Presence of Epstein-Barr Virus and Strain Type Assignment in Argentine Childhood Hodgkin's Disease

By M.V. Preciado, E. De Matteo, B. Diez, J. Menarguez, and S. Grinstein

 Epstein-Barr virus (EBV) has been implicated in the etiology of a large number of malignancies. Most recently several studies have linked EBV to Hodgkin's disease. In this report, formalin-fixed, paraffin-embedded tissues were collected retrospectively from 41 children with Hodgkin's disease treated at our hospital. Lymph node biopsies were examined for the presence of two virus-encoded latent proteins: latent membrane protein (LMP) and Epstein-Barr nuclear antigen-2 (EBNA-2), in Reed-Sternberg (RS) and Hodgkin (H) cells, by peroxidase immunolabeling. Nonisotopic Epstein-Barr encoded RNAs (EBERs) in situ hybridization was also performed and positive labeling in malignant cells was detected. Twenty specimens were EBER+/LMP+, 2 were EBER+/LMP−, and 19 were EBER−/LMP−. However, none of the 41 cases expressed EBNA-2. Twenty-two of 41 (54%) cases were EBV positive including 2 of 6 with lymphocyte predominance, 19 of 25 with mixed cellularity, 0 of 9 with nodular sclerosis, and 1 of 1 with lymphocyte depletion. In the age range of 2 to 6 years, 14 of 17 (82%) samples were EBV-positive, whereas only 8 of 24 (33%) samples from the age range of 7 to 15 years contained EBV. (P = .004, a two-tailed Fisher's test). In 17 samples, polymerase chain reaction amplification was performed using strain specific primers for exon sequences of the EBNA-3C gene of EBV. From 12 positive samples, 8 contained EBV-A and 4 EBV-B. These results support the hypothesis that EBV contributes to the pathogenesis of pediatric Hodgkin’s disease, particularly in mixed cellularity Hodgkin’s disease and in the younger group.

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From the Laboratory of Virology, the Oncology Unit, and the Pathology Service, Ricardo Gutiérrez Children’s Hospital, Buenos Aires, Argentina; the Laboratory of Molecular Biology, Pathology Department, Gregorio Marañon Hospital, Madrid, Spain. Submitted January 3, 1995; accepted July 14, 1995.

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Address reprint requests to Maria Victoria Preciado, Laboratorio de Virología, Hospital de Niños Ricardo Gutiérrez, Gallo 1330, (1425) Capital Federal, Argentina.

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EBPSTEIN-BARR VIRUS (EBV), the etiologic agent of infectious mononucleosis,3 has a well established association with endemic Burkitt’s lymphoma, nasopharyngeal carcinoma,4 secondary B-cell proliferation in immunosuppressed persons,5 and a variety of other clinical conditions.

Since 1970, arguments for a role for EBV in the pathogenesis of Hodgkin’s disease (HD) were based on epidemiologic evidence linking Hodgkin’s patients with increased antibody titers against EBV.6,8 But a small percentage of Hodgkin’s patients were seronegative for EBV. Therefore, the role played by EBV in the lymphomagenesis of HD remained unclear. Then, Southern blot hybridization demonstrated that EBV DNA was present in up to 25% of Hodgkin’s biopsies.8 Because of the limited sensitivity of these approaches, polymerase chain reaction (PCR) analysis of the samples was performed and viral DNA was shown in a considerably larger proportion of cases, ranging between 60% and 80%.9

Most recently, using Epstein-Barr encoded RNAs (EBERs) in situ hybridization, EBV was found in Reed-Sternberg (RS) and Hodgkin (H) cells, which are the malignant cell population of HD. Furthermore, the EBERs are expressed at levels in excess of 106 copies per cell in latently infected lymphocytes,10 so the sensitivity of this technique is very high.

