High Prevalence of Epstein-Barr Virus in the Reed-Sternberg Cells of Hodgkin’s Disease Occurring in Peru

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The Epstein-Barr virus (EBV) has been implicated in the pathogenesis of Hodgkin’s disease (HD). This study was undertaken to determine whether the association of EBV with HD showed geographical variation, as in Burkitt’s lymphoma. We studied 32 formalin-fixed, paraffin-embedded cases of HD occurring in Peru. EBV DNA-RNA in situ hybridization was performed using a 30-base biotinylated antisense oligonucleotide complementary to the EBER1 gene of EBV. EBV immunohistochemistry was also performed, using a monoclonal antibody (MoAb) to the latent membrane protein (LMP1) of EBV. Identification of the precise cellular subset staining with EBV was accomplished via double-labeling with MoAbs directed against Reed-Sternberg cells (LeuM1/CD15) and B cells (L26/CD20). EBV RNA was identified in all or virtually all of the Reed-Sternberg cells and variants in 30 of the 32 (94%) cases of HD by in situ hybridization. LMP1 expression was identified in 83% of the EBER1-positive cases. Double-labeling studies confirmed the localization of EBV RNA to CD15-expressing Hodgkin’s cells. This study found an extremely high prevalence of EBV in Peruvian HD, in contrast to the much lower percentage of EBV-associated cases of HD occurring in “Western” patients.

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MATERIALS AND METHODS

Cases. Thirty-two formalin-fixed paraffin-embedded cases of HD were analyzed. The cases were derived from the files of the Hospital Belen and the Centro de Cito-Patologia of Trujillo, Peru. Frozen tissue was not available for study in any case. The morphologic criteria for the diagnosis and classification of HD were those previously established.21-18 EBV serologic data was not available for any of the patients.

In situ hybridization studies. The EBV RNA in situ hybridization studies were performed using a 30-base oligonucleotide complementary to a portion of the EBER1 gene, a region of the EBV genome that is actively transcribed (up to 107 copies per cell) in latently infected cells.19 The oligonucleotide was biotinylated using methods previously described.20 The procedure used for the in situ hybridization studies has been described fully by us elsewhere.20,36 Briefly, 10-μm sections cut from paraffin blocks of formalin-fixed tissues were deparaffinized, dehydrated, predigested with pronase, prehybridized, and then hybridized overnight at a concentration of 0.25 μg/mL of probe. After washing, detection was accomplished by using avidin-alkaline phosphatase conjugate followed by development of the signal with McIlvaine’s substrate. A blue, black, or blue-black color in the nucleus over background levels was considered a positive reaction. This methodology detected EBV RNA from all cases of known EBV-positive acute infectious mononucleosis, nasopharyngeal lymphoepitheliosis, and post-transplantation lymphoproliferative disorders. Lymphoid tissue from an EBV-seronegative patient and tissues infected with herpes simplex virus 1, papilloma virus 16, and adenovirus showed no cross-reactivity. Although a sense strand oligonucleotide could not be used as a negative control (because of partial identity with adjacent antisense sequences), substitution of the probe with 10 other oligonucleotides of identical length and similar G-C content showed no staining. The staining pattern was abolished under any of the following conditions: preincubation with 9 μg/mL of boiled ribonuclease A (Boehringer Mannheim, Indianapolis, IN) at 37°C overnight using buffer conditions recommended by the manufacturer, omission of the labeled probe; or addition of a 50-fold excess of unlabeled probe to the hybridization solution. Any slide negative for EBV RNA was tested for preservation of total RNA using a poly d(T) probe as described elsewhere by us.21 Immunohistochemical studies. Immunophenotypic studies were performed using several monoclonal antibodies (MoAbs) that are reactive in routine paraffin-embedded sections, using a previously

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published technique without modifications. The MoAbs used were leukocyte common antigen (LCA or CD45RB; Dako, Carpinteria, CA), L26 (CD20; Dako), Leu22 (CD43; Becton Dickinson, Mountain View, CA), and LeuM1 (CD15; Becton Dickinson). We also used the mouse MoAb, LMP1 (Dako), which detects a recombinant fusion protein containing sequences of bacterial β-galactosidase and the EBV-encoded latent membrane protein. Normal lymphoid tissue served as a negative control for the LMP1 studies. Ten cases were also stained with the polyclonal antibody CD3 (Dako). Two cases of the lymphocyte-depleted subtype of HD were stained with epithelial membrane antigen (EMA; Dako).

