The Consistent Association Between Epstein-Barr Virus and Hodgkin's Disease in Children in Kenya


Recent studies have suggested that Epstein-Barr virus (EBV) may play a role in the etiology of Hodgkin's disease (HD). In a previous study, we used latent membrane protein 1 (LMP1)-specific antibodies to examine archival material from 74 British children with HD and found 50% of cases to be positive. It is known that there are geographic and ethnic variations in the incidence of HD. We have investigated LMP1 status in formalin-fixed, paraffin wax-embedded lymph nodes with HD involvement from 53 children and 48 adults from Kenya using immunohistochemical staining. We also developed sensitive and specific in vitro gene amplification protocols for examining the EBV strain type in such material using several combinations of primers derived from the EBNA 2 and EBNA 3 coding regions. LMP1 positivity was present in 100% of the pediatric cases (two lymphocyte-predominant, 25 nodular sclerosis, 16 mixed cellularity, 5 lymphocyte depletion, and 5 unclassified) and in 66% of the adult cases (two of three lymphocyte-predominant, 26 of 39 nodular, sclerosis, two of two mixed cellularity, and two of four lymphocyte depletion). Tests to type the EBV strain were undertaken in 25 EBV-positive pediatric cases. A combination of type-specific polymerase chain reactions for EBNA 2 and EBNA 3C genes indicated that seven patients had type 1, eight had type 2, and 10 had dual infections with both types. Five cases with dual infections were further investigated using a sensitive in situ hybridization for the EBV-encoded, small nuclear nonpolyadenylated RNAs (EBERs). EBER transcripts were detected in Reed-Sternberg and Hodgkin cells and in occasional infiltrating lymphocytes. These observations indicate that in Kenya EBV is consistently associated with pediatric cases of HD, and that biopsies from a number of such cases appear to carry both type 1 and type 2 viral sequences.

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EPSTEIN-BARR VIRUS (EBV) is ubiquitous and has been found as an asymptomatic infection in all human communities. However, geographic and ethnic variation have been recognized by studies of the incidence of EBV-associated malignancies, namely, Burkitt's lymphoma and nasopharyngeal carcinoma. In addition, an association between EBV and Hodgkin's disease (HD) is now supported by a variety of evidence. Thus, serologic and epidemiologic studies in adults first raised the possibility of an association between EBV and HD, followed by molecular detection of EBV in HD biopsies. In this context, Southern blotting and the polymerase chain reaction (PCR) are specific and sensitive methods for detecting EBV genomes, but do not determine which cell population in the biopsy is infected by the virus. This contrasts with in situ hybridization, where viral sequences have been demonstrated selectively in malignant cells. Further evidence for an active role of EBV in the pathogenesis of HD was provided by reports that EBV-positive malignant cells express the virus latent membrane protein 1 (LMP1), one of the key effectors of EBV-induced cell transformation in vitro.
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EBV-Negative Type 1 Type 2
BL 2 KYU-BL CHEP-BL
BL 30 WW-1-BL WW-2-BL
BJ 40 MUTU-BL GOR-BL
BJAB ODHI-BL WAN-BL
MOLT 4 B 96.8-LCL ELI-BL
Ramos [CR + BL 37] LCL AG 876
[CR + OBA] LCL JC5-LCL
Zost-1-LCL [C2 + BL 16] LCL
X 50-7-LCL
I B4-LCL

Abbreviations: BL, Burkitt’s lymphoma; LCL, lymphoblastoid cell lines.