Latent membrane protein (LMP) and the Epstein-Barr nuclear antigen-2 (EBNA-2) are the two EBV latency proteins that have been associated with cellular growth regulation.11 It is well known that LMP has transforming properties in continuous rodent fibroblast cell lines.12 It has also been demonstrated that LMP protects against apoptosis by increasing expression of the bcl-2 protooncogene.13 EBNA-2 has been associated with cellular phenotypic modifications, especially the upregulation of CD21 and CD23 cell surface receptor expression. It has thus been suspected to play a key role in the immortalization process, being a trans-activator of latent infection cell gene expression.14 The in situ detection in malignant cells of the EBV-LMP suggests that EBV is not merely a silent passenger in HD. EBV-encoded genes may be transcribed and translated into proteins in RS and H cells.15

EBV may follow either a latent or a productive life cycle. Whereas genes indicating a latent infection such as EBER-1, EBER-2, or LMP are strongly expressed, replication-associated proteins are only rarely detected in malignant cells suggesting that the switch between latency and replication is impaired.16 This is consistent with the evidence that EBV latency is maintained under strict host control in HD.17

It has also been demonstrated that there are two EBV types (type A and B),18,19 which differ with respect to the nucleotide sequence within the open reading frame (ORF) for several of the genes expressed in latent infection, namely EBNA-2, EBNA-3,20 and EBERs.21 Type A has shown in vitro to be a more potent transformer of B cells than type B. The original observation that EBV-B is frequently detected in cell lines derived from African or New Guinea Burkitt’s lymphoma has been assumed as a reflection of the prevalence of EBV-B in these regions. In contrast, only very few cell lines derived from non- endemic areas for Burkitt’s lymphoma contained EBV-B.22 Several studies have also demonstrated a particularly high prevalence of EBV-B in immunosuppressed patients in both peripheral blood lymphocytes and lymphomas.23,24

As there are not many data on childhood HD related to its potential association with EBV, we have immunohisto-
chemically examined archival biopsy material from a range of pediatric HD and have screened the presence of LMP and EBNA-2 in malignant cells. The expression of these two proteins is of particular interest because in addition to promoting cellular growth regulation, they can serve as a target for T lymphocyte recognition. We have also used nonisotopic in situ hybridization to detect EBERs and we have correlated those results with LMP data. Furthermore, we have determined virus subtype association in a subset of frozen tumor samples by PCR.

MATERIALS AND METHODS

Tissue handling. Formalin-fixed, paraffin-embedded tissues were collected retrospectively from 41 cases of pediatric HD treated at our hospital (32 boys and 9 girls). Initial and recurrence biopsies had been taken from four cases and thus the total number of tissue biopsies that could be analyzed was 45.

Diagnosis was confirmed and histologic classification was performed by two hematopathologists (E.D.M. and J.M.) after review of hematoxylin- and eosin-stained sections according to Rye convention.28 The immunohistochemical characterization of the lymphocyte predominance cases was formalized in fixed-tissue for B and T lymphocytes, as well as RS cells, specific antigens using antibodies to CD45 (LCA), CD20 (L26), CD30 (Ber-H2), EMA, CD45Ro (Biomeda, Foster City, CA), and CD15 (Leu-M1; Becton Dickinson, San Jose, CA).36

All the patients were classified according to the Ann Arbor staging scheme.27 Lymph node biopsies from children with benign lymphoid hyperplasia and tonsils from children with acute infectious mononucleosis were used as negative and positive controls, respectively.

Isolation of DNA. On 17 of the 41 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 sodium dodecyl sulfate (SDS)-proteinase K lysis and phenol-chloroform extraction.28

PCR amplification. The PCR strategy reported by Sample et al.29 was used to detect strain-specific sequences. Briefly, the PCR reaction used a set of primers common to both type A and type B strains: 5'-AGAAGGGGGCGTTGTTGTT-3' and 5'-GCCGACGTCAAA-ACCAAGGCC-3'. However, the priming sites flanked regions of type specific variation, such that the resulting fragments were of different sizes: 153 bp and 246 bp for type A and B, respectively. DNA, 1 μg, was amplified in a 100 μL reaction mixture containing 10 mmol/L Tris-HCl buffer at pH 8.4, 50 mmol/L KC1, 1.5 mmol/L MgCl2, 250 μmol/L of each of the deoxynucleoside triphosphates (Pharmacia/LKB, Biotechnology AB, Uppsala, Sweden), 1 μmol/L of each primer and 2.5 U of native Taq DNA polymerase (GIBCO, BRL, Gaithersburg, MD). Reaction tubes were overlaid with mineral oil (Sigma, St Louis, MO) and placed in a thermal cycler (Hybaid). An initial denaturing reaction was carried out at 95°C for 5 minutes followed by 30 cycles of amplification. Each of the amplification cycles consisted of a 45 s denaturation step at 95°C followed by a 45 s annealing step at 55°C and a 1 minute extension step at 72°C. In the final cycle, the extension step was carried out for 5 minutes. In a separate reaction tube, a second set of primers for the beta-globin gene: 5'-GAAGAGCCTGGTGGCTTGG-3' and 5'-CTGACGTCGACCTTGG-3' were incubated with the template DNA and served as a control to monitor the amplification ability of a single copy gene.