Double labeling immunohistochemical/in situ hybridization studies. Immunohistochemical studies were performed first, using the MoAbs LeuM1, L26, Leu22, and polyclonal CD3. All of the cases were labeled with LeuM1. A subset of 10 cases was labeled with L26, Leu 22, and polyclonal CD3. The in situ hybridization studies followed, using the procedure outlined above.

RESULTS

The age and sex of the 32 patients and the histologic subtype of HD are given in Table 1. The patients ranged in age from 2 to 75 years, with a median age of 9 years. Three-quarters of the patients were less than 40 years old. The male to female ratio was 25:7. The most common histologic subtypes were mixed cellularity (63%) and nodular sclerosis (22%). There were 4 cases of lymphocyte depletion (12%) and only 1 case of diffuse lymphocyte predominance (3%). There were no cases of nodular L&H lymphocyte predominance HD.

The diagnosis of HD was established histologically using accepted diagnostic criteria and was supported by immunohistochemical studies. The Reed-Sternberg cells in 29 of the 32 cases (91%) showed membrane or paranuclear staining with CD15. The Reed-Sternberg cells of 31 of the 32 cases did not stain with CD45RB. The morphologic features and immunologic staining profile was otherwise typical of HD in the sole CD45RB-positive case. CD20 stains were interpretable in 29 of the 32 cases. CD20 staining of Reed-Sternberg cells was observed in 2 cases of mixed cellularity (neither of which were the CD45RB-positive case described above); in these 2 cases, the Reed-Sternberg cells also showed CD15 positivity and the morphologic features were diagnostic of HD. CD43 stains were interpretable in 29 of the 32 cases. No CD43 or CD3 staining of Reed-Sternberg cells and variants was seen in any of the cases.

EBV RNA was identified in all or virtually all of the Reed-Sternberg cells and variants in 30 of the 32 cases of HD (94%) by in situ hybridization (Fig 1A). Rare scattered EBER-positive non-neoplastic lymphocytes were also present in these 30 cases. The 2 EBER-negative cases were both of the lymphocyte depletion subtype. In 1 of these cases, rare small lymphoid cells were EBER-positive, whereas in the second case, no EBER staining was observed, despite adequate RNA shown by hybridization with a poly d(T) probe. Twenty-five of the 30 EBER-positive cases (83%) were also positive for LMP1 (Fig 1B). In contrast to the EBER studies, the LMP1 antibody labeled approximately half of the Reed-Sternberg cells in the positive cases. The 2 cases in which EBER was negative within Reed-Sternberg cells were both negative for LMP1. Double-labeling studies, successful in all 32 cases, confirmed the localization of EBV RNA to Reed-Sternberg cells and variants with the demonstration of copositivity of the Reed-Sternberg cells and variants for CD15 and EBV RNA in 28 cases (Fig 1C). In 2 of the 3 cases with discordant EBER/CD15 staining (1 lymphocyte depletion and 1 nodular sclerosis), the Reed-Sternberg cells were shown to be CD15-negative. The other case with discordant EBER/CD15 staining (lymphocyte depletion subtype) contained Reed-Sternberg cells that were EBER-negative and LeuM1-positive. A single case of lymphocyte depletion contained numerous Hodgkin cells that were EBER-negative, CD15-negative, and LMP1-negative. Both cases of lymphocyte-depletion HD that were EBER-negative showed no staining of the Reed-Sternberg cells or variants with EMA.

