Leukemia (1998) 12, 1272–1276

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Epstein–Barr virus and Hodgkin's disease: further evidence for the three disease hypothesis

AA Armstrong[1], FE Alexander[2], R Cartwright[3], B Angus[4], AS Krajewski[5], DH Wright[6], I Brown[7], F Lee[8], E Kane[3] and RF Jarrett[1]

[1]*LRF Virus Centre, Department of Veterinary Pathology, University of Glasgow, Glasgow;* [2]*Department of Public Health Sciences,* [5]*Department of Pathology, Edinburgh University Medical School, Edinburgh;* [3]*LRF Centre for Clinical Epidemiology, University of Leeds, Leeds;* [4]*Division of Pathology, School of Pathological Sciences, The Royal Victoria Infirmary, Newcastle Upon Tyne;* [6]*Department of Pathology, University of Southampton Medical School, Southampton;* [7]*University Department of Pathology, Western Infirmary, Glasgow; and* [8]*University Department of Pathology, Royal Infirmary, Glasgow, UK*

The epidemiology of Hodgkin's disease suggests that it is a heterogeneous condition comprising more than one disease entity. The Epstein–Barr virus (EBV) is present in the Reed–Sternberg cells of a proportion of cases and is likely to play a role in the pathogenesis of these cases. In this study we show that EBV association rates vary with age at diagnosis. We suggest that Hodgkin's disease can be divided into three disease entities on the basis of EBV association and age, thereby providing biological support for the multiple aetiology hypothesis proposed by MacMahon (*Cancer Res* 1966; 26: 1189–1290).
Keywords: Hodgkin's disease; Epstein–Barr virus; epidemiology

Introduction

In contrast to most other malignancies, the age incidence curve for Hodgkin's disease (HD) is bimodal.[1,2] In developed countries, the first peak in incidence occurs between the ages of 15 and 34 years, the incidence declines in the fourth decade and then peaks or plateaus above the age of 50 years. In contrast, in developing countries the first age incidence peak occurs in childhood, the young adult peak is absent or less pronounced and there is a second peak in the older adult age group.[2,3] Intermediate patterns of age incidence curve have been described in rural areas and in countries undergoing socio-economic development.[2,4,5]

The distribution of the histological subtypes of HD also varies with age; nodular sclerosis HD (HDNS) largely accounts for the young adult age incidence peak whereas the incidence of mixed cellularity HD (HDMC) increases with age and eventually exceeds that of HDNS.[6,7] In comparison to young adult HD, HDMC is relatively more common than HDNS in childhood.[2,6] Lymphocyte-depleted (HDLD) and lymphocyte predominance HD (HDLP) account for only a small proportion of cases. HDLP is now considered to be a disease of B cell origin and is distinguished from other subtypes which are collectively referred to as classical HD.[8–10]

Incidence rates vary by sex and race, and temporal changes in disease incidence have been reported in the United States. Racial differences are largely confined to the HDNS subtype, predominantly HDNS in young adults.[7,11] Overall, HD is more common in males but within the young adult age group the incidence in females equals or exceeds that of males.[6,12,13] Over time there appears to have been an increase in young adult HD, and in HDNS, but changes in the opposite direction have been observed for HD in older persons and for HDMC.[14] Spatial clustering of HD appears to be specific to young adults and absent in older people.[5] Taken together, the data give rise to the idea that the epidemiological features of HDNS in young adults are different from those of other forms of HD.

The heterogeneity of the epidemiological features led MacMahon[1] to propose that HD comprised a group of conditions with different aetiologies and that these conditions could be distinguished on the basis of age at clinical onset of disease. He defined three age groups, 0–14 years, 15–34 years and 50 years and over, and suggested that HD in young adults was caused by an infectious agent. Subsequent studies have provided support for the 'two-disease' hypothesis for adult HD;[5,15] data relating to childhood HD are less extensive. Risk factors associated with the development of HD in young adulthood include single family housing, small family size and a high level of maternal education, all features suggesting a high standard of living in early childhood.[5,16] From these data it has been inferred that HD in this age group arises as a result of delayed exposure to a common infectious agent – the so-called delayed exposure hypothesis or late host response model. Biological support for this model is provided by Paffenbarger *et al*[17] who reported that college students who subsequently died of HD were less likely to have experienced common contagious illnesses in childhood than controls. HD in older adults is not associated with these risk factors and disease development is generally associated with lower socio-economic status.[5,15]

Direct evidence for involvement of an infectious agent in the aetiology of HD has accumulated over the last decade. Epstein–Barr virus (EBV) genomes are consistently detected in affected tissues from a proportion of cases.[18–20] The EBV genomes are clonal and have been localised to Reed–Sternberg (RS) cells, the putative malignant cells in HD.[18,21,22] Furthermore, EBV latent genes encoding the EBNA1 and LMP-1 proteins and EBER RNAs are expressed by RS cells in these cases.[21–24]