Table 1. Sequence of Oligonucleotide Primers Used for Type-Specific PCRs and Restriction Sites for Specific Restriction Enzymes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence 5’-3’</th>
<th>EBNA 3C 5’ primer</th>
<th>EBNA 3C 3’ primer</th>
<th>EBNA 3C Type 1 Product</th>
<th>EBNA 3C Site</th>
<th>EBNA 3C Type 2 Product</th>
<th>EBNA 3C Site</th>
<th>EBNA 2 A 1 Type</th>
<th>EBNA 2 A 1 Site</th>
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<th>EBNA 2 M 1 Site</th>
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<th>EBNA 2 Type 2 Site</th>
<th>EBNA 2 Type 2 Product</th>
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<tr>
<td>EBNA 3C</td>
<td>5’ GGCTCGTTTTTGCAGTCGCTGC 3’</td>
<td>5’ AGAAAGGAGGCGSTGGTG 3’</td>
<td>153 bp</td>
<td>246 bp</td>
<td>119 bp</td>
<td>180 bp</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>EBNA 2</td>
<td>5’ TTGCTGAAGGTTGCTACTCT 3’</td>
<td>5’ GCTGCCCCACCTGAGGTTT 3’</td>
<td>119 bp</td>
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<tr>
<td>EBNA 2</td>
<td>5’ TGCTGCCCCACCTGAGGTTT 3’</td>
<td>5’ GCAGGCCCCAAGGAGACCCTA 3’</td>
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<tr>
<td>EBNA 2</td>
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</tbody>
</table>

DNA preparation: DNA from cell lines of Burkitt’s lymphoma, or from an in vitro-transformed lymphoblastoid cell line (LCL) was extracted using a standard protocol. These were used as EBV-negative or EBV type 1-positive or type 2-positive controls (Table 1). For sensitivity testing, we prepared 10-fold serial dilutions (10^7 to 10^6 cells) of EBV-positive cells in an excess of 10^7 EBV-negative cells, of EBV type 1-positive cells in an excess of type 2-positive cells, and of EBV type 2-positive cells in an excess of type 1-positive cells. DNA samples prepared from these mixtures were used as additional standards.

DNA was extracted from formalin-fixed, paraffin-embedded tissue using a method previously described. Oligonucleotide primers: The strain type of EBV was determined using differential PCR. Three pairs of oligonucleotide primers were used to amplify sequences from strain type 1 and type 2, respectively. Common oligonucleotide primers directed against the polymorphic gene sequence of EBNA 3C detected both type 1 and type 2. To verify specificity of the sequence of amplified EBNA 3C fragments, two unique cutting restriction enzymes were identified: Rsal I for strain type 1 and Hpa II for strain type 2. Two pairs of primers were targeted to the different regions of EBNA 2 type 2 (2B1, 2B2, 2B3, and 2B4). EBNA 2 PCR products were confirmed to be EBV type 2-specific using the presence of a diagnostic Mnl I restriction site. Details are shown in Table 2.

PCR: Each amplification reaction was performed in a final volume of 50 µL containing 0.05 µg DNA extracted from formalin-fixed, paraffin-embedded samples or from cell lines, 200 µg/L of each dNTP, 1 µg/L of each oligonucleotide primer, 3 U Taq polymerase (AmpliTaq; Perkin Elmer, Branchburg, NJ), and the appropriate volume of PCR buffer (Gene Amp; Perkin Elmer). The samples were denatured for 7 minutes at 94°C and subjected to 45 cycles of amplification in a DNA thermal cycler (GeneAmp PCR System 9600; Perkin Elmer). For EBNA 3C primers, one cycle consisted of denaturation for 30 seconds at 94°C, reannealing for 90 seconds at 50°C, and primer extension for 2 minutes at 70°C. For EBNA 2 primers, one cycle consisted of 1 minute at 94°C, 2 minutes at 55°C, and 30 seconds at 72°C. In situ hybridization: Section from formalin-fixed, paraffin-embedded sections from each biopsy were stained with a pool of four mouse monoclonal antibodies (CS 1 to 4) specific for LMP1.
bedded archival HD lymph nodes from children were hybridized with a biotinylated and digoxigenin-labeled probe complementary to EBER\textsuperscript{1 + 2}. The basis of our method has been reported previously.\textsuperscript{30} The hybridization signal was detected with alkaline phosphatase/4-Nitro blue tetrazolium chloride (NBT) 5-Bromo-4-chloro-3-indoyl-phosphate (BCIP) according to Boehringer (Mannheim, Germany). The sense probes to EBER\textsuperscript{1 + 2} served as a control for nonspecific hybridization; these were negative in all cases.