Double-labeling studies for CD20, CD43, and CD3 were performed in a subset of 10 cases. The studies found rare scattered EBER-positive small cells colabeling with CD20 and much less often with CD43 and CD3, consistent with a rare population of EBV-infected B and T lymphocytes (Fig 1D and E).
A statistical comparison of the association between EBV and HD subtypes in the Peruvian versus Western cases of HD was performed using the $X^2$ test with continuity correction. The comparison was performed for the two HD subtypes for which adequate numbers of cases were available: mixed cellularity and nodular sclerosis. The cases that were analyzed included the Peruvian mixed cellularity and nodular sclerosis HD cases reported in Table 1 and Western cases of mixed cellularity and nodular sclerosis HD that were derived from our three previous studies\textsuperscript{10,24,25} that were performed using a probe and an in situ hybridization technique identical to that used in this study. The contingency table $X^2$ analysis of the Western cases showed that distribution was the same in all three studies (mixed cellularity, $P = .67$, and nodular sclerosis,
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P = .76) and, therefore, the data was collapsed. A \( \chi^2 \) test with continuity correction performed on the cases of Peruvian nodular sclerosis HD versus Western nodular sclerosis HD showed that the difference in EBV distribution was statistically significant \( (P = .0021) \). The same analysis performed on the cases of Peruvian mixed cellularity HD versus Western mixed cellularity HD also showed a statistically significant difference in EBV distribution \( (P = .0136) \).

A statistical comparison of the association between EBV and age in the Peruvian versus Western cases of HD was performed using the \( \chi^2 \) test. The comparison was performed for all HD subtypes for the adult age group \((\geq 21\) years old\). The cases that were analyzed included the 12 Peruvian HD cases reported in Table 1 and 28 cases of Western HD that were derived from our previous study \(^{24} \) in which age of patients was reported and that used a probe and an in situ hybridization technique identical to that used in this study. A \( \chi^2 \) test performed on the cases of Peruvian HD versus Western HD of the same age group showed that the difference in EBV distribution was statistically significant \( (P = .0305) \).

**DISCUSSION**

Our cases of Peruvian HD show interesting epidemiologic features that distinguish this series from series reported in the United States and other developed countries. First, the majority of our patients are children, with 63\% less than 21 years old. The age distribution of our patient population is similar to that previously described in HD occurring in Peru. \(^{26,27} \) In one study of 20 cases of HD occurring in Peru, \(^{26} \) 40\% of the patients were less than 19 years old, whereas in another Peruvian study of 475 cases of HD, \(^ {27} \) 46\% of the patients were children. HD in children has been reported to be a relatively common disease in developing countries such as Brazil, Colombia, Lebanon, Mexico, Costa Rica, and some parts of Africa. \(^ {28-33} \) Second, there was an accentuation of the usual 3:2 male to female ratio, with a 25:7 male to female ratio found in the current series. This male predilection has also been reported in studies of HD occurring in Latin America, particularly in children. \(^ {34} \) Third, in the current series, mixed cellularity was the most common histologic subtype and lymphocyte depletion was also relatively frequent, in contrast to the nodular sclerosis predominant and the rarity of lymphocyte depletion in series of HD reported from the United States and other “Western” countries. \(^ {35,36} \) Again, a predominance of mixed cellularity has been reported in some series from Third World countries, including Peru. \(^ {27} \) Lymphoid malignancies account for approximately 11\% of all malignancies seen at a major cancer referral center in Peru; of these, approximately 52\% are lymphomas and the rest are leukemias. \(^ {27} \) Of the lymphomas, HD accounts for approximately 25\%.

We found a statistically significant difference in the association between EBV and Peruvian and Western HD of mixed cellularity and nodular sclerosis subtypes. Pallesen et al. \(^ {12} \) and Vestleve et al. \(^ {17} \) have previously documented a statistically significant correlation between EBV and HD subtypes, with a high prevalence of EBV found in mixed cellularity HD as opposed to other subtypes; however, this association has not been found in some other studies. \(^ {11,38} \) Although there was a high percentage of mixed cellularity cases in our study, a statistically significant higher number of cases of Peruvian HD was found to express EBV than cases of Western HD, even when accounting for differences in EBV expression in the different subtypes.

We also considered the possibility that the high rate of EBER-positivity in the Peruvian HD cases was correlated with the low age of the patients. Such an association has been described previously by Jarrett et al. \(^ {28} \) in Western pediatric patients, but this association has also not been confirmed by other investigators. \(^ {37} \) In our patient sample, 83\% of patients over the age of 21 years had EBER-positive tumors. Our rate of EBV-positivity in Peruvian adult HD was statistically significantly greater when compared with previously reported cases of Western HD in patients over 21 years. \(^ {24} \) We cannot exclude the possibility that the high rate of EBER-positivity may be partially explained by the age of our patient population; however, our findings document an increased prevalence of EBV-positivity in Peruvian HD in all age groups.