Agarose gel electrophoresis and Southern blot. PCR amplified DNA was subjected to electrophoresis on a 2% agarose gel containing ethidium bromide and fragments were subsequently transferred to nylon membranes (Gene Screen, NEN, Boston, MA). After blotting DNA was UV cross-linked, then the sheet was prehybridized at 42°C overnight in a buffer containing 40% formamide, 5x SSC, 50 mmol/L NaH2PO4, 0.4% SDS, 5x Denhardt's, and 100 μg/ml denatured, sonicated salmon sperm DNA. Hybridization was performed at 42°C overnight in fresh prehybridization buffer containing type-specific oligonucleotide probes: type A, 5'-GAAGATTCTACGTCAGTGTC-3'; type B, 5'-CCTGATTCTACCCGAGT-3'. They were 5' end-labeled using [γ-32P]-ATP and polynucleotide kinase. Washings were performed in 2× SSC and 0.1% SDS at 65°C with shaking.28

Immunohistochemistry (IHC). LMP detection was performed using a pool of four mouse monoclonal antibodies (MABS) (C1-4, Dako, Glostrup, Denmark),30 which together recognize at least three different epitopes on the molecule. EBNA-2 was detected using one mouse monoclonal antibody (PE2, Dako). Tissue sections were deparaffinized in two changes of xylene and rehydrated through 100%, 70%, and 50% alcohol and 0.1% distilled water. Sections were digested with protease type XIV (10 mg/mL) (Sigma) in phosphate-buffered saline (PBS) for 15 minutes at 37°C. LMP and EBNA-2 were detected using the streptavidin-biotin complex with HRP (Vectastain Elite ABC, Vector, Burlingame, CA). Blocking agent consisted of 1.5% normal horse serum in PBS. Primary antibodies and biotinylated anti-mouse secondary antibody (Vector) were used at dilutions of 1:25 and 1:200 in blocking agent, respectively. Slides were rinsed in distilled water, dehydrated through 70%, 90%, and 100% alcohol, counterstained with hematoxylin, and mounted.

In situ hybridization (ISH). Fluorescein isothiocyanate (FITC)-conjugated EBERs oligonucleotides (Dako) were used as probes for the hybridization procedure on paraffin sections. Tissue sections were deparaffinized in two changes of xylene and rehydrated through 100%, 70%, and 50% alcohol and 0.1% diethyl pyrocarbonate (DEPC) distilled water. To allow access of probes to tissue RNA sequences, they were digested with pepsin 0.1% (Sigma) in CH for 15 minutes at 37°C. Sections were rinsed in 0.1% DEPC distilled water, dehydrated through 70%, 90%, and 100% alcohol, and allowed to dry. Probes were diluted in a hybridization solution consisting of 30% formamide, 10% dextran sulfate, 0.1% sodium pyrophosphate, 0.2% polynylinpyrophosphate, 0.2% ficoll, 5 mmol/L EDTA, 50 mmol/L Tris/CH pH 7.5. Depending on the size of the section, between 7 and 25 μL of probe was applied and a coverslip placed carefully over the section. Slides were transferred to a tray and incubated for 2 hours at 37°C. Coverslips were then removed and sections were washed twice in 2× SSC (1× SSC = 0.15 mol/ L NaCl, 0.015 sodium citrate) at room temperature for 5 minutes, and finally were rinsed in TBS. Blocking solution consisted of 0.1% Triton X-100 and 0.1% bovine serum albumin in TBS. Detection of the hybridized sites was achieved using an MAb anti-FITC (Dako) 1:100 in blocking solution, followed by a sensitive streptavidin-biotin labeled reagents kit, and peroxidase (LSAB, Dako) performed according to the manufacturer’s instructions. Slides were counterstained with hematoxylin and mounted.

