Epstein-Barr Virus DNA Is Abundant and Monoclonal in the Reed-Sternberg Cells of Hodgkin's Disease: Association With Mixed Cellularity Subtype and Hispanic American Ethnicity

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One hundred twenty-five cases of Hodgkin's disease from the United States (79), Mexico City (31), and Costa Rica (15) were analyzed for the presence of Epstein-Barr virus (EBV) by in situ hybridization to EBER1 transcripts. EBV was more frequently detected in the Reed-Sternberg (RS) cells of mixed cellularity Hodgkin's disease (37 of 48 [77%]) compared with the nodular sclerosis subtype (19 of 71 [27%], \( P < .001 \)). The presence of EBV was also associated with Hispanic ethnicity \( (P < .001) \). In a multivariate analysis, patient age, gender, and geographic location were less predictive of EBV positivity than were mixed cellularity histology (odds ratio = 8.3) and Hispanic ethnicity (odds ratio = 4.3). Southern blot analysis of EBV terminal repeat fragments using the Xho1a probe showed that the viral DNA was monoclonal in 17 of 17 cases having EBER1-positive RS cells. By comparison, EBV DNA was not detected by Southern analysis in 20 cases lacking EBER1 in RS cells, even when occasional background lymphocytes expressed EBER1. Because clonal viral DNA was so readily detected in EBER1-positive cases, the EBV genome is probably amplified at least 50-fold in the infected RS cells. Monoclonality of EBV DNA implies that the RS cells were infected before malignant transformation.

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MATERIALS AND METHODS

Tissue Handling and Clinical Information

Paraffin-embedded tissues were collected retrospectively from 125 cases of Hodgkin's disease. Diagnosis was confirmed and histopathologic classification was made by one of us (P.M.B.) according to standard histopathologic criteria and without knowledge of clinical or experimental data. Patients biopsied in the USA (68 in American children, in contrast with 1 of 5 blacks and 4 of 10 caucasians. In the current study, Hodgkin's disease tissues from US Americans, Mexican Americans, Mexicans, and Costa Ricans were examined for the presence of EBV by in situ hybridization. In cases in which frozen tissue was available, the clonality of the tumors with respect to the structure of the EBV genome was assayed. Our goal was to identify the clinical parameters most strongly associated with EBV in Hodgkin's disease with particular emphasis on the influence of Hispanic ethnicity.

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San Antonio, 6 in Atlanta) were identified as Hispanic or non-Hispanic Americans based on comparison of surnames with the 1980 US census list of Spanish surnames. This method was validated by Hazuda et al.27 and by our own review of medical records on 39 of our patients showing correct assignment of ethnic group in 97%. All patients from Mexico City and Costa Rica had Spanish surnames. Patients known to be infected with human immunodeficiency virus (HIV) were excluded from this study because their Hodgkin's tumors are virtually always EBV-associated.28

Southern Blot Analysis

On 37 of the 67 cases, tissues were snap frozen at the time of biopsy and stored at -70°C until the time of analysis. High molecular weight DNA was isolated by SDS-proteinase K lysis and phenol-chloroform extraction and analyzed by the Southern blot method29 using 32P-labeled riboprobes complimentary to the EBV Xho1 fragment28 (kindly provided by Dr Nancy Raab-Traub of the University of North Carolina at Chapel Hill). DNA from the Raji Burkitt lymphoma cell line30 was used as a standard by which to estimate the amount of EBV DNA in each tumor specimen. Blots were interpreted without knowledge of clinical data or in situ hybridization results.

In Situ Hybridization

Riboprobes complimentary to RNA transcripts were generated by transcribing cloned DNA sequences using T3 or T7 RNA polymerase in the presence of digoxigenin-labeled UTP (Boehringer Mannheim, Indianapolis IN). The DNA constructs that served as templates for probe production were EBER1 (RA386)25 and U6 (RA390), both of which were very kindly donated by Dr Richard Ambinder of Johns Hopkins University (Baltimore, MD). Antisense probe to EBER1 recognizes a nonpolyadenylated pol III transcript characteristic of latent EBV infection.31 Sense probe to EBER1 serves as a control of nonspecific hybridization and was negative in all cases. Antisense probe to U6 recognizes a ubiquitously transcribed small nuclear pol III RNA32 that serves as an indicator of RNA preservation. All cases included in this study expressed U6 RNA, whereas 5 cases were excluded from the study because U6 RNA was absent. Tissues fixed in 10% buffered formalin more consistently expressed U6 by this protocol than did those fixed in B5.

