Epstein-Barr Virus and Childhood Hodgkin's Disease in Honduras and the United States

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In industrialized populations, Hodgkin's disease (HD) has an initial peak in young adulthood, whereas in economically developing populations the initial peak occurs in childhood. This pattern resembles that of infection with poliovirus and suggests an infectious cofactor in the etiology. Serologic studies have linked Epstein-Barr virus (EBV) to young adult and adult HD, and viral nucleic acids and antigens have been detected in a subset of Hodgkin's tumor specimens. To investigate the association of childhood HD with EBV we studied tumor specimens from 11 children treated in Honduras and 25 children treated in the United States using in situ hybridization and antigen detection techniques.

Among the patients from Honduras, tumor specimens from all cases were EBV positive. Among the patients from the United States, tumor specimens from six of seven patients with mixed cellularity histology, 2 of 15 with nodular sclerosis histology, and neither of two patients with lymphocyte-predominant histologies were EBV positive. These findings support the hypothesis that EBV contributes to the pathogenesis of HD in children, particularly in mixed cellularity HD, and raises the possibility that there are important geographic, racial, or ethnic factors in the EBV association with HD.

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PECULIARITIES in the epidemiology of Hodgkin's disease (HD) suggest that HD, like paralytic polio in the prevaccine era, might be associated with delayed infection by a widespread agent ("the polio model").[1-3] Support for this hypothesis has come from observations that in industrialized populations HD has an initial peak in young adulthood, whereas in economically developing populations the initial peak occurs in childhood.[4]

Since 1970,[5-7] serologic studies have linked Epstein-Barr virus (EBV) to HD. In most populations, primary EBV infection is asymptomatic. However, in economically advantaged populations, in which viral exposure is delayed until early adulthood, primary infection is frequently associated with the syndrome of infectious mononucleosis. There is a consistent finding of increased risk of HD among persons diagnosed with infectious mononucleosis.[8-14] Furthermore, in patients with a diagnosis of HD, increased antibody titers against EBV antigens antedate diagnosis by several years.[15]

Early efforts to detect EBV DNA in HD by DNA-DNA reassociation kinetics failed,[16] leading many investigators to search for an indirect association. However, with the advent of cloned viral probes and blot hybridization, EBV DNA was detected in 15% to 25% of mostly adult Hodgkin's tumor specimens from North America, Europe, and Japan.[17-20] Analysis of the viral terminal repeats as described by Raab-Traub and Flynn[21] indicated that the EBV genome was present in a monoclonal proliferation of cells.[18,19,22] Polymerase chain reaction (PCR) amplification has identified EBV in a larger fraction of adult HD cases.[20,23-25] In situ hybridization provided direct evidence that the virus was present in the malignant cells in Hodgkin's tumors (Reed-Sternberg cells and their variants).[19,22,26,27] Most recently, EBV gene expression has been detected in Reed-Sternberg cells, indicating that the viral genome is not merely a silent passenger.[26,28-32] In all of these studies EBV has been detected most commonly in mixed cellularity HD.

Given the paucity of data on childhood HD, we undertook to investigate the potential association with EBV by using recently developed detection techniques applicable to fixed specimens. Specimens from patients in Honduras (11 children) and the United States (25 children) were studied.

MATERIALS AND METHODS

Patients and tissues. Formalin- or B5-fixed paraffin-embedded diagnostic biopsy and staging laparotomy specimens (with and without tumor involvement) from 36 children under the age of 15 with HD were studied. The children were treated at the Hospital Escuela (Tegucigalpa, Honduras) or in an institution that is affiliated with a US national collaborative clinical trials group, the Pediatric Oncology Group. The diagnosis was confirmed by a hematopathologist (R.B.M.) after review of hematoxylin and eosin-stained sections in accord with standard criteria.

