybridization signals were detected in most tumor cells of all samples (Fig 3) and were not identified in small lymphocytes or in overlying squamous epithelium. Frozen sections from samples 2 and 3 showed characteristic granular nuclear staining for EBNA species 1 through 4 and 6 within the tumor populations (Fig 4) and diffuse nuclear staining for EBNA-2 in a proportion of cells. Both samples contained low numbers of large atypical cells that exhibited strong cytoplasmic/membrane staining for EBV-LMP as defined with MoAb CS1-4. No EBV lytic cycle antigens were shown unequivocally in either of these tumors, although atypical weak cytoplasmic

Fig 2. Tongue biopsy in case 3 showing CD2+ tumor cell infiltration between muscle fibres (PAP staining; original magnification X 400).
EBV-VCA staining was seen in most tumor cells from case 3. Gel electrophoresis of PCR reaction products showed bands of amplified EBV DNA in each sample (Fig 5A).

The HL sample analyzed from case 1 showed intense EBV DNA signals (not shown) confined to the upper spinous layer of vacuolated epithelium in a pattern that is characteristic for this lesion and that has been widely reported. There was no evidence of EBV infection in adjacent morphologically normal suprabasal and basal epithelium.

Gene rearrangement studies. PCR analysis of tumor DNA extracts with primers to TCR γ chain gene produced a single discrete band of amplified DNA of similar (approximately 270 bp) but not identical size in each sample, indicating the presence of a clonal T-cell population (Fig 5B). No reaction product was detected with IgH chain primers and negative control DNA showed no evidence of contamination.

DISCUSSION

The presence of EBV in three high-grade malignant oral TCLs described in this study and the development of these lesions in the context of HIV immunosuppression provides another dimension to the newly emerging group of T-cell diseases associated with EBV. EBV has been documented in individual cases or small patient series of transient benign T lymphocytosis associated with acute EBV infection (infectious mononucleosis),9 large granular lymphoproliferative disease (LGLPD) of T-cell origin,10 angioimmunoblastic lymphadenopathy (AILD) of T-cell origin,20 Kawasaki-like disease,21 and nodal and peripheral TCL,10-14 including nasal TCL,15 formerly known as lethal midline granuloma. Recently, Ott et al22 have shown EBV DNA in 35% TCL of various histologic types arising in nonimmuno-compromised patient groups, and a high proportion of these tumors have been shown to occur at extranodal sites.22,23 T-cell proliferative disease has been reported in HIV-infected patients, although less commonly than the occurrence of AIDS-related B-cell lymphoma, of which 40% to 77% are EBV associated.34 T-cell malignancies in AIDS patients encompass a wide range of pathologies, including LGLPD, described as Tγ lymphoproliferative disease, CD8 T-cell lymphocytosis, T-cell chronic lymphocytic leukemia,
AILD, Sezary syndrome, and angiocentric T-cell lesions consistent with postthymic TCL, as well as anaplastic/large-cell TCL. Some of these tumors may also contain integrated viral sequences for HIV and/or HTLV-1. The presence of EBV in AIDS-related TCL has not been reported widely. An EBV DNA containing immunoblastic TCL shown by dot blot hybridization was reported by Borisch-Chappuis et al. and by Pallesen et al, who identified limited EBV gene expression in an AIDS-CD30 lymphoma with restricted T-cell phenotype, although virus DNA was not detectable by ISH in this case. Highly variable T-cell phenotypes are well documented in peripheral TCLs in both HIV-infected and noninfected patients. In the present study, notwithstanding the partial activity of the tumor cells with pan-T- and T-cell subset markers, particularly in case 2, all three patient biopsies were phenotypically and genotypically assigned to the T-cell lineage and B-cell markers were negative in each case. All samples contained a single clonal TCR chain rearrangement without evidence of IgH chain rearrangement, which is in keeping with a clonal T-cell proliferation. In addition, the TCR-amplified products were all of a similar size, which may reflect selective use of TCR V and J regions in this type of malignancy. Evidence of clonal T-cell proliferation also excluded misdiagnosis of a T-cell–rich B-cell lymphoma, a distinct clinicopathologic entity, in which malignant B-cell populations mixed with abundant reactive T cells may mimic a T-cell neoplasm. These tumors exhibit germline TCR gene configuration and clonally rearranged Ig genes, and may contain EBV DNA.