RESULTS

The cases analyzed included 25 cases of mixed cellularity (MC), 9 cases of nodular sclerosis (NS), 1 case of lymphocyte depletion (LD), and 6 cases of lymphocyte predominance of the diffuse subtype (LP). Specifically, the diagnosis of the LP was established histologically using accepted diagnostic criteria and was supported by immunohistochemical studies.31 All samples were negative for the RS cell-associ-
ated antigens CD15 and CD30 as well as for the T-lymphocyte-associated antigen CD45 and for CD45RO; meanwhile samples were positive for the B-lymphocyte-associated antigen CD20 and for the EMA.

According to the clinical stage, 5 cases were stage I, 23 cases were stage II, 10 cases were stage III, and 3 cases were stage IV. Patient age ranged from 2 to 15 years (median 7). Application of the anti-LMP pool of MAbs showed strong cytoplasmic and membrane labeling in RS and H cells in 20 (49%) of the 41 cases (Fig 1). LMP-positive staining was also detected in L and H cells of LPHD cases (Fig 2). In no instance could we detect LMP positive small lymphocytes, but, as previously described by Pallesen et al, a weak cross reactivity with a small fraction of plasma cells was detected. The easy morphologic visualization of H and RS cells on adjacent sections revealed that the proportion of LMP-positive cells was nearly 90% and this figure was not merely due to a high number of RS or H cells in positive tissues since many LMP-negative ones had numerous malignant cells.

Application of the anti-EBNA-2 MAb revealed absence of detectable levels of this protein in all HD biopsies studied. The subcellular localization of the EBERs is a helpful indication of the specificity of the reaction. A nuclear localization of the hybridization signal was detected with a characteristic
labeling of the inner nuclear membrane and around the nucleolus. Among sections of 41 pediatric cases of HD hybridized with the EBERs probe, 22 showed clear reaction products in the nuclei of RS and H cells (Fig 3). When initial and recurrent biopsies were available from the same patient, concordant results were uniformly observed.

The clinical stage, sex, and histologic subtype distribution of EBV-positive cases by ISH and IHC were compared (Table 1). The comparison showed an excess of EBV-positive cases in males by both techniques ($P = .2$, a two-tailed Fisher's test), but it did not attain statistical significance. Meanwhile, a significantly high proportion of EBV-positive samples were noticed in MC subtype ($P = .0006$, a two-tailed $\chi^2$ test).

The EBV-positivity in two age groups, 2 to 6 years and 7 to 15 years, was analyzed. The incidence of EBV positivity was significantly higher ($P = .004$, a two-tailed Fisher's test) in patients of the younger age group than in patients of the older age group (Fig 4).

EBV genome were detected by PCR in 12 (71%) of the 17 samples tested, with 8 being type A and 4 being type B (Fig 5). Amplification with the EBNA-3C primers resulted in the expected 153 bp fragment in samples containing EBV type A.
and a 246 bp fragment in samples containing EBV type B. HD samples were analyzed in parallel with B95.8 and Ag876 cells.

DISCUSSION

This study was carried out to assess the possible role of EBV in the lymphomagenesis of pediatric HD. A definite bimodal age peak present in the incidence of HD is not seen for most other lymphomas. In developing countries, the early peak occurs in childhood, whereas in industrialized countries it occurs in young adults. The second peak occurs in late adulthood in both types of countries. Besides, in developing countries there is usually a marked male preponderance and a high proportion of MC histologic subtype. NS, the predominant histologic subtype in the United States and Western Europe, is generally much less common in developing countries.

Concerning the EBV infection, in developing countries it occurs very early in life; more than 90% of children are infected by 6 years of age. Meanwhile, in developed countries only 30% to 40% are seropositive at that age. Al-
though a relationship between childhood HD and EBV in developing countries has been hypothesized, direct detection studies have not been systematically performed.

We performed LMP and EBNA-2 IHC and EBERs ISH to identify those cases in which EBV is exclusively expressed in malignant cells. This is normally related to a direct role of the virus in the neoplastic process. We demonstrated LMP-specific labeling without EBNA-2 expression in RS and H cells in 20 of 41 HD biopsies. ISH for the detection of EBERs showed positive staining of malignant cells in 22 of 41 cases analyzed. This figure supports the view that previous studies using LMP immunohistology slightly underscored the exact proportion of HD cases with EBV-positive tumor cells. The high sensitivity of the EBERs ISH, due to the number of viral transcripts per cell, represents a great technical advancement because it allows the detection of practically every EBV latently infected cell.