In North American and European Union countries (considered representative of developed countries) between 26 and 50% of HD cases are EBV associated.[25–27] There is a clear relationship between histological subtype and EBV with cases of HDMC being more likely to be EBV associated than HDNS cases.[21,28] Data with regard to age are more controversial. We have previously reported that paediatric and older adult (>49 years) cases of HD are more likely to be EBV positive than young adult cases[29,30] but others have found a more uniform distribution of EBV associated cases.[31–33] The reasons for such differences are not clear but in some cases they may have arisen because of small case series, inclusion of few cases at extremes of age, differing laboratory techniques and inclusion of HDLP cases in the overall analysis. In order to test the hypothesis that EBV association in HD is related to age we investigated a large series of adult HD cases, including at least 10 cases in each 5 year age group. EBV assays were

Correspondence: AA Armstrong, LRF Virus Centre, University of Glasgow, Bearsden Road, Glasgow G61 1QH, UK; Fax: 0141 330 5733
Received 30 December 1997; accepted 20 April 1998

performed in a single laboratory and all cases were subjected to histological review.

Materials and methods

Clinical samples

A total of 381 cases of HD resident in the UK at time of diagnosis were examined. Cases were recruited into the study until there were at least 10 cases of classical HD in each 5 year age group from age >14 years. Two hundred and six cases, including 17 paediatric cases, were referred to our laboratory as part of ongoing, population-based studies. One hundred and fifty cases were selected on the basis of age. Age selection occurred for two reasons. First, 107 cases were part of an epidemiological study of young adult lymphoid malignancies (15–24 years). Secondly, cases aged >44 years were selected for age in order to augment numbers in older adult age groups. A further 23 cases were selected on the basis of age and histological subtype as part of a previous study. Selection of cases in the young adult age group resulted in an overall bias toward HDNS cases.

None of the cases was included in our previous study investigating the association between EBV and age and histological subtype;[29] however, 198 cases were included in the international data set analysed by Glaser *et al*.[28]

Detection of EBV

Sections of paraffin-embedded biopsies were examined for the presence of EBV using EBV EBER *in situ* hybridisation and/or LMP-1 immunohistochemistry. The EBER *in situ* hybridisation assay utilised a biotinylated oligonucleotide probe specific for the EBV EBER-1 RNA as described previously,[22] or, in later experiments, a commercially prepared mixture of FITC-conjugated EBER oligonucleotide probes (Dako, High Wycombe, UK). Hybridisation was detected using either avidin–biotin complexes or an alkaline phosphatase-conjugated anti-FITC antibody as appropriate, and nitro-blue tetrazolium was used as the substrate for the alkaline phosphatase catalysed reaction. The expression of LMP-1 was examined using a cocktail of monoclonal antibodies (CS1–4) reactive with the LMP-1 protein[34] as described previously.[22] Sections from EBV-associated HD cases were used as positive controls in both assays. Cases were classified as EBV associated or EBV positive if EBV latent gene products were detected in RS cells using either of the above assays.

Statistical analysis

Multivariate logistic regression was applied using EGRET. The EBV status of the samples was analysed for evidence of association with age, sex and histological subtype with or without inclusion of HDLP cases. Two series of analyses were performed. In the first, the effect of histological subtype was investigated after allowing for the effect of age, both as a linear trend across 10 adult age groups (Table 1) and following division into age groups (0–14 years, 15–24 years, 25–34 years, 35–44 years, >44 years; Table 2). In the second series, the effect of age was determined after allowing for the effect of histological subtype. Both sets of analyses were repeated with sex included in the null model; minimal differences in the results were obtained and these analyses have not been reported. The statistical analysis compared tested models by using the asymptotic χ^2 distribution for the change in deviance.

Table 1 EBV positivity by age group in classical Hodgkin's disease

Age group (years)	*Proportion EBV associated*	*Percentage EBV associated*
0–9	4/5	80
10–14	1/11	9
15–19	10/57	18
20–24	27/98	28
25–29	7/31	22
30–34	4/16	25
35–39	1/9	11
40–44	1/10	10
45–49	7/13	54
50–54	9/11	82
55–59	5/15	33
60+	28/40	70

Cases which were selected on the basis of both age and histological subtype, and HDLP cases have been excluded from the data presented in this table.