**RESULTS**

**LMP1 staining.** Of 101 Kenyan HD specimens examined, all 53 pediatric cases were found to be LMP1-positive by monoclonal antibody staining (Table 3), with clear signals over the majority of the malignant cells. A typical example is shown in Fig 1. The histological subtypes of these pediatric cases were lymphocyte-predominant (n = 2), nodular sclerosis (n = 25), mixed cellularity (n = 16), lymphocyte depletion (n = 5), and unclassified (n = 5). However, only 32 of 48 adult cases were LMP1-positive: two of three lymphocyte-predominant, 26 of 39 nodular sclerosis, two of two mixed cellularity, and two of four lymphocyte depletion.

**EBV strain types.** We then sought to classify the EBV strain type involved in these cases. To optimize the PCR typing protocol, EBV-negative, EBV type 1-positive, and EBV type 2-positive cell lines provided control DNA preparations; in addition, DNA was made from known mixtures of these cell lines. An amplification with EBNA 3C primers resulted in the expected 153-bp fragment for type 1 and 246-bp fragment for type 2. The type 1 EBV 3C detection was very efficient, with the ability to detect as few as 10\textsuperscript{4} type 1 cells either in 10\textsuperscript{7} type 2 cells or in 10\textsuperscript{7} EBV-negative cells (data not shown). However, insufficient sensitivity for type 2 diagnosis was noted, despite our extensive variation of the reaction parameters. Thus, the type 2 detection limit using EBNA 3C primers exceeded 10\textsuperscript{5} type 2 cells in 10\textsuperscript{7} type 1 cells (an effect possibly due to the mutual competition between EBNA 3C primers) and was approximately 10\textsuperscript{6} type 2 cells in 10\textsuperscript{7} negative cells (data not shown). We examined type 1 and type 2 products using diagnostic restriction enzyme sites identified from the prototype B 95.6 (type 1) and Ag 876 (type 2) sequences. The enzyme \textit{Rsa I} recognized a specific restriction site in type 1 sequences amplified from all four lines tested (Fig 2), but in only one of four type 2 lines tested (JC 5; Fig 2). Conversely, the enzyme \textit{Hpa II} recognized a specific restriction site in all four type 2 lines tested, but not in any type 1 lines (Fig 2).

PCR analysis was extended to provide a more sensitive assay for type 2 viral strains based on amplification of regions of the EBNA 2 gene. Two different primer combinations were used (2B1/2B2 and 2B3/2B4), and both combinations detected type 2-positive cells as low as 10\textsuperscript{3} cells in a background of 10\textsuperscript{7} type 1-positive cells or 10\textsuperscript{7} EBV-negative cells (Fig 3). The specific restriction enzyme \textit{Mnl I} was used as a diagnostic test for PCR-amplified product of the 2B1/2B2 primer combination, and all EBV type 2 cell lines tested produced a digestion pattern (Fig 4).
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Using these protocols on the clinical material, EBV strain typing was performed in samples from 25 of the children with LMP1-positive HD using EBNA 3C-specific primers. Representative data are illustrated in Fig 5. Seventeen of 25 HD biopsies (eg, 4, 5, and 14) yielded a clear type 1 product identifiable from both its size of 153 bp and its sensitivity to Rsa I digestion. One biopsy appeared to yield both type 1 and type 2 amplification products (no. 20; Fig 5), whereas three of 25 biopsies had only a type 2 signal (data not shown). A small fraction of biopsies (three of 25) had no detectable EBNA 3C amplification.

