American Journal of Pathology, Vol. 142, No. 6, June 1993

Short Communication

Association of Epstein–Barr Virus with Pediatric Hodgkin's Disease

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A bimodal age incidence curve has been shown for Hodgkin's disease (HD). In developing countries, the first age incidence peak occurs in childhood; however, this peak is delayed until young adulthood in developed countries. This difference may reflect differences in the age of exposure to infectious agents involved in the development of HD or may suggest different etiological agents. Epstein–Barr virus (EBV) has been implicated in the pathogenesis of a proportion of HD cases. In this study, EBV association was investigated in a series of 55 pediatric HD cases from three geographical locations (United Kingdom, Brazil, and Saudi Arabia) and the relationship between country, age, sex, histological subtype, and EBV positivity was evaluated. EBV was detected in 38 cases using RNA in situ hybridization, Southern blot, or immunohistochemical analysis. No significant difference in EBV positivity by country, age, or sex was observed; however, children under 10 years of age were particularly likely to be EBV-associated. The difference in EBV association in the pediatric group compared with that observed previously for young adult HD was highly statistically significant ($P < 0.0001$). These results are consistent with the hypothesis that pediatric and young adult HD have different etiologies and suggest that EBV is likely to be involved in the pathogenesis of pediatric HD. (Am J Pathol 1993, 142:1683–1688)

Epidemiological studies have shown a bimodal age incidence curve for Hodgkin's disease (HD).[1–3] In developed countries, incidence rises through childhood and peaks between the ages of 15 and 34 years. In contrast, in developing countries the first age incidence peak occurs in childhood between the ages of 7 and 12 years, and no young adult age peak is observed.[1,4] In both developed and developing countries, the incidence of HD increases or reaches a plateau from 45 years of age.[3,4] An intermediate pattern of age incidence, in which the childhood peak moves to an older age, has been observed in countries undergoing socioeconomic development and in rural communities.[2,4–6] The proportion of the four different histological subtypes varies between age groups; nodular sclerosis HD (HDNS) predominates in young adults whereas mixed cellularity HD (HDMC) is relatively more common in children and older adults.[3,4,7]

On the basis of the epidemiological data, MacMahon proposed that the three distinct age groups 0 to 14 years, 15 to 34 years, and >49 years may have different etiologies. He further suggested that HD in

Supported by the Leukaemia Research Fund. ASK is funded by the Scottish Hospital Endowment Research Trust.

Accepted for publication February 10, 1993.

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young adults may be caused by an infectious agent of low infectivity.[1] Recent studies have supported the "two-disease" hypothesis in adult HD; however, there is little additional data available for the pediatric age group.[8,9]

There is persuasive evidence that the risk of developing HD in young adult life is increased in individuals with a high standard of living in early childhood.[2,10,11] It has been inferred from these data that HD in young adults may be due to delayed exposure to a common pathogen.[8,9,12] An analogy has been drawn between HD and paralytic poliomyelitis (polio model).[13,14] This suggests that the prevailing socioeconomic conditions determine the age of exposure to the causative agent and that this in turn determines the likelihood of developing disease. In contrast to MacMahon's hypothesis, the polio model therefore predicts that the same infectious agent is involved in pediatric cases in developing countries and young adult cases in developed countries.

One candidate infectious agent is Epstein–Barr virus (EBV). Early studies indicated that patients with a past history of infectious mononucleosis associated with EBV had an increased risk of developing HD.[15–17] Recent molecular evidence indicates an association between EBV and HD; EBV genomes have been detected in 17 to 41% of tumor biopsies using Southern blot analysis.[18–23] Analysis of the terminal repeats of the viral genome has shown that the EBV is clonal, and therefore the EBV-infected cells have arisen from a single infected cell.[18–20,22–24] In addition, the EBV latent genes LMP-1 and EBER-1 and 2 are expressed in Reed–Sternberg (RS) cells, the putative malignant cells.[25–33]

We have investigated previously the EBV status of tumors from 95 HD patients using Southern blot analysis.[24] EBV-positivity rates varied significantly between different age groups. EBV was detected in the majority of cases in the pediatric and older adult age groups, whereas a low incidence of EBV-positivity was found in young adult HD, in particular in HDNS.

In the present study we have increased the number of pediatric cases under investigation and included cases from different geographical locations. This has allowed us to test the polio model and MacMahon's hypothesis in relation to EBV.

Materials and Methods

Clinical Samples

Tumor samples from 55 non-selected HD cases under the age of 15 years at clinical presentation were examined. These included 22 cases from the United Kingdom, 25 cases from Sao Paulo, Brazil, and 8 cases from Saudi Arabia. All cases from Brazil and Saudi Arabia were native to those countries, whereas one of the cases from the United Kingdom was of Asian origin. With the exception of a single United Kingdom case, all cases were reviewed by one pathologist (ASK). One case was considered to have a differential diagnosis of lymphocyte-depleted HD (HDLD) and anaplastic large cell lymphoma but was retained in the study. The cases from Brazil were all HIV-negative; however, HIV testing was not carried out on the remaining cases for ethical reasons.