Those four cases (two positive and two negative), from which we obtained two biopsies, rendered identical results for the initial specimens and for the ones taken during recurrence. This would indicate that the histologic subtype and the EBV status had remained unchanged. Besides the finding that multiple biopsies from the same patient were consistent in terms of EBV, detection supports the hypothesis that EBV plays a role in the lymphomagenesis.

As in our previous report, statistically significant association between EBV-associated HD and a younger age was also evident (82%) ($P = .004$, a two-tailed Fisher's test). This result is in keeping with the reports from developing countries (Honduras and Peru) in contrast to others from developed ones, with some even reporting an association between EBV-associated HD and older age.

Our results strengthen the argument that EBV is involved

### Table 1. Comparative Results of the EBV Detection According to Histologic Subtype, Clinical Stage, and Sex

<table>
<thead>
<tr>
<th>Histologic subtype</th>
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<th>LMP</th>
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<td>Lymphocyte predominance</td>
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### Fig 4. Distribution of EBV positivity by age groups. □ EBV⁺; □ EBV⁻.
in the pathogenesis of an important proportion of pediatric HD as a causative agent or at least as a cofactor. When we correlated the presence of EBV with histologic subtypes, MC showed a high association (76%) with it, while NS had none. This high incidence in MC has been also reported in other studies of adult and pediatric patients. The absence of EBV in NS has not been previously reported. Our findings of 33% of positivity in LP confirm that EBV-infected tumor cells can occur in LP.32

In our study, no statistically significant association between the presence of EBV and either the clinical stage or sex could be found. Interestingly, neither LMP nor EBNA-2 are expressed in endemic Burkitt’s lymphoma, so we can assume that the EBV role played in both malignancies may be different.

It has been suggested that PCR leads to false positive results and is unable to distinguish between EBV-infected RS and H cells and background EBV-infected lymphocytes. For this reason we combined this technique with IHC and ISH to confirm if EBV was actually within the malignant cells in those PCR-positive cases. LMP labeling and EBER ISH showed the presence of EBV in the RS and H cells of 12 EBV-positive samples by PCR. Moreover, inasmuch as we identified only a single strain of virus in each sample, we are confident in our conclusion that EBV was present in malignant cells in these cases. Jarret et al39 and Gledhill et al38 reported typing of EBV strains in adult cases; Ambinder et al40 and Weinreb et al41 strain-typed pediatric cases of EBV-associated HD and found that all of them were type A. In another study, Boyle et al32 communicated the presence of EBV-A in all tumor DNAs except those from two immunocompromised patients with EBV-B. From the 12 EBV-positive patients described in our study, 8 were EBV-type A and 4 EBV-type B and all of them were immunocompetent. The association of EBV-B with immunocompetent patients is not consistent with previous reports.32,33 It may correlate with a high incidence of EBV-B in our healthy population too, but it must still be determined in future studies. It is well known that healthy Western people rarely carry EBV-B, neither in B cells nor in oropharyngeal epithelium, in contrast to immunosuppressed patients and to individuals from areas endemic for Burkitt’s lymphoma.31,32 This suggested that the altered immunity allows EBV-B to be shed from the oropharynx and to infect B cells. Once infected, these cells have the same potential to become malignant regardless of what EBV type they carry.

The role of EBV in HD remains to be clarified and the clinical implications of the presence of EBV need further studies. Although therapy of HD is largely successful, the prospective and retrospective detection of EBV may alter our approach to the mechanism and management of this disease.

REFERENCES

Fig 5. Molecular EBNA-typing of HD from the EBNA-3C coding region. Lane 2 corresponds to a type A positive control, lane 3 corresponds to a type B positive control, and lanes 4 to 9 correspond to patient samples tested. A Southern blot of a gel hybridized with a mixture of the 32P-labeled type A and B specific oligonucleotide probes.
EBV-ASSOCIATED PEDIATRIC HODGKIN’S DISEASE

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