Because EBNA 3C type detection is not optimal with these particular primers, the experiments were then extended using the more sensitive EBNA 2 type 2-specific primer combinations, 2B1/2B2 and 2B3/2B4. This showed that, in fact, 18 of the original 25 biopsies did contain detectable EBNA 2 type 2 sequences; this included nine biopsies already clearly identified as type 1-positive using the sensitive EBNA 3C primers. Results for five representative biopsies (2, 3, 7, 9, and 14) with evidence of such dual infection are shown alongside appropriate controls in Fig 6. In each case, the type 2 specificity of the 2B1/2B2-amplified product was confirmed using diagnostic Mnl I digestion (Fig 7).

PCR typing data for all 25 biopsies are shown in Table 4.

In situ hybridization. We therefore asked to what extent EBV-positive cells in HD biopsy samples might consist of two populations: (1) malignant cells and (2) other bystander cells in the nonmalignant infiltrate. In situ hybridization was therefore performed on five biopsies for which PCR analysis had shown evidence of dual type 1/ type 2 EBV infection. In situ hybridization showed strong nuclear reactivity in Reed-Sternberg and Hodgkin cells in all five biopsies of formalin-fixed, paraffin-embedded archival lymph nodes using a probe complementary to...
Fig 5. Amplification of DNA from EBNA 3C coding region of the EBV genome from DNA extracted from Formalin-fixed, paraffin-embedded lymph nodes with HD. Restriction enzymes Rsal and Hpa II confirmed specificity of the PCR product. EBV-negative cell line Ramos, EBV type 1-positive cell line ODHI-BL, and EBV type 2-positive cell line JC5 are shown as control. Biopsies 4, 5, and 14 gave a clear type 1 product, and biopsy 20 gave both type 1 and type 2 amplification products.

EBER 1 + 2 (Fig 8A). Although the number of EBER-positive cells was higher than the number of LMP1-positive cells in parallel sections, the vast majority of these EBER-positive cells appeared to be Reed-Sternberg and Hodgkin cells, recognized by their characteristically large nuclear size and by Ki-1 positivity in consecutive sections (Fig 8B). In each of the biopsies, only a few small lymphocytes (Ki-1-negative) were hybridized with the EBER 1 + 2 probe (Fig 8A v B).

DISCUSSION

In developing countries such as those in Africa, HD is a relatively common malignant lymphoproliferative disorder with an earlier primary peak of incidence and a higher propor-

Fig 6. Amplification of DNA from EBNA 3C coding region and EBNA 2 coding region of the EBV genome from DNA extracted from Formalin-fixed, paraffin-embedded lymph nodes with HD. EBNA 3C type primers were not optimal for EBV strain type 2 detection. EBNA 2 type 2-specific primer combinations, 2B1/2B2 and 2B3/2B4, showed that biopsies 2, 3, 7, 9, and 14 did in fact contain detectable EBNA 2 type 2 sequences.
tion of cases with advanced disease and unfavorable histology than in other parts of the world. In Kenya, as in other sub-Saharan countries, HD is primarily a pediatric problem, with 49% of all patients aged less than 20 years and approximately 20% of the patients less than 10 years old.13

Although a link between this pattern of HD in developing countries and EBV has been proposed, only a few direct detection studies have been performed in children.4,14 It is therefore of great interest that our results have shown that 100% of the Kenyan children tested had EBV in their malignant cells. Interestingly, in Kenyan adults with HD, we found that only 63% were LMP1-positive.