Table 2 Statistical analysis of age and EBV status in classical Hodgkin's disease

Age group	*Odds ratios and 95% confidence intervals*	
0–14	1.29 (0.38–4.45)	1.53 (0.46–5.08)
15–19	0.61 (0.26–1.43)	1.00*
20–24	1.00*	
25–29	0.46 (0.16–1.31)	0.73 (0.33–1.63)
30–34	0.99 (0.30–3.20)	
35–44	0.22 (0.05–1.04)	0.26 (0.05–1.20)
45–54	3.36 (1.26–8.94)	
55–64	1.42 (0.55–3.62)	3.07 (1.62–5.83)
65+	4.42 (1.46–13.38)	

Cases were stratified into 9 or 5 year age groups and odds ratios and confidence intervals for EBV association, which have been adjusted for the effect of histological subtype, are presented with respect to the indicated reference group*.

Results

Following histological review the breakdown of cases by histological subtype was as follows: 45 cases of HDLP; 231 cases of HDNS; 87 cases of HDMC; and 10 cases of HDLD. It was not possible to subclassify eight cases. In total, 111 of the 381 cases of HD were EBV associated. This included 66 of the 206 non-selected cases, giving an overall positivity rate of 32%. Of the 45 HDLP cases included in the study only six cases were EBV associated. As HDLP cases are considered a distinct entity the following analysis has concentrated on classical HD.

The distribution of EBV-associated cases by age, excluding HDLP cases, is shown in Figure 1 and Table 1. Among young adults aged 15–24 years only 37/155 of the cases were EBV associated and between 15 and 45 years the EBV positivity rate did not exceed 30%. In contrast, in the older age group, >49 years, the majority of cases were EBV associated (42/66 cases). The number of paediatric cases included in this study is small but the data are consistent with the findings of our

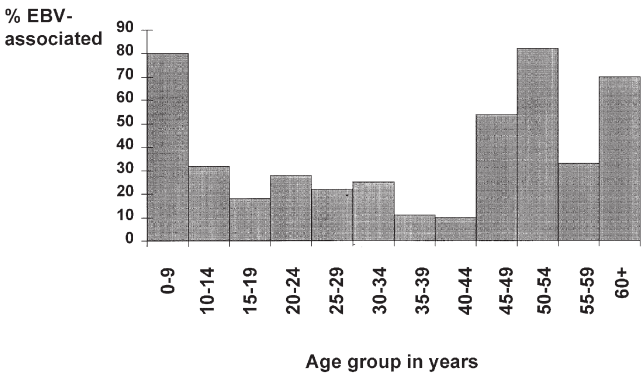

Figure 1 EBV association in Hodgkin's disease by age group. As the number of cases in the childhood age group was small, results from an additional 13 non-selected paediatric UK cases have been included. Cases which were selected on the basis of both age and histological subtype, and HDLP cases, have been excluded from the data shown above.

previous study which showed higher EBV association rates for children under 10 years of age.[30]

Differences by age are statistically highly significant and persist after allowing for the effect of histological subtype (Table 2). The findings are not consistent with a simple linear trend; children, particularly younger children, and older adults are more likely to have EBV-associated disease than cases in the young adult (15–34 years) and intermediate (35–44 years) age groups.

All the analyses showed a statistically highly significant association between EBV and histological subtype after adjusting for the effect of age ($P < 0.001$). A smaller proportion of HDNS cases (25/111) were EBV associated compared with HDMC cases (33/60). The majority of cases within the young adult age range (15–24 years of age) were HDNS, however, only 20/128 (15.6%) of these cases were EBV positive (Figure 2).

Discussion

Results from this study provide clear evidence for a relationship between EBV association and age in HD, confirming previous findings from our laboratory, but using independent data.[29] Older adult cases were found to be more frequently EBV associated than young adult cases or cases in the intermediate age group (35–44 years). Consistent with previous studies from many groups, EBV positivity also correlated with histological subtype with HDMC cases being more frequently EBV associated than HDNS cases.[28] Age and histological subtype are confounding variables, however, the association between age and EBV positivity remained significant following adjustment for the effects of histological subtype and vice versa.

The association between EBV and childhood HD was not specifically addressed in the present study, therefore the number of paediatric cases examined was small. However, results in this age group were consistent with our previous data,[30] and suggest that cases below the age of 10 years are almost invariably EBV associated. EBV positivity rates decrease significantly in the 10–14 year age group. Overall, there is therefore a non-linear association between age and EBV with young and older cases being more likely to be EBV associated than cases in the 10–44 year age bracket.

Studies of childhood HD provide consistent evidence that EBV-associated disease is common in this age group and that young children are most likely to have EBV-positive disease.[30,35–38] Data from studies investigating adult disease or non-selected series are less consistent but there are now several studies, from diverse geographical locales, which report similar age distributions of EBV-associated cases to the present study.[28,39–42]

The data provide biological support for the 'two-disease model' which suggests that HD in young and older adults has different aetiologies.[5,15] Furthermore, inclusion of the paediatric data provides support for MacMahon's hypothesis[1] which suggests that HD in children, young adults and older adults has different aetiologies. From these findings we propose that HD can be divided into three entities, on the basis of age and EBV status, as outlined in Figure 3.

