

*Short Communication*

## Epstein-Barr Virus and HLA-DPB1\*0301 in Young Adult Hodgkin's Disease: Evidence for Inherited Susceptibility to Epstein-Barr Virus in Cases that Are EBV<sup>+ve</sup>

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**Abstract**

Cases of Hodgkin's disease (HD) may be distinguished by whether they do [EBV-positive (<sup>+ve</sup>) cases] or do not [EBV-negative (<sup>-ve</sup>) cases] have evidence of EBV DNA in the Reed-Sternberg cells. Only one study has attempted to distinguish epidemiological risk factors for EBV<sup>+ve</sup> and EBV<sup>-ve</sup> HD, and none have compared inherited susceptibility. The present study involves a population-based case series of HD, diagnosed in patients between 16–24 years of age in the United Kingdom ( $n = 118$ ), of whom 87% were classified by EBV status (EBV<sup>+ve</sup>, 19, EBV<sup>-ve</sup>, 84). History of infectious illness, EBV antibody titers, and HLA-DPB1 type have been compared in EBV<sup>+ve</sup> and EBV<sup>-ve</sup> cases. Reported infectious mononucleosis was more frequent in EBV<sup>+ve</sup> cases (odds ratio (OR), 5.10; 95% confidence interval (CI), 1.12–24.4). EBV antibody titers to viral capsid antigen were significantly higher in EBV<sup>+ve</sup> cases ( $P$  for trend = 0.02). Higher proportions of EBV<sup>+ve</sup> (43%) than EBV<sup>-ve</sup> (31%) cases typed positive for HLA-DPB1\*0301, but this was not statistically significant; the association of infectious mononucleosis with EBV<sup>+ve</sup> cases was stronger in this HLA subgroup (OR, 17.1; 95%CI, 1.06–1177) than in other cases (OR, 1.24; 95% CI, 0.02–15.4). Although these results are based on small numbers of HD cases, they provide suggestive evidence that the etiology of EBV<sup>+ve</sup> HD may involve inherited susceptibility to EBV.

**Introduction**

Only one infectious agent, EBV, has been consistently associated with HD.<sup>2</sup> This is based on three strands of evidence: (a) past medical history of IM; (b) serology; and (c) molecular biology. Of these, the most direct evidence is molecular biology, from which it is now established (1) that EBV is associated with approximately 40% of cases of HD in developed countries. In these (EBV<sup>+ve</sup>) cases, EBV genomes are present in the Reed-Sternberg cells and in EBV-latent gene products, including the oncogenic LMP-1 protein (2), are expressed. These associations have been interpreted as indicating that EBV plays a causal role in HD, although it may not necessarily be the dominant cause. Nested case-control studies using stored serum (3, 4) have shown that cases of HD have elevated antibody titers to the VCA and EA of the EBV antigens several years before diagnosis. At the time of a 1999 review (5), prospective cohort studies involving more than 40,000 young adults with serologically proven IM had demonstrated a subsequent 3-fold excess incidence of HD compared with general population rates. More recently, a prospective study of cancer incidence in 38,652 subjects with IM in Sweden and Denmark (6) has reported a specific excess risk for HD (OR, 2.55; 95%CI, 1.87–3.40); the excess decreased with time from diagnosis of IM and was highest when IM was diagnosed between the ages of 15 and 34 years (OR, 3.49; 95%CI, 2.46–4.81).

In HD, EBV<sup>+ve</sup> cases are not randomly distributed. HDMC cases are more likely to be EBV<sup>+ve</sup> than HDNS cases, and cases presenting in early childhood and in older adults (>50 years) are more likely to be EBV<sup>+ve</sup> than those presenting in the young adult peak (7). Furthermore, males are more likely to be EBV<sup>+ve</sup> than females (8). The relative paucity of EBV<sup>+ve</sup> cases in young adults is an epidemiological conundrum, because the evidence for an infectious etiology is strongest for these cases (9).

Although a significant familial risk of HD is firmly established (5), evidence for inherited susceptibility to HD is difficult to disentangle from the effects of shared environment. However, a large twin study (10) has clearly demonstrated the importance of inherited factors. Interestingly, this specifically applies to disease in young adults.

There is a body of evidence suggesting that the HLA-class II region is associated with susceptibility to HD (11). Although it is not yet clear which is the main susceptibility locus, current evidence points to a role for HLA-DPB and, specifically, for an association of DPB1\*0301 with susceptibility (11–13). Inasmuch as susceptibility and resistance to infections are, in part,

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<sup>2</sup> The abbreviations used are: HD, Hodgkin's disease; IM, infectious mononucleosis; +ve, positive; -ve, negative; VCA, viral capsid antigen; EA, early antigen; OR, odds ratio; CI, confidence interval; MC, mixed cellularity; NS, nodular sclerosing.

controlled by HLA class II genes, the increased frequency of DPB1\*0301 in HD may be associated with susceptibility to an infectious agent involved in the etiology.