Previous studies have shown evidence of EBV in the malignant tissues of 50% to 90% of adults with HD from Denmark, the United Kingdom, and the United States,5,12,15 In children, EBV positivity was present in 37 of 74 (50%) cases from the United Kingdom,11 nine of 16 (56%) from the United States,15 seven of eight (87%) from Saudi Arabia,16 18 of 25 (72%) from Brazil,17 17 of 19 (87%) from Peru,18 and 11 of 11 (100%) from Honduras.19 Armstrong et al19

Table 4. Results of EBV Strain Typing From LMP1-Positive HD in Children From Kenya

<table>
<thead>
<tr>
<th>Identification No.</th>
<th>Histology No.</th>
<th>Age (yr)</th>
<th>Histologic Subtype</th>
<th>EBNA 3C</th>
<th>EBN A2</th>
<th>Strain Type</th>
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<td>+</td>
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have suggested that HD in children and young adults has different etiologies and that EBV is more likely to be involved in the pathogenesis of pediatric cases. Our studies from Kenya would support this hypothesis. It seems probable that EBV is acquired at a very young age in children in tropical Africa, and this may account for the epidemiological features of HD in this setting, particularly its higher incidence in the young age group.

At least two EBV strain types have been identified, and they differ in the latent infection cycle genes EBNA 2,37 and EBNA 3A, 3B, and 3C.38 Initial seroprevalence studies and analyses of EBV DNA in lymphoblastoid cell lines indicated that the EBV strain type 2 is unusual in Western Europe and the United States but common in Africa and New Guinea.38 Results of recent investigations have indicated that EBV strain 2 infection may be more prevalent in the United States than previously believed.39 The prevalence of type 1 virus infection in the healthy adult population in Kenya was studied by Young et al.,22 who showed that 19 of 25 established spontaneous lymphoblastoid cell lines carried type 1 virus and six cell lines had type 2 virus. However, because type 2 cells are difficult to establish in vitro, this may have underestimated the true incidence of type 2 virus in the population.

Studies of EBV in HD in adults from various countries have shown that type 1 virus was generally present.40 However, type 2 virus was reported in three of 10 Australian adult immunocompromised patients with HD.41 A study of Algerian adults with HD, in which DNA extracted from fresh lymph nodes was tested using a PCR technique, was the first report to reveal the coexistence of both types 1 and 2 EBV, in 14 cases.42

The EBNA 3C gene primers we used were efficient for detecting type 1 virus, but they failed to detect type 2 virus as efficiently, whether present alone or as a dual infection with type 1. We believe that a mutual suppression may exist whereby the two strain types compete for the common primers, such that the tendency is for the more abundant strain type to inhibit amplification of the other strain; this is particularly apparent when type 2 is in the minority. Using EBNA 2 gene primers, we were able to increase the efficiency of detecting the type 2 strain and thereby identified dual infection in 10 of 25 children with HD. It therefore seems likely that previous studies using different primers have failed to detect dual infections, either because of priming inefficiency or because of mutual competition as experienced with the EBNA 3C primers.

There have been no previous reports of EBV strain-typing in pediatric HD. In the Kenyan children studied, we found only type 1 virus in seven cases and only type 2 in eight cases, but 10 (40%) of the Kenyan children tested had dual infections. Our results are contradictory to the supposed monoclonal EBV origin of HD. However, a recent report has detected a polyclonal origin of malignant cells within HD tissues. These results suggest that the polyclonal population of Reed-Sternberg cells arise from continuous recruitment of unrelated B lymphocytes.43

HD is now one of the malignancies believed to be associated with human immunodeficiency virus (HIV) infection,44,45 and there are reports of individuals with acquired immunodeficiency syndrome (without HD) who have had dual EBV infections.46 Therefore, our further studies of the Kenyan cases will include screening for HIV.

Early acquisition of EBV infection may account for the high incidence and earlier presentation of pediatric HD in tropical Africa. The presence in many patients of dual infections with both type 1 and type 2 virus is of great interest. The prevalence of strain type 2 EBV in African Burkitt’s lymphoma has been described previously.29 This could reflect poor socioeconomic status leading, for example, to immunocompromise due to malnutrition. Malaria and HIV infection are the other factors that provide an opportunity for the less aggressive type 2 virus to establish an infection.

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