Thirty of the 32 cases (94\%) of Peruvian HD were found to contain EBV RNA. This percentage is similar to the rate of EBV-positivity in endemic Burkitt’s lymphoma. However, it is in sharp contrast to the much lower percentage of EBV-associated cases of HD occurring in “Western” patients. For example, using an identical in situ methodology, we found only 10 of 22 cases (45\%) of classical HD occurring in immunocompetent North Americans to express EBV RNA within Reed-Sternberg cells and variants. \(^ {10} \) It is not surprising that the paraffin immunohistochemical studies for LMP1 were slightly less sensitive than the EBER in situ hybridization studies for the detection of EBV; a similar observation has been previously reported. \(^ {12,13} \) Denaturation of the LMP1 antigen during formalin fixation may account for some EBER-positive cases not staining with LMP1. \(^ {12} \) Both of the EBV-negative cases were of the lymphocyte depletion subtype, a histologic subtype notorious for confusion with non-Hodgkin’s lymphoma; \(^ {29} \) therefore, it is possible that these two cases may not actually represent HD, but rather, pleomorphic non-Hodgkin’s lymphoma mimicking HD. However, both cases showed no staining with EMA and, thus, were considered unlikely to be anaplastic large-cell lymphomas. \(^ {39} \) Additionally, the morphologic features were felt to be more compatible with HD than non-Hodgkin’s lymphoma, and the phenotypes (CD15\(^ + \), CD45RB\(^ + \), CD20\(^ - \), CD43\(^ - \) and CD15\(^ + \), CD45RB\(^ - \), CD20\(^ - \), CD43\(^ - \)) were considered either to favor HD (former phenotype) or be equivocal (latter phenotype).

We also observed EBV RNA within smaller cells that were identified by double-labeling studies to be predominantly B cells or, less frequently, T cells. Although we cannot exclude the possibility that these cells, or a subset of these cells, represent a population related to the neoplastic population (such as a precursor population), we think that it is much more probable that these cells represent an expanded pool of EBV-positive non-neoplastic B cells because of the immunosuppression present in these individuals. We have noted similar EBV-positive small cells in benign lymph node biopsies. \(^ {10,41} \) Interestingly, rare CD43-positive or CD3-positive cells, presumably T lymphocytes, were also found to be EBV positive in addition to the expected CD20-positive B-lineage.
EBV-positive cells. Recent studies have shown that normal as well as neoplastic T cells may be infected with EBV.\textsuperscript{10,42,43}

The exact significance of identifying a rate of EBV-positivity in cases of Peruvian HD higher than the rate in Western HD is not known. However, the nearly uniform association suggests a role for EBV in the pathogenesis of these cases. One may speculate that HD in Peruvian patients arises in a background of chronic immunosuppression, similar to the development of African Burkitt’s lymphoma in a chronically malaria-infected population. This chronic immunosuppression may be attributable to low socioeconomic factors present among the patient population in Peru, or may be the result of other, not yet recognized, factors. In support of the role of chronic immunosuppression, we have found a similar high association of EBV with HD occurring in HIV-infected patients.\textsuperscript{44} However, the number of EBER-positive non-neoplastic lymphocytes in the cases of Peruvian HD appeared to be similar to that previously described in Western HD occurring outside of the setting of HIV-infection.\textsuperscript{10}

One possible role for EBV in HD could be in determining the epidemiologic features of the neoplasm. A high rate of EBV positivity has been found in cases of HD occurring in patients under the age of 15 years\textsuperscript{48} and of the mixed cellularity subtype.\textsuperscript{12} For example, in our study of HD occurring in the United States, mixed cellularity was the most frequent subtype found to be EBV-associated.\textsuperscript{10} It is possible that the high incidence of pediatric cases of HD, mixed cellularity histology, and the marked predominance of males found in Peruvian HD in the current study, is a reflection of the presence of EBV within the neoplastic cells. Study of other populations of HD patients, including patients from other geographic regions, may yield helpful and interesting data.

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