The association between EBV antibody titer and HD shows little evidence of variation by age or tumor EBV-status (3). The relationship between prior IM and EBV status in HD scarcely has been explored. Cases in the same family need not be concordant for EBV status, and EBV<sup>-ve</sup> disease is frequent in familial cases (10, 14). Numerous anecdotal reports (e.g., Ref. 15) note EBV<sup>-ve</sup> HD in people with a past history of IM. The total number of HD cases in the existing literature with a past history of IM and with EBV-status known is extremely small. The only study of reasonable size in which both these pieces of information have been systematically collected is a case series of 100 cases of HD (16) at ages 15–55 years. This study found no association between prior IM and EBV status. The authors had predicted that EBV<sup>+ve</sup> cases would show evidence of childhood experience conducive to late exposure to infectious agents but failed to find this.

Apart from this case series, the epidemiological risk factors for EBV<sup>+ve</sup> and EBV<sup>-ve</sup> HD have not been compared. One study of HD clustering (17) found EBV<sup>+ve</sup> and EBV<sup>-ve</sup> cases occurring within the same clusters, from which we may conclude that, if an etiological agent was responsible for the clustering, then it was common to both EBV<sup>+ve</sup> and EBV<sup>-ve</sup> cases and unlikely to be EBV. This scenario is, perhaps, to be expected from the lack of association of EBV with a clustering of endemic Burkitt's lymphoma (18).

The descriptive epidemiology of HD by EBV status is extremely limited and almost entirely restricted to the examination of proportions (rather than rates) of cases EBV<sup>+ve</sup>. Available results suggest a bimodal distribution of EBV<sup>+ve</sup> cases having peaks in early childhood and in adults >50 years, with EBV<sup>+ve</sup> cases in young adults being either the older tail of the pediatric cases or the younger tail of the older adult cases. We have, for the first time, estimated age-specific incidence rates of EBV<sup>+ve</sup> HD.

This study compares a past history of IM, other epidemiological risk factors, and HLA-DPB1 type (\*0301 and other type) in a prospective series of HD cases classified by EBV status (EBV<sup>+ve</sup> and EBV<sup>-ve</sup>). This case-series comparison uses data collected for HD cases recruited into an epidemiological case-control study of newly diagnosed young adults in selected regions of United Kingdom. Results of case-control comparisons have been presented separately (19).

## Materials and Methods

The geographical region for the present study is the Yorkshire, Wessex, and South-Western Health Board areas and the counties of Cumbria and Lancashire (part) in England. These areas have been covered by a specialist registry of hematological malignancies collecting high-quality incidence data since 1982 (20). All cases of HD diagnosed in young adults (16–24 years of age) between October 1, 1991, and May 31, 1995, were eligible for inclusion in the epidemiological study, and 118 (90%) agreed to take part. The present study relates to 103 cases for which EBV status could be obtained (for the remainder, suitable sample material was not available). For the most part, this report is restricted to cases included in the category of "classical HD" after histopathological review (in practice, HDNS or HDMC subtypes ( $n = 90$ ); the single lymphocyte-depleted case was not classified by EBV status). Cases answered a structured questionnaire at face-to-face interviews with trained inter-

viewers. This elicited, in particular, history of IM (possible answers were "yes," "no," "suspected," and "do not know"). In the present analysis, "do not know" responses are taken as missing and suspected as "no." Thus, our analyses are of definite subject reports of IM but without evidence of serological confirmation.<sup>3</sup> Subjects were also asked to report fact and age of common childhood infectious illness<sup>4</sup>; these have been analyzed in combination (total number of childhood infectious illnesses within 5-year age groups) and interpreted as dichotomies ("none"/"at least one").

**Serological Analyses.** Antibodies against EBV VCA and EA were detected using indirect immunofluorescence assays as described previously (21–23). Sera (collected after diagnosis) were screened at a dilution of 1:10, and samples scoring positively were diluted 2-fold until the end titer was reached. The antibody end titer was defined as the reciprocal of the serum dilution at which specific immunofluorescence was last seen.

**EBV Status.** The EBV status of HD lesions was determined by performing EBER *in situ* hybridization and also, in the majority of cases, LMP-1 immunohistochemistry on sections of paraffin-embedded material as described previously (24). Cases were classified as EBV<sup>+ve</sup> if the Reed-Sternberg cells stained positively in either of these assays.