The first of these entities is largely a disease of childhood. It is an EBV-associated disease which is usually, but not

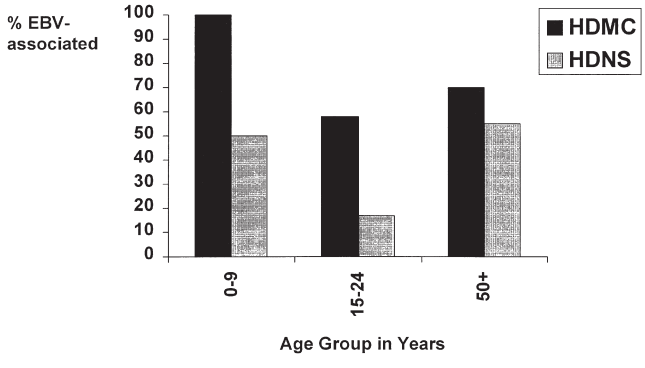

Figure 2 EBV association in classical Hodgkin's disease by age group and histological subtype. HDNS, nodular sclerosis Hodgkin's disease; HDMC, mixed cellularity Hodgkin's disease; HDLD cases were omitted due to the small number of cases in this category.

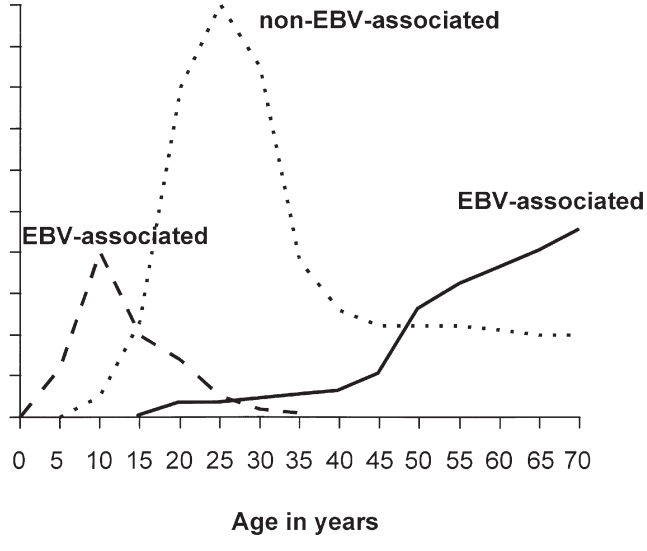

Figure 3 Three-disease model for Hodgkin's disease. The model proposes that HD cases can be divided into three groups on the basis of age and EBV status. It is postulated that the magnitude of the childhood and young adult disease peaks will vary independently of each other and be dependent on the prevailing socio-economic conditions, consistent with Macfarlane *et al*.[3] In contrast, we predict that the magnitude of the older adult peak will show less variation.

invariably, of mixed cellularity subtype. We speculate that disease development is associated with primary infection by EBV, and that the absolute incidence of this disease is higher in developing countries.

The second disease entity is also EBV associated and is also usually associated with HDMC but this disease predominantly affects older adults. It is well documented that diminished immune function is associated with ageing and so it would appear likely that this entity is related to EBV reactivation events.

The third entity is not EBV associated. This disease predominantly affects young adults, it is usually but not always HDNS, and accounts for the young adult age incidence peak. This disease is more prevalent in developed countries. It is in the young adult age group that there is most epidemiological evidence for an infectious aetiology; it would therefore appear plausible that this disease entity is associated with infection by another agent for which the delayed exposure hypothesis or late host response model applies.

Although the epidemiological features of HD are complex, the above model fits with most data sets. The model predicts that the overall proportion of EBV-associated cases in any study will reflect the relative contributions of the three disease entities. EBV positivity rates are higher in Oriental and Latin American countries than in North America and European Union countries.[11] This can be explained by a deficit of cases corresponding to the third disease entity described above, which is non-EBV associated, in the former countries. Belkaid *et al*[43] found that French cases were less likely to be EBV positive than Algerian cases, particularly among young adults, consistent with the idea that in France the non-EBV-associated disease entity is relatively more prevalent.

Differences in risk factors for the development of HD by age group are well established and there is now a body of evidence suggesting that EBV association rates differ by age group. In order to test the model proposed above it is now necessary to determine whether the risk factors associated with young adult HD correlate with the presence of non-EBV-associated disease and similarly, whether risk factors associated with childhood and older adult disease correlate with EBV-associated HD.

Acknowledgements

This work was supported by the Leukaemia Research Fund.

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