The in situ hybridization method has been described previously.[34,35] Digoxigenin-labeled riboprobes were prepared by in vitro transcription of linearized recombinant plasmids in the presence of digoxigenin-UTP (Boehringer Mannheim, Indianapolis, IN). The plasmid, RA386, contains the entire Epstein-Barr early RNA 1 (EBER1) sequence (amplified from B95-8 strain EBV by PCR) subcloned into BlueScribe M13 (Stratagene, La Jolla, CA) between T7 and T3 polymerase promoters such that T7 generates sense and T3 generates antisense transcripts. A human cytomegalovirus (CMV) riboprobe was selected as a control because CMV is a widespread herpesvirus that has not been implicated in the pathogenesis of HD.[7] The plasmid, TCMP10, contains human CMV immediate early 1 exon-4 sequences in a similar vector, as previously described.[36] Cells from the two EBV-transformed B-cell lines (B95-8 and Namalwa) and an EBV-negative B-cell lym-

phoma line (BJAB) were fixed in buffered formalin, embedded in paraffin, and used as positive and negative controls for in situ hybridization experiments.

For LMP-1 detection, tissue culture supernatant containing the mouse monoclonal antibody S12 was used.[37] Dewaxed sections were digested in 0.1% trypsin (GIBCO, Grand Island, NY) in 50 mmol/L Tris, pH 7.6, 0.1% $CaCl_2$ for 10 minutes at room temperature. LMP-1 was detected by a standard immunoperoxidase technique (Vectastain Elite ABC; Vector, Burlingame, CA).[33] Blocking agent consisted of 2% normal horse serum in phosphate-buffered saline (PBS) with 0.5% dried milk. S12 primary antibody and biotinylated horse antimouse secondary antibody (Vector) were used at dilutions of 1:2 and 1:200 in blocking agent, respectively. Slides were counterstained with hematoxylin.

For strain typing virus only formalin-fixed specimens could be studied. Sections (5 μm) were cut from tissue blocks and placed in microtubes. The microtome blade was changed between specimens to avoid carryover. Sections were deparaffinized with xylene, digested with proteinase K, extracted with phenol and chloroform, ethanol precipitated, and resuspended in PCR amplification buffer as previously described.[38] Amplification of a strain-variable region of the Epstein-Barr nuclear antigen 2 coding sequence (EBNA-2) and Southern blot hybridization of the amplification products with type-specific probes was performed as described by Sixbey et al.[39] DNA from the Raji and AG876 African Burkitt's lymphoma cell lines that carry EBV type A and type B, respectively, were used as positive controls. Human placental DNA was used as a negative control.

RESULTS

HD tumor specimens from 11 children treated in Honduras and 25 children treated at Pediatric Oncology Group-affiliated institutions in the United States were studied. The characteristics of the children studied are presented in Table 1. The mean age at diagnosis was 8 years among the Honduran patients and 10 years among the United States' patients studied. Mixed cellularity and nodular sclerosis were the most common subtypes in the Honduran and United States' patients, respectively. The distribution of histologies in childhood HD in the United States is similar to that reported from the Pediatric Oncology Group previously, with mixed cellularity representing about one-third of the disease in children less than 10 years but only about 15% in patients aged 11 to 15 years.[40] The male to female ratios were close to unity in both countries.

Tumor specimens from all of the Honduran patients showed intense hybridization with the EBV EBER1 antisense probe (Fig 1 and Table 1). These patients' tumors included six with mixed cellularity histology, three with nodular sclerosis histology, one with lymphocyte predominant histology, and one that was interfollicular and therefore not subclassified (Fig 2). Only 9 of 25 United States' patients were hybridization positive. These tumors included six of seven with mixed cellularity histology, 2 of 15 with nodular sclerosis histology, neither of two with lymphocyte predominant histology, and one that was interfollicular. The hybridization signal was clumped with margination against the nuclear membrane and nucleolus, and was predominantly associated with Reed-Sternberg cells and their variants (Fig 1). EBER-positive cells of the size of small lymphocytes were also detected in nine cases with EBV-positive Reed-Sternberg cells. Small EBER-positive cells were not detected in any of the specimens in