**HLA Typing.** Genomic DNA was extracted from the blood samples using established methods, and a 288-bp fragment of HLA-DPB1 exon 2 was amplified using the PCR primers DPB1–5' (GAG AGT GGC GCC TCC GC TCA T) and DPB1–3' (GCC GGC CCA AAG CCC TCA CTC), under PCR conditions described previously (25). DPB1 typing was carried out by the method of Bugawan *et al.* (26) using 24 sequence-specific oligonucleotide probes. DPB1 alleles were assigned from patterns of sequence-specific oligonucleotide probe reactivities according to the 11th HLA workshop (27) and as detailed in the Report of the HLA Nomenclature Committee (28).

**Statistical Analyses.** Statistical analyses have used exact methods (29) for univariate analyses and logistic regression (30) for multivariate analyses with both reporting ORs and 95% CIs as well as *P*s (all being 2-sided). Where *P*s are reported for logistic regression, the likelihood ratio test was used. Analyses have been adjusted for gender (logistic regression) or stratified by gender (exact analyses). Additional adjustment/stratification for age was examined but did not alter the ORs. The statistical package EGRET has been applied exclusively except for one application of the Wilcoxon test, which used SPSS.

EBV VCA antibody titers were grouped into five levels with, as nearly as possible, equal numbers in each (negative, 20–640, 1280, 2560, and 5120 or more), and EA antibody titers (with many negatives) were grouped into four levels (negative, 10–20, 40–80, and 160 and higher).

Both serology and HLA typing required additional consent from subjects to obtain blood samples, and this was available for only a proportion of cases. We have checked that agreement to ensure that this consent is not subject to bias by socioeconomic status.

To assist in the interpretation of the present results, we have made a crude estimation of the age-specific incidence curve of EBV<sup>+ve</sup> and EBV<sup>-ve</sup> disease in the study region. The age-specific proportions of EBV<sup>+ve</sup> (0–9, 10–14, 15–19, 20–

<sup>3</sup> Taking "suspected" as "yes" was analyzed; but the results, which were similar, though with ORs closer to the null, have not been reported.

<sup>4</sup> Measles, pertussis, rubella, chicken-pox, and mumps.

Table 1 Cases available for analysis

	EBV <sup>+</sup> ve	EBV <sup>-</sup> ve
Male	14	43
Female	5	41
HDLP <sup>a</sup>	2	10
HDMC	7	6
HDNS	10	67
HDNOS <sup>b</sup>	0	1
With HLA-DPB1 known	14	59
With EBV antibody titers known	10	44
With both HLA-DPB1 and EBV antibody titers known	10	38

<sup>a</sup> LP, lymphocyte predominant.

<sup>b</sup> NOS, not otherwise specified.

Table 2 EBV status<sup>a</sup> by history of reported IM and childhood infectious disease<sup>b</sup>

	EBV <sup>-</sup> ve	EBV <sup>+</sup> ve
IM <sup>c</sup>		
No	65	11
Yes	8	6
Childhood infection, ≥10 yr <sup>d</sup>		
No	62	11
Yes	11	6
Childhood infection, 5–9 yr <sup>e</sup>		
No	24	2
Yes	49	15
Childhood infection, <5 yr <sup>f</sup>		
No	48	12
Yes	25	5

<sup>a</sup> Numbers available for analysis = 90; restricted to classical HD.

<sup>b</sup> Measles, mumps, chicken-pox, rubella, and pertussis.

<sup>c</sup> OR, 5.10, 95% CI, 1.12–24.4; results are stratified by sex.  $P = 0.03$ .

<sup>d</sup> OR, 4.94; 95% CI, 1.06–26.3; results are stratified by sex.  $P = 0.04$ .

<sup>e</sup> OR, 4.41; 95% CI, 0.87–44.3; results are stratified by sex.  $P = 0.08$ .

<sup>f</sup> OR, 0.80; 95% CI, 0.19–2.86; results are stratified by sex.  $P = 0.94$ .

24, 25–34, 35–44, 45–49, and 50+ year) from Armstrong *et al.* (7) have been applied to age-specific HD incidence rates for a slightly larger area reported in Cartwright *et al.* (20).

The epidemiological study from which these data derive (19) included two controls/case matched by age and gender. The controls are not included in any results reported here.

## Results

EBV<sup>+</sup>ve HD cases in this series are more likely to be male and HDNS (Table 1). There is some evidence that EBV<sup>+</sup>ve cases are older than the EBV<sup>-</sup>ve cases; the Wilcoxon test comparing age at diagnosis by EBV status gives a marginally significant result for the total series ( $P = 0.053$ ), but when restricted to classical HD, the differences are less evident ( $P = 0.19$ ). Results below are restricted to classical HD except where explicitly stated otherwise.

