Subtypes of Epstein-Barr Virus (EBV) in Hodgkin's Disease: Association Between B-Type EBV and Immunocompromise


Epstein-Barr virus (EBV) has been associated with Hodgkin's disease (HD) in up to 50% of cases, but the subtype of EBV involved has only recently been studied. In this report, biopsy samples from 30 patients with HD were assessed for EBV sequences using both the polymerase chain reaction (PCR) and in situ hybridization (ISH). EBV sequences were localized to the malignant Reed-Sternberg cells and their mononuclear variants (Hodgkin's cells) in 9 of the 30 cases, with 7 demonstrating A-type and 2 B-type EBV sequences. Both of the patients with B-type EBV-associated HD had features to suggest pre-existing immune compromise: one was infected with human immunodeficiency virus (HIV) and had severe CD4+ T-lymphocyte depletion; the other was a debilitated elderly patient with dementia. A previous study suggested that A-type EBV alone is associated with HD and the finding of predominantly A-type EBV in the present series is in keeping with this report. The presence of B-type EBV in the HD of patients with pre-existing immunodeficiency, taken together with the recent report that B-type EBV occurs in HIV-associated non-Hodgkin's lymphoma, suggests that B-type EBV may be an important human pathogen in immunocompromised patients.

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Materials and Methods

Patient characteristics. The clinical details of 30 patients were collated from the hospital medical records and from discussions with the attending physician. The classification and staging of HD was performed using the Rye classification and the Cotswold modification of the Ann Arbor system.22-24 Immunohistology was performed on fresh frozen or formalin-fixed tissue for B- and T-lymphocyte-specific, as well as Reed-Sternberg cell-specific antigens, using, where possible, Dako antibodies to CD45 (LCA), CD20 (L26), CD3 (Ber-H2), IgM, IgG, IgA, CD3 (UCHT1 and T3-485) (Dako, Carpinteria, CA), and CD15 (Leu M1); Becton Dickinson, Mountain View, CA). HIV infection was detected in serum by multiple enzyme-linked immunosorbent assays (ELISAs; Wellcome Diagnostics, Dartford, UK; Genetic Systems Corp, Seattle, WA) and confirmed by Western immunoblot (BioRad, Richmond, VA).25 Flow cytometric analysis (Epics Profile; Coulter Corp, Hialeah, FL) of T cells and their subsets was performed as previously described.26

ISH. ISH for EBV sequences was performed using a commercially available kit (Pathogene: Enzo Diagnostics Inc, Syosset, NY). The EBV probe was derived from the 3.1-kb BamHI V, internal repeat sequence of the virus, which is distinct from the EBNA-2 gene. Formalin-fixed, paraffin-embedded sections were deparaffinized with xylol and rehydrated before proteinase K digestion (0.25 to 1 μg/
mL) for 5 to 15 minutes at 37°C. The kit method was modified by increasing the amplification cascade using monoclonal mouse antibody and biotinylated goat antimouse antibodies (Dako), 3,3-Di-aminobenzidine was used for the color reaction and haematoxylin counterstaining was used before dehydration and mounting of the section.

Preparation of DNA. DNA was extracted from formalin-fixed, paraffin-embedded tissues as previously described. Briefly, 10 × 20 to 30 μm sections were cut, finely diced, and sequentially washed in xyol (3 times), 99% ethanol (2 times), and 95% ethanol (1 time) before dehydration in water (30 minutes for each step). Control DNA was extracted from unfixed JS/B95-8 cells (A-type EBV infected), and HUT cell lines transfected with HIV for EBNA-2A, EBNA-2B, and HIV experiments, respectively. All samples were incubated in DNA lysis buffer (50 mmol/L NaCl, 10 mmol/L EDTA, 10 mmol/L TrisCl, pH 8, 1% sodium dodecyl sulfate [SDS], 100 μg/mL proteinase K) for 3 hours at 37°C, with HIV for EBNA-2A, EBNA-2B, and HIV experiments, respectively. All samples were incubated in DNA lysis buffer (50 mmol/L NaCl, 50 mmol/L KCl, 1.5 mmol/L MgCl2, 0.01% [wt/vol] gelatin, 200 μmol/L of each dNTP, 2 U of Taq polymerase (Amersham International, Amersham, U.K.), and 0.25 μg of amplification primer.