Table 1. Characteristics of Children With HD and Reed-Sternberg Hybridization Results

	EBV Honduras		EBV United States	
	+	−	+	−
No. of patients	11	0	9	16
Age ≤9 yr	8	0	4	3
Age 10-14 yr	3	0	5	13
Male	6	0	6	6
Female	5	0	3	10
Race				
White			4	10
Black			1	4
Hispanic			3	1
Other			1	1
Histologic subtype				
Mixed cellularity	6	0	6	1
Nodular sclerosis	3	0	2	13
Lymphocyte predominant	1	0	0	2
Interfollicular	1	0	1	0

which large malignant cells were not EBER positive. In some cases, the intensity of hybridization was so strong as to obscure the nuclear morphology.

Hybridization with EBER1 sense probe, complementary only to DNA, did not lead to hybridization signal in any specimen. The failure of the sense probe to hybridize to tumor cells is consistent with the interpretation that only RNA, and not DNA, was being detected by the antisense probe. Selective hybridization to RNA was anticipated because of the nondenaturing conditions used. Thus, the sense RNA probe served as a negative control, indicating that Reed-Sternberg cells are not nonspecifically "sticky."

Further investigations were undertaken to better characterize the high EBV association with HD in the Honduran patients. Detection of EBV by in situ hybridization was confirmed in each case by immunoperoxidase detection of the EBV-transforming protein, LMP-1 (Fig 3). This protein was seen to be cytoplasmic and membranous and associated with Reed-Sternberg cells and their variants. In several cases, both techniques were performed in succession on the same tissue section (Fig 3, inset). Lymphoid tissues obtained at diagnostic staging laparotomy from patients with EBV-positive tumor specimens were studied. At least one further tumor specimen was available from five patients. All of these tumor specimens were EBV positive. Ten specimens of lymphoid tissue, from these same patients but uninvolved by tumor, were also available. These were EBV negative. A CMV probe did not lead to hybridization in any specimen. PCR amplification of DNA extracted from formalin-fixed paraffin sections from each of these patients, followed by Southern blot hybridization, detected type A EBV in each tumor specimen. Type B was not detected in any tumor specimen. Two specimens from young adults with nodular sclerosis HD were also studied and these were EBV negative.