EBV status is significantly and positively associated with reported prior IM (Table 2). Infectious illnesses under 5 years of age appear to be equally common in EBV<sup>+</sup>ve and EBV<sup>-</sup>ve HD, whereas those at older ages are more frequent in EBV<sup>+</sup>ve cases. Multivariate logistic regression confirms significant independent contributions for IM (OR, 4.96; 95% CI, 1.25–20.1;  $P = 0.03$ ) and childhood infections over 10 years (OR, 4.73; 95% CI, 1.13–19.9;  $P = 0.03$ ) in distinguishing EBV<sup>+</sup>ve from EBV<sup>-</sup>ve HD.

Small numbers of subjects limit analyses of EBV antibody

Table 3 EBV tumor status<sup>a</sup> by EBV antibody titers

	EBV <sup>-</sup> ve	EBV <sup>+</sup> ve
VCA antibody titers		
Negative	10	0
20–640	8	1
1280	9	2
2560	7	4
Higher	3	2
Exact $P$ (trend)		0.02
EA antibody titers		
Negative	18	3
10, 20	10	2
40, 80	6	2
Higher	3	2
Exact $P$ (trend)		0.22

<sup>a</sup> Numbers available for analysis = 46; restricted to classical HD.

Table 4 Associations<sup>a</sup> of EBV status, HLA-DPB1 type and reported IM

	Other type	DPB1*0301	Other type		DPB1*0301	
			IM: no	IM: yes	IM: no	IM: yes
EBV <sup>-</sup> ve	39	13	35	4	11	2
EBV <sup>+</sup> ve	8	6	7	1	1	4
OR (95% CI)	3.13 (0.57–18.9)		1.24 (0.02–15.4)		17.1 (1.06–11.77)	
Exact p-value	0.23		1.00		0.043	

<sup>a</sup> Numbers available for analysis = 66 (restricted to classical HD), with 65 also having prior known IM.

titers, but EBV<sup>+</sup>ve cases have higher antibody levels to both VCA and EA, with differences for the former being statistically significant (Table 3). These positive results have been checked using log (geographic mean titer). These cases all had a known IM history, and all of the EBV<sup>+</sup>ve cases (100%) had a positive history of IM or VCA antibody titers >640 (or both) compared with 20 of the EBV<sup>-</sup>ve cases (52%); this difference is statistically significant ( $P = 0.02$ ).

EBV<sup>+</sup>ve cases are more likely to type for HLA-DPB1\*0301 (Table 4); the difference is substantial but based on small numbers and not statistically significant (although of marginal statistical significance in the total series;  $P = 0.07$ ). Reported IM as a risk factor for EBV<sup>+</sup>ve compared with EBV<sup>-</sup>ve disease was examined for cases with and without the DPB1\*0301 phenotype. IM was strongly and statistically significantly associated with EBV-status in cases with the DPB1\*0301 phenotype only.

Multivariate analyses of cases where both serology and HLA-DPB1 type were known found VCA antibody titers (trend across five levels;  $P = 0.02$ ) and reported that IM ( $P = 0.04$ ) and typing positive for HLA-DPB1\*0301 ( $P = 0.049$ ) made significant independent contributions to EBV<sup>+</sup>ve status. However, the data are sparse, and  $P$ s based on the asymptotic  $\chi^2$  distribution will not be entirely reliable. Testing of interactions of HLA-DPB1 phenotype with reported IM and VCA antibody titers were similarly impaired by the small numbers but did not provide evidence of formal statistical significance ( $P = 0.14$  and  $0.53$ , respectively).

Fig. 1 gives estimated age-incidence curves for EBV<sup>+</sup>ve and EBV<sup>-</sup>ve HD in the study region. The anticipated peaks are seen in children (5–9 years of age) and in older adults, but, in addition, there is clear evidence of a young adult peak.

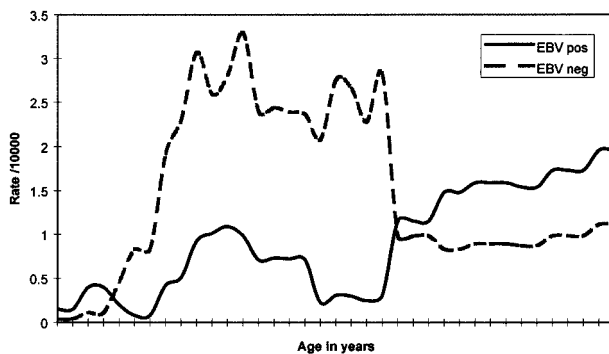


Fig. 1. Estimated age incidence curves.