In the Sao Paulo area of Brazil, the incidence of pediatric HD in males is highest in the age group 5 to 9 years, the incidence of acute lymphoblastic leukemia is low, and a high incidence of non-Hodgkin's lymphoma in young children is observed.[34] These features suggest that this region may be representative of developing countries. In Saudi Arabia, no cancer registry exists but the features of acute lymphoblastic leukemia are similar to those described for Western countries.[35]

We have compared previously several different techniques for detecting EBV in HD. The optimal method was found to be *in situ* hybridization for the detection of the EBV EBER-1 RNA, which is abundantly transcribed in cells latently infected by EBV.[31] Fifty-three of the cases including all cases from Saudi Arabia and Brazil were examined using EBER *in situ* hybridization.

The results of the Southern blot analysis of 13 of the United Kingdom cases have been reported previously.[24] In the present study, 12 of these cases were investigated further using *in situ* hybridization, and the remaining case from which paraffin-embedded material was not available was EBV-positive by Southern blot analysis. One of the additional 9 United Kingdom cases was investigated for the presence of EBV using immunohistochemical staining for the detection of EBV LMP-1 protein. Cases were considered EBV-associated if Southern blot analysis was positive or RS cells expressed EBER-1 or LMP-1.

Detection of EBV

All investigations were carried out on formalin-fixed, paraffin-embedded material.

In Situ *Hybridization*

Sections from HD biopsies were hybridized with a biotinylated oligonucleotide probe, specific for the EBER-1 RNA using methodology that has been de-

scribed previously.[30,31] The hybridization signal was detected using an ABC method (Dako Ltd.) incorporating nitro blue tetrazolium as chromogenic substrate. Sections from paraffin-embedded L591 cells (EBV-infected HD-derived cell line) and J-Jhan cells (EBV-negative T cell line) were used as positive and negative controls, respectively.

Immunohistochemical Analysis

The expression of EBV LMP-1 was examined using a cocktail of monoclonal antibodies (CS1–4) reactive with the LMP-1 protein, as described previously.[31,36]

Statistical Methods

Equality of proportions in single tables was tested using Fisher's exact test. To adjust for the influence of other factors (eg, age and subtype) the tables were stratified and more general tests applied. The statistical package EGRET was used throughout.

Results

In total 38 of the 55 pediatric cases were shown to be EBV-associated. The results are summarized in Table 1 and Figure 1. EBV was detected in RS cells in 35 of 53 cases analyzed by EBV EBER-1 *in situ* hybridization (Figure 2). This included 7 of 8 and 18 of 25 cases from Saudi Arabia and Brazil, respectively. In many of the remaining cases, scattered lymphocytes stained positively in the EBV EBER-1 *in situ* hybridization assay.

Thirteen of the 22 United Kingdom cases, including the case with the differential diagnosis of anaplastic large cell lymphoma, were EBV-positive. One case examined solely by immunohistochemical staining was positive and, as stated above, the case examined by Southern blot analysis only was EBV-positive. EBER-1 expression in RS cells was detected in a single case scored previously as negative on the basis of Southern blot analysis.

Table 1. *Analysis of EBV Association by Country and Histological Subtype*

HD Subtype	United Kingdom	Brazil	Saudi Arabia	Total
HDNS	4/12	7/10	2/2	13/24
HDMC	7/7	10/12	5/5	22/24
HDLP	0/1	0/2	0/1	0/4
HDLD	2/2	1/1	0/0	3/3
Total	13/22	18/25	7/8	38/55

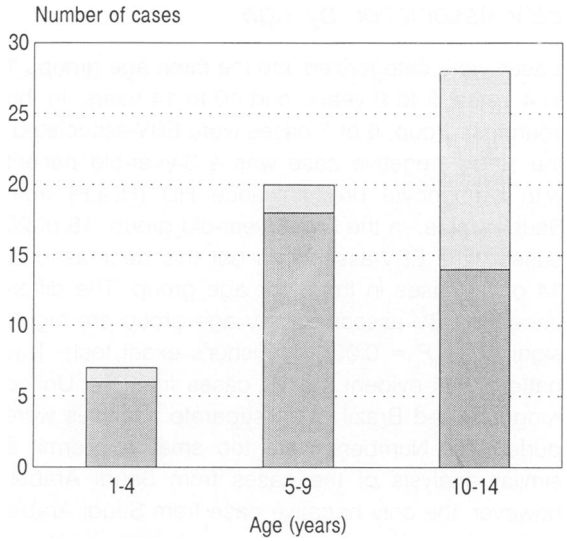

Figure 1. *Analysis of EBV association by age. Dark shading, EBV-positive cases; light shading, EBV-negative cases.*

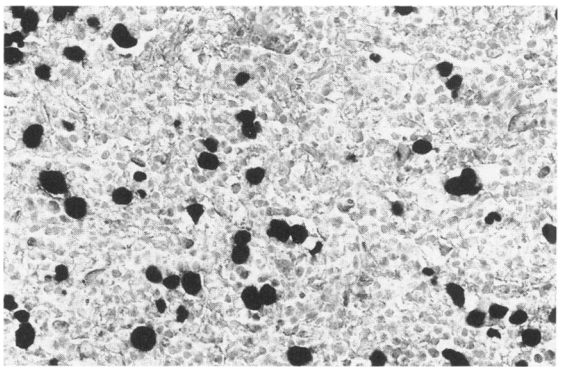

Figure 2. *EBV RNA in situ hybridization. EBV EBER-1 localized to RS cells in a paraffin-embedded lymph node biopsy from an 11-year-old child with HDNS from Saudi Arabia.*