DISCUSSION

Although a link between childhood HD in underdeveloped countries and EBV has been hypothesized,[41] direct detection
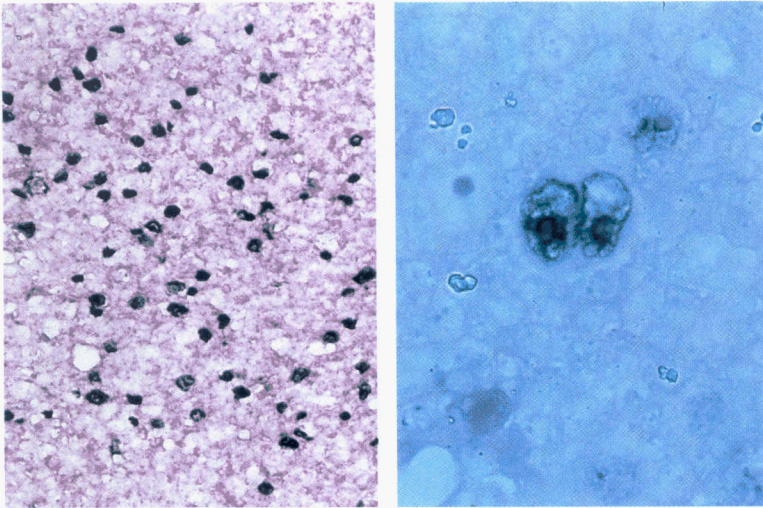
Fig 1. Hybridization with EBER1 antisense probe. A biopsy section of mixed cellularity HD was hybridized with EBER1 antisense probe and counterstained with eosin. (Left) Intense hybridization is seen over numerous neoplastic cells and occasional small cells. (Right) Hybridization signal is clumped and marginated against the nuclear membrane and nucleolus of a Reed-Sternberg cell. The nucleolus and areas of nucleoplasm are devoid of signal.
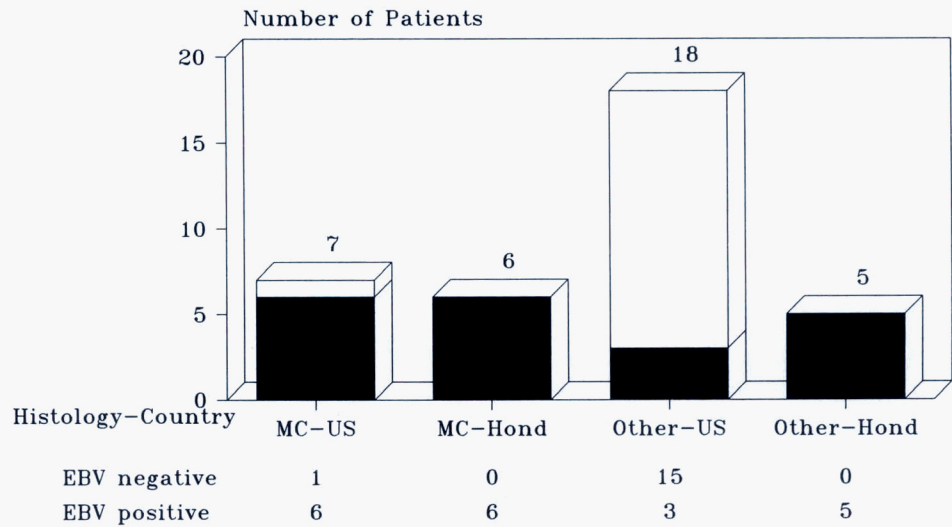

Fig 2. Bar graph showing the relationship between histologic subtype, country, and EBV association. MC, mixed cellularity; Other, nodular sclerosis, lymphocyte predominant, interfollicular; US, United States; Hond, Honduras. (■) EBV positive; (□) EBV-negative.

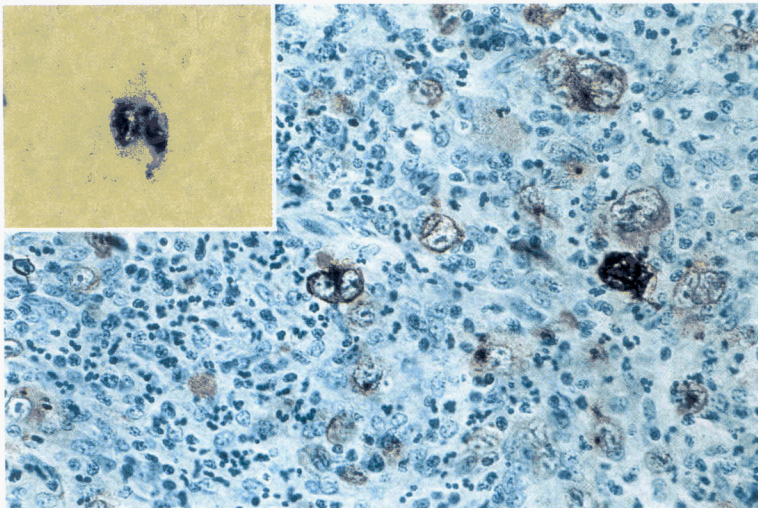| Histology-Country	MC-US	MC-Hond	Other-US	Other-Hond
EBV negative	1	0	15	0
EBV positive	6	6	3	5

Fig 3. LMP-immunoperoxidase staining. A biopsy section of mixed cellularity HD shows cytoplasmic and membranous labeling of Reed-Sternberg cells and their variants. The section is counterstained with hematoxylin. (Inset) Combined in situ hybridization and antigen detection. EBER1 hybridization signal is nuclear; LMP-immunoperoxidase staining is cytoplasmic.