Denaturing and extension steps of 1 minute each at 93°C and 72°C were used for all amplifications. The annealing step was performed for 2 minutes at 55°C for EBV sequences and for 10 minutes at 50°C for HIV and β-actin sequences. For EBV and β-actin sequences, 25 cycles were employed using a Thermal Reactor (Hybaid, Teddington, U.K.), whereas for HIV sequences, 36 cycles were used due to its relatively low abundance.

The amplified products were electrophoresed in 1.2% agarose gels and transferred to Hybond N+ membranes (Amersham) and transfered to Hybond N+ membranes (Amersham) for cold deoxycytidine 5'-triphosphate (dCTP) in the reaction mix. HIV and β-actin probes were prepared by end-labeling oligonucleotides designed to anneal to the PCR products at sites distinct from the amplification primers using 32P-γ-ATP (Amersham) and polynucleotide kinase (Pharmacia, Uppsala, Sweden). After overnight hybridization, the membranes were washed before autoradiography, which was performed at −70°C using Cronex x-ray film (DuPont, Boston, MA). The final wash was in 150 mmol/L NaCl, 15 mmol/L Na citrate, pH 7, 0.1% SDS for 30 minutes at 65°C for EBNA-2A and -2B, and for 10 minutes at 50°C for HIV and β-actin.

Statistics. Nonparametric statistical analyses were performed using the SAS statistical package.

RESULTS

Patients. The median age of the patients was 35 years (range, 16 to 82 years), with 13 females and 17 males. Fifteen of the 30 patients had nodular sclerosing HD, 10 had mixed cellularity HD, and 5 had lymphocyte-depleted HD. All samples were positive for the Reed-Sternberg cell-associated antigens CD15 (Leu M1) and CD30 (Ber-H2), although the findings in patient 30 were unusual as the tissue was also positive for the B-lymphocyte-associated antigen CD20 (L26). Of the 30 patients, 2 (patients 1 and 2) were confirmed HIV positive by ELISA and Western immunoblot and 13 were HIV negative. Both of these patients had CD4+ lymphocyte counts less than 100 and one (patient 2) had a prior history of Pneumocystis carinii pneumonia. HIV antibody testing could not be performed on the other 15 patients, but PCR of DNA extracted from their HD biopsies did not show any HIV sequences. HIV sequences were detected in patient 2 only after prolonged exposure of the autoradiograph (2 weeks) and were not detected in patient 1 (data not shown). This would suggest that HIV infection of Hodgkin’s and Reed-Sternberg cells is unlikely in HIV-infected patients, but HIV-ISH studies were not performed and the numbers reported in this study are too small to be definitive on this point. A summary of the patient details and their stage of disease at the time of diagnosis is given in Table 1.

EBV subtypes in HD. EBV sequences were detected by PCR in 10 of the 30 samples, with 7 being of A-type EBV (patients 7, 11, 13, 19, 26, 27, and 28) and 3 of B-type virus (patients 1, 15, and 30) (Fig 1). ISH confirmed the presence of EBV DNA in the Reed-Sternberg cells and their mono-nuclear variants (Hodgkin’s cells) of 9 of these patients (Fig 2), but was not successful in patient 30. The Hodgkin’s and

Table 1. Patient Details

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The patient numbers refer to the same patients as in the figures. Abbreviations: +, positive; -, negative; ND, test not performed; MC, mixed cellularity HD; LD, lymphocyte-depleted HD; NS, nodular sclerosing HD.
Reed-Sternberg cells in patient 30 were rapidly degraded during the ISH procedure, despite multiple attempts with a variety of protocols, including ones that altered the degree of proteinase K digestion used before hybridization. Also, a significant number of background lymphocytes in the biopsy were positive for EBV, making a firm conclusion of EBV-associated HD impossible in this case. The other two patients with B-type EBV detected in their tumor biopsies (patients 1 and 15) had features to suggest pre-existing immunocompromise. Patient 1 was infected with HIV and had severe CD4⁺ T-lymphocyte depletion (CD4⁺ lymphocyte count, 57 × 10⁶ cells/L) at the time of the diagnosis of HD. Patient 15 was a 82-year-old woman confined to a nursing home; such advanced age and infirmity are often associated with immunocompromise. The patient was unlikely to be HIV infected as she was a sister in a religious order, she had not received a blood transfusion since the beginning of the HIV epidemic in Australia, and PCR of her tumor DNA did not show any HIV sequences.

