Detection of Epstein-Barr virus genomes in Hodgkin’s disease: Relation to age


Abstract
An investigation as to whether any particular subgroup of patients with Hodgkin’s disease was particularly likely to be Epstein-Barr virus (EBV) genome positive was made on samples from 95 patients. These were grouped according to age and Hodgkin’s disease subtype, and analysed using Southern blot analysis. Most samples from children or adults aged 50 years or over contained detectable EBV genomes; samples from young adults were only rarely positive. The differences in EBV positivity by age were highly significant, but there was no significant association between EBV and histological subtype after allowing for the effect of age.

The results support the hypothesis that Hodgkin’s disease in different age groups may have different aetiologies, and suggest that EBV does have a pathogenic role in Hodgkin’s disease in children and older age groups.

Accumulating evidence suggests that the Epstein-Barr virus (EBV) has a role in the pathogenesis of Hodgkin’s disease. Initial studies found raised antibody titres to EBV in such patients, and later it was shown that increased antibody titres occurred before Hodgkin’s disease was diagnosed. Using sensitive molecular probes, EBV genomes have been detected in tumour biopsy specimens from a proportion of cases. The EBV genomes contained within the tumours have been shown to be clonal, indicating that the EBV infected population has arisen from a single infected cell. Furthermore, the EBV genomes have been localised to Reed-Sternberg cells by in situ hybridisation, and recent studies have shown that the EBV latent genes EBER 1 and LMP are expressed by Reed-Sternberg cells (unpublished observations). These latter observations support the argument that EBV has an aetiological role in Hodgkin’s disease and is not simply reactivated during the course of the disease.

A consistent feature of the molecular studies is that EBV is detectable in only a proportion of cases. This figure varies from 18–41% using Southern blot analysis. The failure to detect EBV in all cases does not detract from the importance of the viral association as the pathological and epidemiological features of Hodgkin’s disease suggest that it is a heterogeneous condition. The disease is usually subdivided histologically into the four subtypes described in the Rye classification. Epidemiologically, however, three groups of cases can be distinguished and these correspond to the age groups 0–14 years, 15–34 years, and 50 years and over. There is some concordance between these two methods of sub grouping Hodgkin’s— for example, nodular sclerosis Hodgkin’s disease (HDNS) accounts for the peak incidence in the age range 15–34 years and mixed cellularity Hodgkin’s disease (HDMC) shows an increasing incidence with increasing age.

Most groups studying the role of EBV in Hodgkin’s disease have reported the subtype distribution of their cases. Using Southern blot analysis, four groups, including ourselves, have found a lower proportion of EBV genome positive cases within the HDNS subtype when compared with HDMC or all the other subtypes grouped together. These differences, however, have not reached statistical significance. Significant associations may have been masked either by the small numbers of cases included in some studies or the subjectivity of the histological classification system used. In a larger series of cases Pallesen et al, using immunohistochemistry, found a significant association between EBV positivity and HDMC.

In our initial study of the association between EBV and Hodgkin’s disease it was found that the EBV positivity was significantly associated with increasing age. It was also noted that the only two cases aged younger than 15 years in our study were EBV positive.

The purpose of the present study, therefore, was to determine whether the association between age and EBV genome positivity remained following the analysis of a larger number of cases in the age groups 50 years and over and younger than 15 years. Cases of HDMC disease were also positively selected to ascertain whether EBV was significantly associated with this histological subtype. We also EBV subtyped the positive cases to extend our previous observation that the EBV genomes were of subtype A, which is thought to be the prevalent strain of EBV in the United Kingdom.