## Discussion

Our present results confirm the case-control comparisons (19) in providing the first evidence that increased risk of HD after relatively recent infection with EBV is specific to EBV<sup>+</sup> HD. The study is limited by small numbers, especially of EBV<sup>+</sup> cases and of subjects with blood samples available for analyses. The total numbers and the general objectives of the study were similar to that of another recent case series (16), the main differences between the two studies being (a) different age ranges; (b) the inclusion in our study of the HLA-DPB1 type; and (c) the results. Sleckman *et al.* (16) found no association between EBV status and reported IM, whereas an association was found in the present study.

The data for reported IM for both case series should be uninfluenced by recall, because HD cases do not know whether their disease is EBV<sup>+</sup> or EBV<sup>-</sup>. Other artifacts (IM being a misdiagnosis of early HD; elevated EBV antibody activity in postdiagnosis serum being a consequence of HD) should affect both EBV<sup>+</sup> and EBV<sup>-</sup> HD in the same way. The case-case comparisons, which we present here, are more direct than comparisons of each case subgroup with separate controls, as we have reported previously (19); avoid recall and selection bias in controls; and permit analyses of additional data (serology and HLA type).

Differences in the results of the two case series need not be seen as contradictory, because Sleckman *et al.* (16) has a broader age range (16–55 years). EBV<sup>+</sup> cases in their study are concentrated at the lower and upper ends of the range, with the latter, we would suggest, likely to form the “younger tail” of older adult EBV<sup>+</sup> disease in whose etiology immune competence may be critical (7). However, IM diagnoses in young adults (16–34 years of age) are most likely to lead to HD and to do so relatively soon (6). Our study supports Sleckman *et al.* (16) and the anecdotal reports mentioned earlier, which indicated that EBV<sup>-</sup> HD was quite frequent in subjects who had had IM; our data suggest that may be attributable to the high population frequency of IM.

The difference between EBV<sup>+</sup> and EBV<sup>-</sup> cases in frequency of reported childhood infection in school-age children is statistically firm but difficult to interpret. Sero-conversion rates to EBV, but not HHV6 and HHV7, are low in children of these ages (31) suggesting that the frequency of exposure to infectious agents may have little relevance to age at EBV infection; however, low frequency might indicate increased risk of late infection, including that by any etiological agent(s) for EBV<sup>-</sup> HD. Previously, we had hypothesized that EBV<sup>+</sup> cases in young adults would resemble pediatric HD,

with markers of younger ages of childhood infection in general and EBV in particular. The present data suggest that ages of exposure to infectious agents are somewhat older in EBV<sup>+</sup> cases and, in any event, the EBV<sup>+</sup> cases were older when infected with EBV (*i.e.*, when they had IM).

The present study also finds evidence relating high EBV antibody activity to EBV<sup>+</sup> HD, and this may be interpreted as additional evidence of a causal role for recent EBV infection.

There is a strong suggestion that HLA-DPB1\*0301 is associated specifically with EBV<sup>+</sup> HD, although this is not statistically significant in the univariate analyses. It is of marginal statistical significance in the multivariate analyses and is supported by the effect modification that we have observed for reported IM. These results provide the first evidence that the increase in HLA-DPB1\*0301 observed in HD may be associated with a specific infectious agent, namely EBV. The findings are preliminary, and there is clearly a need for larger studies including all age groups to test the hypotheses that this study has generated:

(a) There is an excess of HLA-DPB1\*0301 in EBV<sup>+</sup> HD, or specifically, EBV<sup>+</sup> HD in young adults; (b) history of IM increases the risk of EBV<sup>+</sup> HD in young adults; and (c) these two items interact so that reported IM is a specific risk factor for EBV<sup>+</sup> disease in those who have inherited the HLA-DPB1\*0301 phenotype.

Consideration of the information presented in Fig. 1 suggests that the three-disease hypothesis for HD (7) may also apply to EBV<sup>+</sup> disease. Previously, we have suggested that early exposure to EBV may be associated with pediatric EBV<sup>+</sup> HD and an age-related decline in immune competence may be associated with EBV<sup>+</sup> HD in older adults (7). It now appears that late first exposure to EBV may increase the risk of EBV<sup>+</sup> HD in young adults.

Recently, some of us have completed data acquisition for a new study capable of testing these hypotheses. This study includes typing of *HLA-DQ* and *-DR* alleles. Other studies outside of the United Kingdom clearly are required. Furthermore, the results emphasize the need to include EBV status in any study investigating risk factors for the development of HD.

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