Twenty samples were negative for EBV sequences by both PCR and ISH. However, weak signals of both EBV types could be detected by PCR in some EBV-negative samples on prolonged exposure of the autoradiograph. Occasional EBV-positive lymphocytes were seen scattered throughout the tissue sections on ISH both in samples that had EBV signals from Reed-Sternberg cells and those that did not. The weak signals at the limit of detection of the PCR in the EBV-negative HD cases are probably due to this EBV infection of nonmalignant cells.

There is often significant and variable degradation of DNA derived from fixed tissues, so PCR for human β-actin sequences was performed to assess that each sample contained DNA of suitable quality for use in PCR. As expected, amplification for β-actin sequences was variable between samples, and no β-actin sequences were detected in nine samples. However, significant EBV signals were detected in samples with both weak and strong β-actin signals (Fig 1). Even after normalization of the samples for excessive degradation of the DNA, it was apparent that only patients 1, 7, 11, 13, 15, 19, 26, 27, 28, and 30 contained significant amounts of EBNA-2 gene sequence. Although it must be accepted that seven patient samples had no detectable sequences by PCR, the ISH studies of these patients were technically satisfactory and all were negative for EBV sequences.

Patients with EBV-associated HD were significantly younger (median age, 25 years) than those in whom EBV was not detected (median age, 36 years; \( P = .045 \)), whereas there was no significant difference between the groups in disease stage \( (P = .1) \), patient sex \( (P = .42) \), tumor histology \( (P = .74) \), or the presence of B symptoms \( (P = .11) \). In the small cohort studied, it was not possible to compare the relationships between EBV type and these variables. However, both B-type EBV-associated cases presented at stage IV B, whereas only two of the seven patients with A-type EBV-associated HD (patients 7 and 28) had stage IV disease and only patient 28 reported B symptoms.

**DISCUSSION**

The frequency of the association between EBV and HD varies widely in different reports. Studies using PCR have found up to 65% of cases that harbor the virus. Those using ISH and traditional Southern blotting have reported a lower frequency of 20% to 40%. Although the use of probes to EBV small RNAs (EBERs) in ISH studies may detect EBV in a higher proportion of HD, it has been suggested that PCR overinterprets positive signals and is unable to distinguish between EBV-infected Reed-Sternberg cells and background EBV-infected lymphocytes. For this reason, a
Fig 2. ISH for EBV of patients 26 (positive sample; A) and 3 (negative sample; B). The Reed-Sternberg cells of both patients are indicated by the solid arrows. The positive EBV signals of patient 26 are indicated by the open arrows.

combination of PCR and ISH was used in this study to determine whether EBV was incorporated into the malignant Hodgkin’s and Reed-Sternberg cell DNA. Nine of the 30 patients (30%) reported in this study had EBV-associated HD, consistent with previously published reports.12

One patient (patient 30) was excluded from the analysis. Patient 30 was a 24-year-old woman with a history of severe infectious mononucleosis 3 years before the onset of her HD. The immunophenotypic features of the tumor, although unusual, have certainly been reported in HD17 and the patient’s clinical presentation and response to therapy have been unremarkable. She is currently in remission from her HD now 8 months after her diagnosis. ISH failed to demonstrate EBV in the malignant cells as they were rapidly degraded during
the process and a significant number of background lymphocytes, which were less susceptible to the ISH process, harbored EBV. Some investigators have used PCR criteria alone to determine that a tumor is EBV associated, arguing that ISH is too insensitive but, in our view, it is impossible to be certain that the PCR result of patient 30 was due to EBV-infected Hodgkin’s and Reed-Sternberg cells and not background EBV-infected lymphocytes.

Gledhill et al recently reported that 40% of HD cases harbor monoclonal EBV, exclusively of A-type. Seven of the nine patients reported in this study had A-type EBV in their tumor DNA. All seven were previously healthy young people and all were HIV negative as determined either by serology or by PCR of tumor DNA. The two cases with B-type EBV in their tumor biopsies each had conditions associated with immune compromise. Patient 1 was a homosexual male infected with HIV and had severe CD4+ lymphocyte depletion. Patient 15 was an 82-year-old woman with dementia who was confined to a nursing home. Immunocompromise presumably allows the virus to spread beyond its restricted confines in the oropharynx and facilitates B-type EBV infection of the PB. As the relative frequency of EBV subtypes in PB alters, so too does the frequency of the endemic BLs and 20% of the population’s PB B cells and facilitates B-type EBV infection of the PB. As the relative frequency of EBV subtypes in PB alters, so too does the frequency of the association between EBV subtypes and EBV-associated malignancies. Fifty percent of the EBV-associated NHLs occurring in HIV-infected patients contain B-type virus, and the PB of HIV-infected patients regularly carries B-type EBV.