Methods
Samples of lymph node or spleen from a total of 95 cases of Hodgkin’s disease were examined. Thirty five of these have been reported previously. The cases were grouped accord-
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EBV positivity by age and histological subtype

| Selection procedure | Age and histological subtype | | | | |
|---------------------|------------------------------|------------------|------------------|------------------|------------------|------------------|------------------|
|                     | 15-34 y                      | > 50 y           |
| Paediatric          | 2/22                         | 2/4              | 0/2              | 3/8              | 6/7              | 2/2              | 2/2              |
| HDNS                | 5/11                         | 10/16            | 7/11             | 16/23            | 9/13             | 2/2              |
| HDMC                | 2/8                          | 0/2              | 1/2              | 2/2              | 2/2              | 2/2              |
| Other               | 7/13                         | 4/12             | 0/2              | 3/8              | 16/23            | 9/13             | 2/2              |

"Other" includes cases of lymphocyte predominance and lymphocyte depleted Hodgkin's disease. Only non-selected cases or those selected on the basis of age were included in the age analysis. The histological subtypes of cases in the young adults and the 35-49 year age brackets are not given as the numbers in these groups were small; positive results were obtained from both HDMC and HDNS cases.

ing to age at date of biopsy and histological subtype (table). Forty seven of the samples were from non-selected patients with Hodgkin's disease; 40 were selected on the basis of age; and a further eight were selected for both age and subtype to increase the numbers in appropriate groups. These particular age groups were chosen on the basis of the epidemiological data cited above.17 Cases aged 35 to 49 years were not specifically selected as they are thought to represent an overlap between the young adult (15-34 years) and older (50 years and over) age groups. Cases aged less than 15 years were classified as paediatric. Control samples included 35 random lymph nodes, 12 reactive lymph nodes from paediatric patients, and 14 lymph nodes from patients with non-Hodgkin's lymphoma aged over 60; the latter samples have been reported previously.13 DNA was extracted from all samples using standard procedures, and Southern blot hybridisation was performed as described elsewhere.20 Southern blot hybridisation was chosen as the method of analysis in these studies as EBV genomes are only rarely detected in non-Hodgkin's lymphomas using this technique.5 21 Notable exceptions to the above are Burkitt's lymphoma and lymphomas in the immunosuppressed.22-26 To assess the sensitivity of our Southern blot technique we analysed 10 μg of serial 10-fold dilutions of DNA from the RAJI cell line. This cell line is estimated to contain 45 copies of the EBV genome.27 Two EBV probes were used. All samples were hybridised with the BamHI-W fragment which represents the internal repeat of EBV and therefore provides a sensitive indicator for its presence.28 For this analysis 10 μg of DNA were digested with the restriction endonuclease BamHI.

Twenty six of the positive samples, including samples from all groups, were subsequently hybridised with the EcoRI-D fragment of EBV.28 This probe contains the terminal repeats of EBV and by virtue of this can be used to assess the clonality of the infected population.29 For this analysis 20 μg of DNA were double-digested with BamHI and EcoRI. Following digestion of circular EBV genomes with BamHI and EcoRI, the EcoRI-D probe detects two fragments. One is a fragment of 6.5 kilobase pairs which is of constant size in all EBV genomes; the second varies in size from one viral genome to another, depending on the number of terminal repeat sequences which it contains. If the cell population infected with EBV has arisen from a single cell, infected at a low multiplicity of infection, then all the genomes will contain identical numbers of terminal repeats.29 This will result in the visualisation of a single, discrete band in addition to the 6.5 kilobase pair band.

In the above analyses DNA from placenta and from the infected cell line, B95-8, were used as negative and positive controls, respectively.

The polymerase chain reaction (PCR) was used to determine whether the EBV genomes detected were of subtype A or B. The primers used in this assay and the reaction conditions have been described elsewhere.13 30 Logistic regression was applied using GENSTAT V.31 Statistical testing used analysis of deviance and the asymptotic χ² distribution for the deviance difference under the null hypothesis. Each analysis was applied to the largest number of results which were appropriate from the table. In some analyses HDMC cases were grouped together with lymphocyte predominance and lymphocyte depleted cases in a category designated all other subtypes (HDAOs).

Results

SENSITIVITY OF SOUTHERN BLOT ANALYSIS FOR THE DETECTION OF EBV
Following Southern blotting and hybridisation with the BamHI-W probe, EBV DNA was detectable in 10 μg of the 10⁻⁴ dilution of RAJI DNA (data not shown). This suggests that we can detect a single EBV genome present in less than 0.45% of the cells in any 10 μg sample. This figure is only an approximate value as this calculation does not allow for the variation in the number of BamHI-W repeat sequences (usually seven to 12) present in different viral genomes.