studies have not been systematically performed in children. Lack of study has, in part, reflected a lack of tumor tissue. The initial studies detecting EBV in adult HD all relied on Southern blot hybridization of DNA extracted from snap-frozen or fresh tumor tissue—a detection method not readily applicable to a rare disease. The techniques applied here have been optimized for application to routinely fixed tissue. In situ hybridization studies with the EBV large internal repeat probe using ^{35}S label was the method initially used to identify the cellular locus of EBV in HD and is applicable to formalin-fixed paraffin-embedded material, but appears to be less sensitive and to be associated with more background.[19,22,26] The intensity of the hybridization signal here and the absence of background are attributable to the very high copy number of the EBER transcripts in infected cells and their resistance to autolysis. Whereas most of the EBV genome is either expressed at very low copy numbers (10 to 100 copies per cell) or not expressed at all during latent infection, the EBV EBER1 and 2 RNAs are expressed in great abundance (up to 10^7 copies per cell).[42,43] Thus, the technique is sufficiently sensitive to consistently detect EBV in formalin-fixed paraffin-embedded Namalwa cells, a Burkitt's cell line with only two copies of the EBV genome per cell, used as a positive control in these experiments. The EBERs are complexed with cellular proteins and exhibit extensive intramolecular base pairing.[44,45] These characteristics may account for their stability even in specimens stored at room temperature without fixation for 24 hours and in autopsy specimens.[35] The EBERs do not cross-hybridize with other viruses, including human immunodeficiency virus (HIV), CMV, and herpes simplex viruses, or with normal tissues under the conditions used in the assay described.[35]

The finding that multiple tumor specimens from the same patient were consistent in terms of EBV detection supports the hypothesis that EBV plays a role in tumorigenesis. Furthermore, the absence of detectable viral transcripts in uninvolved lymphoid specimens suggests that virus is largely confined to tumor cells in lymphoid tissues.

The significance of the population of EBV-positive cells the size of small lymphocytes is uncertain. The existence of this population of cells, not noted in studies using the EBV large internal repeat probe,[19,22,31] was only recognized with the increased sensitivity achieved by targeting the abundant EBER transcripts.[30,32] We have detected such cells in lymph nodes from patients with infectious mononucleosis, in lymphoid tissue from HIV-infected patients, and in patients with congenital immunodeficiencies. In this series, these small EBER-positive cells were detected only in patients who also had EBER-positive Reed-Sternberg cells, and with two exceptions were quite rare. Other investigators have reported detection of these cells in HD tissues in which the Reed-Sternberg cells were EBV negative; we have seen such cases in adults, but not in this series of children. Whether they are related to the disease process or are epiphenomena remains to be determined.

LMP-1 is one of two EBV latency genes that have been associated with cellular growth regulation.[46] When overexpressed in rodent cells, this protein is transforming.[47] Recent evidence also suggests that this protein protects against apop-

tosis (programmed cell death), perhaps by increasing expression of the bcl-2 protooncogene.[48] Expression of the LMP-1 has been previously reported in adult HD.[28,29,31] Its detection here in children provides confirmation of the in situ hybridization results and further support for the hypothesis that, when present, EBV is not merely a silent passenger in HD, but is contributing directly to its pathogenesis. Interestingly, this protein is not expressed in endemic (African) Burkitt's lymphoma.[49] Thus, it is reasonable to suppose that the role of EBV is likely to be very different in EBV-associated HD and EBV-associated Burkitt's lymphoma.

Mixed cellularity HD in children in Honduras and the United States was strongly associated with HD (92%). Other investigators have reported a strong histologic association in series composed largely of adult patients. LMP-1 was detected in 23 of 24 (96%) cases of mixed cellularity HD in a Danish series.[28] Other histologies of HD in children in Honduras are also strongly associated with EBV. In contrast, in children in the United States, only a minority of other histologies were EBV associated (Fig 2). Interestingly, one of two cases of EBV-positive nodular sclerosis HD in the United States sample was in a hispanic child and tumors from three of four hispanic children in the United States sample were EBV positive. The numbers studied are too small to allow any firm conclusions to be drawn, but suggest the possibility that the association of EBV with childhood HD may vary as a function of histology, geography, or ethnic background.