EBV investigation of the lesions in the present study was prompted by presentation of lymphoma in close proximity to clinically suggestive HL on the lateral tongue border in two cases. ISH identification of EBV-DNA in each tumor sample was confirmed by PCR analysis. By comparing the frequency of ISH signals in the tumor cells with reference control EBV-infected cell lines, the viral genome content was estimated to be in the region of 50 genome copies/tumor cell. Relatively low viral copies are associated with the latent/nonproductive type of EBV infection in B cells. This finding contrasts with the intense DNA ISH signals associated with high levels of virus production shown in the HL biopsy from case 1. Evidence of EBV latent gene expression and absence of lytic cycle antigens in the cryopreserved lymphoma samples strengthens the concept of latent EBV infection in these T-cell lesions. The extent and biologic effects of EBV infection in normal T cells in vivo are far from clear, and the significance of the present findings can be conjectured only by parallels with established data on EBV latency in B cells and the fully permissive type of virus infection associated with squamous epithelium.

In vitro, EBV polyclonally activates B cells, resulting in the generation of immortalized B-lymphoblastoid cell lines. These are latently infected with virus and typically express eight latent viral proteins, EBNA species 1 through 6 and LMP 1 and 2. Expression of EBNA2 and LMP 1 are essential for continued B-cell proliferation and persistence of the latent viral cycle. The EBNA 1, 2, LMP-positive tumor cell phenotype shown in two tumors of this study has been reported also in nasal TCL. This finding suggests that EBV may have an immortalizing role in T cells in some cases, similar to the direct causal role suggested for EBV in transplantation-associated B-lymphoproliferative diseases. In posttransplantation B-cell tumors, full latent viral gene expression is consistent with sustained EBV-induced B-cell proliferation due to the loss of effective EBV-specific T-cell surveillance in the immunocompromised host. Using Southern blot analysis, Bonagura et al. have shown one case of EBV-TCL to contain circular/episomal EBV DNA, consistent with a latent infection, as well as linear EBV DNA fragments, suggesting that concomitant virus production might occur in these lesions. However, no clear evidence of lytic cycle antigen expression was shown in the tumors of the present study, although weak anti-VCA staining was present in the sample from case 3. On the other
hand, immediate early EBV replicative cycle antigen, BZLF1, thought to be involved in disruption of virus latency and activation of lytic cycle genes, has been shown in a minority population of tumor cells in one AIDS-related TCL. The cells lacked expression of late lytic cycle antigens (EA, VCA, and MA), suggesting incomplete or defective virus production in the tumor.

Serum Ig abnormalities and serologic evidence of abnormally persistent EBV infection have been reported in some EBV T-cell-related diseases, suggesting that immune impairment, or excessive T-cell activation, might predispose to the development of these lesions in some patients. The present finding of EBV-related TCLs arising with severe HIV immunosuppression endorses this notion. The increased frequency of EBV reactivation and high levels of orally excreted virus in HIV-seropositive patients may increase the susceptibility of T cells to EBV infection, and give rise to T-cell lesions located in oral mucosa. The functional T-cell abnormalities in early HIV infection, before T-cell numbers decline, might favor these cells as targets for infection. EBV entry into T cells has been suggested to involve the CD21 antigen/EBV-binding B-cell receptor, which is expressed on normal T cells, or a CD21-related EBV-binding antigen identified on phenotypically immature T-cell lines in vitro. The weak or negative staining for CD21 antigen on the tumors in this study, and on EBV-TCL in non-HIV-infected subjects, reflects previous observations that CD21 expression is transient on T cells and lost after EBV internalization in B cells in vitro. Alternatively, co-infection with HIV or other T-cell tropic viruses might facilitate EBV T-cell entry, although there was no evidence of HIV p24 antigen in two tumors and cases 2 and 3 were HTLV-1 seronegative.

AIDS-related TCLs are not common and are not routinely subjected to virus investigation. This study highlights the occurrence of EBV involvement in AIDS-related oral TCL and particularly the association of HL as a possible risk factor. Studies are now needed to determine the prevalence of EBV as well as other co-infecting viral agents in these lesions. Detailed clinicopathologic investigation in conjunction with in vitro functional analyses will provide further insight into the role of EBV and severe immunosuppression in the development of these tumors.

REFERENCES


Epstein-Barr virus-related oral T-cell lymphoma associated with human immunodeficiency virus immunosuppression

JA Thomas, F Cotter, AM Hanby, LQ Long, PR Morgan, B Bramble and BM Bailey