Biopsy specimens were routinely fixed, processed, and paraffin-embedded. Tissue sections were cut onto silane-coated glass slides and stored at room temperature. The sections were deparaffinized in xylene for 6 minutes and rehydrated in graded ethanol (100%, 95%, 70%, and 50%) and phosphate-buffered saline (PBS) for 1 minute each. After digestion with proteinase K (20 μg/mL in 100 mmol/L TRIS, 50 mmol/L ethylenediaminetetraacetate [EDTA], 0.5% Triton X-100) for 10 minutes at 37°C, the sections rinsed in PBS and covered with 20 μL of hybridization solution (50% formamide, 5X salted sodium citrate [SSC], 5X Denhardt's, 1% sodium dodecyl sulfate [SDS], 1 mmol/L EDTA, 0.1 mg/mL calf thymus DNA, 7% dextran sulfate) containing 1 μL of riboprobe. Paraffin coverslips were applied and the slides were placed in a humid chamber at 55°C for 2 hours. Until this point, all solutions and glassware were handled in ribonuclease-free conditions.

After hybridization, slides were washed for 10 minutes in 2X SSC plus 0.1% SDS. Excess unhybridized probe was removed by treating with RNase A (10 μg/mL in 2X SSC) at 37°C followed by a 5-minute wash in 0.1X SSC at 55°C and a rinse in 2X SSC. To localize the digoxigenin-labeled riboprobe, sections were covered with a 1:500 dilution of antidigoxigenin antibody linked to alkaline phosphatase (Boehringer Mannheim) in 100 μL of 1% sheep serum and 0.3% Triton X-100 in buffer 1 (100 mmol/L TRIS, 150 mmol/L NaCl, pH 7.5) for 1 hour in a humid chamber. The slides were agitated for 1 minute in buffer 1 and then for 1 minute in filtered buffer 2 (100 mmol/L TRIS, 100 mmol/L NaCl, 50 mmol/L MgCl2, pH 9.5). Adequate color development was achieved after treating with 200 μL of color solution (10 mL buffer 2, 45 μL NBT, 35 μL o-phosphate; Boehringer-Mannheim) for 90 minutes in the dark. The reaction was stopped by treating with 10 mmol/L TRIS, 1 mmol/L EDTA for 5 minutes. Tissues were counterstained with eosin or methyl green, dehydrated in graded ethanol, washed twice in xylen, and coverslipped using Permount (Fisher Scientific, Fair Lawn, NJ). Histologic interpretation of EBER1 staining was performed without knowledge of clinical data or other experimental results.

Statistical Analysis

Because 119 of the 125 cases in the study fell into only two histologic categories, the remaining 6 cases were too few for statistical evaluation and were excluded from all statistical analyses. Sociodemographic data were described using means and frequencies. Univariate associations between clinical variables and EBV-associated Hodgkin's disease (defined as EBV in RS cells) were examined using logistic regression for continuous data and χ2 analysis for categorical data. Variables with univariate associations (P < .25) were entered into a multivariable logistic regression model to identify clinical parameters independently associated with EBV positivity. The strength of association is expressed as an odds ratio that approximates how much more likely it is for EBV to be found in RS cells of patients with than without a given clinical parameter.3435 Data were analyzed using the SAS (Research Triangle Park, NC) package of statistical calculations.

RESULTS

This series was composed of 125 cases of Hodgkin’s disease from the USA (79), Mexico City (31), and Costa Rica (15). Age ranged from 15 to 79 years with a mean of 39 and bimodal peaks in young adulthood and again at advanced age. Histologic subclassification showed 7 cases of lymphocyte predominant, 2 cases of lymphocyte depleted, and 1 unclassified case. Epstein-Barr virus was detected by in situ hybridization to EBER1 transcripts in 19 of 67 cases. Statistical analysis showed that EBER1 positivity was 46% in Hispanic patients compared with 28% in non-Hispanic patients (P < .05).

EBER1 In Situ Hybridization

EBER1 transcripts were localized to RS cells in 58 of 125 cases (46%). In these 58 cases, virtually all RS cells were EBER1 positive. Infected RS cells comprised only a small fraction (<1%) of all tissue cells by our own estimation and by previously published criteria.36 The EBER1 signal was confined to the nucleus with sparing of the nucleoli (Fig 1).