Early reports suggested that B-type EBV was limited to Central Africa and New Guinea, where approximately 40% of the endemic BLs and 20% of the population’s PB B cells carry B-type EBV. Later studies showed that B-type virus regularly infects the oropharyngeal epithelial cells of both normal and HIV-infected persons in a healthy Western population. However, B-type virus is rarely found in the PB B cells of healthy individuals, although it regularly infects the PB of HIV-infected patients and cardiac transplant recipients. Immuno-compromise presumably allows the virus to spread beyond its restricted confines in the oropharynx and facilitates B-type EBV infection of the PB. As the relative frequency of EBV subtypes in PB alters, so too does the frequency of the association between EBV subtypes and EBV-associated malignancies. Fifty percent of the EBV-associated NHLs occurring in HIV-infected patients contain B-type virus, and the PB of HIV-infected patients regularly carries B-type EBV. All of the previously healthy individuals with HD reported in this study and in that of Gledhill et al were A-type EBV-associated, and the PB B cells of such individuals carry almost exclusively A-type EBV. HD itself is associated with defects in cell-mediated immunity that become worse with therapy for the disease. The weak background signals of EBNA-2A and EBNA-2B seen on PCR of the tumor biopsies suggests that the PB B cells of these patients carry both A- and B-type EBV. Thus, the frequency of EBV types in EBV-associated lymphoid malignancies simply reflects the relative frequency of EBV types in PB and, by implication, their frequency in other lymphoid compartments.

Such an hypothesis implies that EBV-infected cells have a similar chance of becoming malignant regardless of which EBV type they carry, and that in vitro differences in the transforming capacity of A- and B-type EBV do not correlate with their in vivo ability to contribute to the neoplastic process. The EBNA-2 protein is thought to be crucial for in vitro B-cell immortalization and to contribute to the biologic differences between A- and B-type viruses, yet it is not expressed in HD.

Human PB lymphocyte/SCID mice that develop EBV-carrying immunoblastic lymphomas also do not express EBNA-2 unless put into culture. However, it is still possible that EBNA-2 is expressed at crucial times in the neoplastic process. EBV latent proteins (EBNA-1 alone is not sufficient) allow EBV-infected B cells to pass into the long-lived memory B-cell pool by preventing apoptosis at key stages in B-cell maturation, a function similar to the bel-2 oncogene. EBNA-2 is recognized as an important target for CTL responses to EBV. The limited expression of EBNA-2 during crucial stages in the neoplastic process may allow the protein to perform tumor-promoting functions, such as preventing cell death by apoptosis, while allowing the tumors to escape immune surveillance. This suggestion requires further testing as the specific EBV protein(s) that influences apoptosis has not been defined.

A significant association between EBV-associated HD and younger age was evident in this study. However, this may be a function of our relatively small sample size. Others have not reported such an association, with some even reporting an association between EBV-associated HD and older age. In the present study, there was no association between the stage of HD and presence of EBV. In other studies, EBV has been found in 23 of 24 cases, 12 of 15 cases, and three of seven cases of mixed cellularity HD compared with three of nine cases in the present study. The reason for these discrepancies is not clear, but the relatively small numbers of patients in some of these studies, including the present study, may limit comparison. Both patients with B-type EBV-associated HD had advanced-stage IVB HD. An association between advanced-stage HD and HIV infection has been extensively reported, as has a higher frequency of EBV in the HD that occurs in HIV-infected subjects. It is likely that the advanced stage of the two cases of B-type EBV-associated HD reflects the pre-existing immunodeficiency of these patients rather than any specific effect of the EBV subtype. However, this group of subjects is too small for further interpretations to be made.

These studies suggest that EBV is an important cofactor in the development of HD. The finding that B-type EBV is associated with HD occurring in the setting of immunocompromise suggests that B-type EBV may be an important human pathogen in this group of patients.

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EBV SUBTYPES IN HODGKIN’S DISEASE

473


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Subtypes of Epstein-Barr virus (EBV) in Hodgkin's disease: association between B-type EBV and immunocompromise [see comments]

MJ Boyle, E Vasak, M Tschuchnigg, JJ Turner, T Sculley, R Penny, DA Cooper, B Tindall and WA Sewell