EBV Association by Country

The majority of cases from each of the three countries were EBV-associated (Figure 1). The proportion of EBV-associated cases was higher in Saudi Arabia and Brazil than in the United Kingdom, but differences between the three countries are not statistically significant ($P = 0.30$ by Fisher's exact test). The patients from Brazil were younger than those from the United Kingdom (15 of 25 and 8 of 22, respectively, being aged <10 years) and this was reflected in increased proportions of the HDMC subtype. The odds ratio for EBV association in Brazil compared with the United Kingdom decreased to <1 after adjustment for age, sex, and histological subtype, so that the increased frequency of EBV-associated cases in Brazil could be entirely explained by the patterns of age and subtype.

EBV Association by Age

Cases were categorized into the three age groups 1 to 4 years, 5 to 9 years, and 10 to 14 years. In the youngest group, 6 of 7 cases were EBV-associated; the single negative case was a 3-year-old patient with lymphocyte predominance HD (HDLP) from Saudi Arabia. In the 5 to 9-year-old group, 18 of 20 cases were EBV-associated but this decreased to 14 of 28 cases in the older age group. The differences in EBV association by age group are highly significant ($P = 0.004$ by Fisher's exact test). This pattern was evident for the cases from the United Kingdom and Brazil when separate analyses were performed. Numbers were too small to permit a similar analysis of the cases from Saudi Arabia; however, the only negative case from Saudi Arabia was in the youngest group as mentioned above.

EBV Association by Histological Subtype and Sex

A smaller proportion of HDNS cases were EBV-associated as compared with HDMC and this difference is highly significant (odds ratio = 0.11 with 95% confidence limit, 0.01–0.63, $P = 0.008$). EBV was not detected in the 4 HDLP cases examined; however, all 3 of the HDLD cases were EBV-associated. Females were less likely to be EBV-positive than males (11 of 18 compared with 27 of 37); however, this difference is not statistically significant (odds ratio = 0.59 with 95% confidence limits, 0.15–2.32).

Discussion

Examination of a large series of HD cases from different geographical locations revealed that EBV was present within the RS cells of affected tissues in the majority of cases. These results substantiate our previous findings in this age group and suggest that EBV is likely to play a role in the pathogenesis of childhood HD.[24] Differences in EBV association rates in different geographical locations, with different ethnic and socioeconomic backgrounds, were not significant. A striking feature of the present study was that the risk of EBV association was much higher in patients under 10 years of age. Of the 27 cases examined in this age category, 24 cases were EBV-associated. Thus HD in this group is clearly an EBV-associated disease. Overall, this study provides no evidence that pediatric HD, within each of the 5-year age groups defined above, is more likely to be EBV-associated in developing countries than in developed countries. It is important, however, that the age distribution of cases is taken into account when performing international comparisons.

Similar analyses performed in our laboratory on non-selected young adult (15 to 34 years) cases from the United Kingdom indicated that only 17 of 67 cases in this age group were EBV-associated.[24,27] The proportion of EBV-associated cases in the pediatric and young adults is markedly different ($P < 0.0001$). Fourteen years of age is conventionally taken as the upper limit of the pediatric age group, but this need not reflect a biological distinction and there is likely to be an overlap between the childhood and young adult disease patterns around this age. In this study, patients aged 13 and 14 years predominated within the 10- to 14-year age bracket; given the decline in the proportion of EBV-associated cases in this group, these cases may represent the start of the young adult age incidence peak.

Several studies including largely adult cases have shown an excess of EBV-positive cases within the HDMC subtype as compared with HDNS,[18,19,21,25,28,30,32] and in three this difference has attained statistical significance.[25,28,30] The results of the present study indicate that this difference is also found in pediatric populations. In keeping with other studies, all of the HDLD cases were shown to be EBV-associated; however, the HDLP cases were EBV-negative.[22,25,30]

There is no definitive evidence that EBV plays a causal role in HD, but the available data suggest that it is likely to play a role in the pathogenesis of a proportion of cases. The above data clearly show that the distribution of EBV-associated cases is not random. HD occurring in childhood, particularly under the age of 10 years, is predominantly an EBV-associated disease in both developed and developing countries. The finding that EBV association is significantly less frequent in young adults in a developed country indicates that an analogy between EBV and poliovirus cannot be used to explain the epidemiological features of HD. It remains possible, however, that HD in young adults is related to an infectious agent for which the polio model does hold. This study does provide supportive evidence for the multiple etiology hypothesis as proposed by MacMahon.

Acknowledgments

We thank Dr. L. S. Young for the gift of the LMP-1 (CS1–4) antibodies and Mrs. M. Riddell for help with the preparation of this manuscript.

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