EBER1 was found in occasional small background lymphocytes in 10 of 58 cases that had EBER1-positive RS cells and 17 of 67 cases that had EBER1-negative RS cells. These small cells always comprised less than 5% of total cells and frequently less than 1%. EBER1 was localized to the nucleus of the small cells, where nucleoli were inapparent. Transcripts of the U6 cellular gene were detected in the majority of nucleated cells (Fig 2) in all cases included in this study, confirming that RNA integrity was preserved.
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Fig 1. In situ hybridization to EBER1 transcripts in paraffin sections of Hodgkin's disease case no. 197. RS cell (large cell with bilobed nucleus and prominent nucleoli) and RS variants exhibit nuclear localization of EBER1 with sparing of the nucleoli. The tissue is counterstained with eosin. (Original magnification × 1,000.)

transcripts represent an abundant small nuclear RNA that is similar in copy number, size, and cytologic distribution13 to EBER1, but is encoded by a cellular gene whose transcription is apparently independent of EBV infection. U6 transcripts are encoded by a "housekeeping gene" whose expression is essential for RNA splicing; therefore, U6 transcripts are thought to be expressed in all living cells. In our Hodgkin's tissues, U6 RNA was detected in all cytologically identifiable large lymphocytes and RS cells, in at least 80% of small lymphocytes, but in only a small percentage of eosinophils. The lack of U6 detection in some cells could reflect focal cell-specific RNA degradation, perhaps as a result of endogenous RNase action. In our series, there was no evidence that such focal RNA degradation interfered with the interpretation of EBER1 in situ localization to RS cells.

Southern Blot Analysis of EBV DNA Content and Structure

Frozen tissue was available from 37 cases of Hodgkin's disease that had been characterized by in situ EBER1 hybridization. This tissue was used for Southern blot analysis
of EBV DNA structure using the Xho1a probe that detects EBV DNA in clonal populations of lymphocytes (Fig 3). EBV DNA was present and monoclonal in all 17 cases having EBER1-positive RS cells. By comparison, EBV DNA was not detected by Southern analysis in 20 cases lacking EBER1 in RS cells, even when occasional background lymphocytes expressed EBER1. The average amount of clonal EBV DNA in the Hodgkin’s disease tissues varied from 0.5 to 5 copies per cell.

![Figure 3](image)

**Fig 3.** Southern blot autoradiograph of BamHI-digested DNA extracted from Hodgkin’s disease tissues and hybridized to the Xho1a probe representing unique sequences from the right terminal end of the EBV genome. Tumors no. 11, 12, and 13 each contain monoclonal EBV DNA, as indicated by the presence of a single band. The variable migration of each patient’s band is a function of the number of terminal repeat sequences in each terminal fused restriction fragment. Tumor no. 14 has no evidence of clonal EBV DNA. Lanes are equally loaded so that viral copy number can be compared with dilutions of Raji Burkitt lymphoma cell line DNA (5 and 1 copies per cell).

**Univariate Associations Between Clinical Parameters and EBV-Infected RS Cells**

**Histologic subtype.** EBV was strongly associated with mixed cellularity Hodgkin’s disease in this series. EBV was detected in the RS cells of mixed cellularity Hodgkin’s disease in 37 of 48 cases (77%) as compared with nodular sclerosis histology, in which only 19 of 71 cases (27%) were EBV-associated ($P < .001$). There were too few Hodgkin’s cases ($n = 6$) of other histologic subclassifications for mean-
ingful statistical analysis. These included 3 cases of lymphocyte-predominant Hodgkin's disease and 1 unclassified Hodgkin's disease that were EBER1 negative, and 2 cases of lymphocyte-depleted variant that were both EBER1 positive in the RS cells.

**Geographic location and ethnicity.** There was significant geographic variability in the incidence of EBV in Hodgkin's disease. EBV was detected in the RS cells of 27 of 74 (36%) cases from the USA, 24 of 31 (77%) cases from Mexico City, and 5 of 14 (36%) cases from Costa Rica ($P < .001$). Many of the US cases were Hispanic Americans living in San Antonio. When ethnic group was examined, EBV was found in 47 of 79 (59%) Hispanic cases, including 18 of 34 (53%) cases in Hispanics living in the USA. In contrast, EBV was found in only 9 of 40 (23%) non-Hispanic cases ($P < .001$).

**Age and gender.** There was a significant relationship between advancing age and EBV-positive Hodgkin's disease ($P < .05$). EBV was found in the RS cells more commonly in males (40 of 74 [54%]) than in females (16 of 45 [36%], $P < .05$).