Geographic or ethnic variation in the incidence and viral association of malignancies in which EBV has been detected is not a new phenomenon.[50] Although EBV is a ubiquitous virus, there is geographic variation in the incidence of nasopharyngeal carcinoma and Burkitt's lymphoma. Nasopharyngeal carcinoma is common in Southern Chinese, including those who emigrate, but rare in most of the rest of the world. There appears to be no geographic variation in its apparent 100% association with EBV.[34,50,51] In contrast, there is geographic variation in the incidence and viral association of Burkitt's lymphoma.[52] In North America and Europe, Burkitt's lymphoma is a relatively uncommon tumor and is only occasionally associated with EBV, whereas in Equatorial Africa, the tumor is common and is usually associated with the virus.[52] Evidence has been marshalled to suggest that, despite similar histology and karyotype, EBV-negative and EBV-positive Burkitt's lymphoma may differ in the locus of the chromosomal breakpoints within the c-myc gene.[52]

Age may also be an important determinant in the viral association. In a recent series from Glasgow, Jarrett et al reported that Southern blot hybridization demonstrated EBV DNA in the tumors of 71% of patients with HD aged 50 years or more and 54% of patients with HD aged 14 years and younger, but less than 15% of patients aged 15 to 34 years.[55]

Recently, it has been demonstrated that, much like herpes simplex viruses 1 and 2, EBV is comprised of at least two common strains, A and B. In vitro assays show clear differences between these two strains. The type A strain (prototype B95-8) transforms lymphocytes much more efficiently than the type B strain (prototype AG876), and the lymphocyte transformants containing the type A strain are more easily maintained in culture than transformants carrying the type

B strain.[53] Sixbey et al have designed PCR primers for a strain-variable region that he has used to assess EBV type in throat washings, and has reported detection of type B virus in 41% of healthy EBV shedders, type A virus in 50%, and both strains in 9%.[39] However, immune function may selectively restrict systemic expression of EBV type B infection. Thus, Sculley et al has reported that type B EBV lymphoblastoid cell lines could be established from the peripheral blood of HIV-infected patients sixfold more frequently than from the peripheral blood of normal controls.[54] Our previous work has shown that adult HD in the United States is associated almost exclusively with type A EBV (14 of 15 cases studied; Ambinder, unpublished results). Jarrett et al has reported strain typing 30 cases of EBV-associated HD and all were type A.[55] The present study indicates that in children in Honduras, type A EBV also predominates. It should be noted that the existence of EBV-positive small, possibly reactive lymphocytes makes the interpretation of PCR without correlative in situ hybridization hazardous insofar as viral sequences amplified might derive from reactive lymphocytes rather than tumor cells; it is certainly possible that we amplified virus from both subpopulations of cells. However, inasmuch as we identified only a single strain of virus, we are confident of our conclusion that type A EBV was present in the Reed-Sternberg cells in these cases.

The absence of EBV from lymphoid tissues not involved by tumor and the absence of type B EBV in children with EBV-associated HD in Honduras suggests that the association is not a consequence of global immunocompromise, but must represent some more subtle and complex interaction between virus and host.

These findings support the hypothesis that EBV contributes to the pathogenesis of HD in children, particularly in mixed cellularity HD. If geographic, racial, or ethnic variation in the association of EBV with HD is confirmed in other locales, this may serve as a starting point for studies involving other important etiologic cofactors. The techniques of EBER1 RNA and LMP1 protein detection, here applied to conventionally fixed tissues, should facilitate future epidemiologic studies of the viral-tumor association in diverse settings.

ACKNOWLEDGMENTS

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