**Multivariate Analysis of Clinical Parameters Associated With EBV**

Multivariable logistic regression analysis was performed to identify the clinical parameters most closely associated with the presence of EBV in RS cells. The strongest predictor of EBV positivity was mixed cellularity histology (odds ratio, 8.3; 95% confidence interval [CI], 3.4, 20.3). This means that mixed cellularity Hodgkin's disease was 8 times more likely to contain EBV-positive RS cells than was the nodular sclerosis subtype.

Hispanic ethnicity was strongly and independently associated with EBV positivity (odds ratio, 4.3; 95% CI, 1.6, 11.4) even when controlling for histologic subtype and other clinical variables in a statistical model. In contrast, geographic location was not predictive of EBV status ($P > .5$) in the multivariate analysis. Similarly, neither patient age nor gender were significantly associated with EBV positivity when controlling for other parameters ($P > .15$).

Multivariable logistic regression analysis showed that the probability of EBV in RS cells was 37% for Hispanics with nodular sclerosis histology and 83% for Hispanics with mixed cellularity type. Among non-Hispanics, the predicted probability was 12% for nodular sclerosis and 54% for mixed cellularity type. The influence of ethnic group and histologic subtype is graphically depicted in Fig 4, in which the parallelism of the lines indicates that there was no interaction between ethnic group and histologic subtype in predicting EBV positivity.

**DISCUSSION**

This study showed a high prevalence of EBV in mixed cellularity Hodgkin's disease. Hispanic ethnicity was independently associated with EBV positivity. Patient age, gender, and geographic location were less predictive of the presence of EBV. Another significant finding of this study was that viral DNA was always monoclonal, consistent with the malignant nature of the tumor. Quantitative analysis of viral burden suggested that the EBV genome is amplified at least 50-fold in infected RS cells, which is consistently higher than any other EBV-associated tumor type.

Recent reports have indicated a high frequency of EBV in Hodgkin's cases from children in Honduras, predominantly children in Peru. In those series, mixed cellularity histology was at least twice as common as nodular sclerosis, and EBER1 transcripts were localized to RS cells in 100% of either histologic subtype. The implication is that ethnic or local environmental cofactors to EBV infection predispose to the development of Hodgkin's disease, either mixed cellularity or nodular sclerosis type, among children in those locations. Our data from Mexico City are similar to those from Honduras and Peru in showing a predominance of mixed cellularity histology (74%) and EBV positivity (77%). In contrast, our cases from the South Central USA and Costa Rica were predominantly nodular sclerosis histology (72%) and only 36% EBV-associated, more closely resembling series reported from other parts of the USA, Europe, and Japan. Regardless of geographic location, the strongest determinant of EBV positivity in our series was mixed cellularity histology. Hispanic ethnicity was independently associated with the presence of EBV, indicating that even nodular sclerosis cases were more likely to contain EBV in Hispanic individuals.

The population of Hispanics in the Americas is comprised of a complex mixture of genetic heritages, including European and Amerindians. Even more complex are the
potential environmental cofactors influencing tumorigenesis among individuals in varying geographic locations or ethnic groups. Other EBV-related malignancies such as Burkitt’s lymphoma and nasopharyngeal carcinoma exhibit geographic or ethnic variability. In the malaria belt of central Africa, Burkitt’s lymphoma is common and is virtually always EBV-positive, whereas in the USA, Burkitt’s lymphoma is rare, only 20% EBV-associated, and lacks an ethnic bias.2 Immunodeficiency, either as a consequence of malaria or HIV infection, is thought to contribute to the pathogenesis of Burkitt’s tumors in Africa and the USA. Dietary factors as well as ethnicity appear to contribute to the prevalence of the EBV-associated nasopharyngeal carcinoma in Southern China, the Middle East, and the Eskimos of Alaska and Greenland.28 Whether dietary factors, genetic traits, or coinfection with another agent contribute to the pathogenesis of EBV-associated Hodgkin’s disease remains to be elucidated.

We considered whether variations in the reported rate of EBV-associated Hodgkin’s disease could be a consequence of technical pitfalls or problems in histologic classification. In terms of technical detection of EBV, we used two independent tests of EBV that yielded identical conclusions in 37 of 37 cases. In relation to the morphologic diagnosis of Hodgkin’s disease, histologic subclassification was made only in cases in which sampling and slide quality were adequate to confidently diagnose and subtype the tumor. Therefore, we are convinced that EBV is not restricted to one histologic subtype of Hodgkin’s disease, but that it is much more commonly associated with the mixed cellularity as compared with nodular sclerosis subtype.

Other investigators29,30 have found an association between EBV-positive Hodgkin’s disease and patient age (either pediatric or advanced age, but not young adults). Our data on the association between EBV positivity and age are displayed in Table 1. It should be noted that our study included no patients younger than 15 years of age. In a univariate analysis, increasing age was associated with a higher prevalence of EBV ($P < .05$). However when controlling for other parameters in a multivariate analysis, age was not associated with EBV positivity ($P > .15$) even when analysis was confined to only one histologic subtype or one ethnic group.

The high prevalence of EBV in Hodgkin’s disease implies an etiologic role for the virus in Hodgkin’s tumorigenesis. This pathogenetic theory is supported by the monoclonality of EBV DNA in these tumors.6,19,29,41-43 In our series, monoclonal EBV DNA was detected in all 17 cases having EBER1-positive RS cells. Because tumor-associated viral DNA is monoclonal, it is likely that virus infection preceded clonal expansion. This reinforces the hypothesis that the virus is not an innocent bystander but rather plays a role in the pathogenesis of the Hodgkin’s disease and the other tumor types in which it is found.30,49 Our observation of EBER1 expression in the RS cells of clonally infected cases indicates that the clonal virus is localized to these cells and suggests that Hodgkin’s disease results from the transformation of an EBV-competent cell.

Previous investigations into the biology of EBV infection have shown that only one viral particle successfully infects a given cell.50 Once the viral DNA is established inside the cell, it circularizes and reproduces itself to yield multiple identical copies of viral DNA.51 In this way, tumors derived from infected cells can have multiple copies of EBV per cell while maintaining clonal viral DNA structure. The average amount of clonal EBV DNA in our series of Hodgkin’s disease tissues varied from 0.5 to 5 copies per cell. Because RS cells comprised only a small fraction (<1%) of all tissue cells, the content of EBV DNA in each RS cell is estimated to be at least 100 times higher than the measured average copy number per cell, or at least 50 copies of viral DNA per RS cell. This is comparable with or greater than the viral burden in infected non-Hodgkin’s lymphomas.29 The high copy number of EBV in RS cells may relate to the pathobiology of this complex lymphomatous disorder.

This study showed that EBV DNA is abundant and monoclonal in infected RS cells. The presence of EBV in RS cells was strongly and independently linked to mixed cellularity histology and Hispanic ethnicity. The association of EBV with certain subsets of Hodgkin’s disease could provide impetus for novel approaches to tumor prevention or therapy.

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| Table 1. Association of Clinical Characteristics With EBV in 119 Cases of Hodgkin’s Disease |
|----------------------------------|-------------------------------|
| Histologic subtype ($P < .001$) | % EBV Positive |
| Mixed cellularity ($n = 48$)    | 77               |
| Nodular sclerosis ($n = 71$)    | 27               |
| Ethnic group ($P < .001$)       |                 |
| Hispanic ($n = 79$)             | 59               |
| USA Hispanic ($n = 34$)         | 53               |
| Non-Hispanic ($n = 40$)         | 23               |
| Geographic location ($P < .001$)|                 |
| United States ($n = 74$)        | 36               |
| Mixed cellularity ($n = 21$)    | 67               |
| Nodular sclerosis ($n = 53$)    | 25               |
| Mexico City ($n = 31$)          | 77               |
| Mixed cellularity ($n = 23$)    | 87               |
| Nodular sclerosis ($n = 8$)     | 50               |
| Costa Rica ($n = 14$)           | 36               |
| Mixed cellularity ($n = 4$)     | 75               |
| Nodular sclerosis ($n = 10$)    | 20               |
| Age ($P < .05$)                 |                 |
| 15-24 ($n = 30$)                | 43               |
| 24-34 ($n = 31$)                | 32               |
| 35-55 ($n = 31$)                | 52               |
| >55 ($n = 27$)                  | 63               |
| Gender ($P < .05$)              |                 |
| Male ($n = 74$)                 | 54               |
| Female ($n = 45$)               | 36               |

Only cases with mixed cellularity or nodular sclerosis histology are listed. Six additional cases were assigned to other histologic subtypes but were too few for statistical evaluation and were excluded from this table.
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