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The results are summarised in the table. A total of 43 positive samples were identified; in these a fragment of about 3.1 kilobase pairs, which hybridised with the Bam HI-W probe, was detectable by autoradiography following two days of exposure. Seventeen of the 47 non-
selected cases were EBV genome positive (fig 1). Two of the random lymph nodes were EBV positive, one was a secondary nasopharyngeal carcinoma and the other was a B-cell immunoblastic lymphoma; both malignancies have been associated with EBV.\textsuperscript{6,21-23} One of the 14 samples from patients over the age of 60 with non-Hodgkin’s lymphoma was positive. This sample, which was also from a B cell immunoblastic lymphoma, gave a weakly positive autoradiographic signal following a 10 day exposure of the Southern blot.\textsuperscript{1} None of the reactive nodes from paediatric patients contained detectable EBV DNA.

**EFFECT OF AGE**

In the oldest age group 27 of the 38 (71\%) cases of Hodgkin’s disease were positive and in the paediatric group seven of the 13 (54\%) samples were EBV positive. In contrast to these results, only four of the 28 (<15\%) cases in the young adults aged 15 to 34 were positive. Samples which were selected on the basis of both age and subtype were excluded from the above analyses. EBV was detected in three of the eight samples from patients in the 35–49 year old age range; this proportion of EBV positive cases is intermediate between that for the young adult and older cases.

Statistical testing of the above results, performed without adjustment for disease subtype, indicated a trend for increasing EBV positivity with age: $\chi^2 = 15.7$ on 1 degree of freedom; $p < 0.001$. The departure from linear trend was tested by adding a quadratic term to the model: $\chi^2 = 9.6$ on 1 degree of freedom; $p < 0.01$. This indicates that the trend for an increase in positivity with increasing age is not linear and infers that as a group the paediatric cases are different. The age groups 15–34 and 50 years and over were then compared after adjustment for subtype. The finding of increased positivity in the older age group was highly significant: $\chi^2 = 24.8$ on 1 degree of freedom; $p < 0.0001$.

**EFFECT OF HISTOLOGICAL SUBTYPE**

There was a slight excess of positive cases in the HDMC group (table). This was statistically tested in the two age groups 15–34 and 50 years and over; all cases in these age ranges were included and adjustment for age was made. The effect of subtype was not significant when either HDNS, HDMC, and other subtypes were compared ($\chi^2 = 0.73$ on 2 degrees of freedom; $p > 0.5$), or when HDNS and HDAOS cases were compared ($\chi^2 = 0.73$ on 1 degree of freedom; $p > 0.5$). In the 15–34 year age group there was an excess of positive cases in the HDAOS group, but this did not reach significance (Fisher’s exact test, 1 tail; $p = 0.14$).

**CLONALITY OF EBV GENOMES**

The clonality of the EBV genomes in 26 of the positive cases was assessed by hybridisation with the Eco RI-D probe, as outlined above. In 25 of the 26 cases examined a single discrete fragment, which varied in size from case to case, was detected in addition to the invariant 6.5 kilobase pair fragment (fig 2A). This suggests that the cell population infected with EBV in these cases is a clonal expansion of a single infected cell. In the remaining sample, which was from a 59 year old man with HDNS (case 60), an intense invariant band at 6.5 kilobase pairs was present, but no additional bands were visualised (fig 2B). A similar result was obtained when this sample was digested with BamHI alone; the latter observation indicates that the fragment containing the terminal repeats was not comigrating with the 6.5 kilobase pair band in the original analysis.

**EBV SUBTYPE**

Thirty of the EBV positive samples were EBV subtyped using a PCR strategy; in all cases the genomes were subtype A (data not shown).

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**Figure 1**  Southern blot analysis of representative Hodgkin’s disease samples using the BamHI-W probe. Case 37: HDMC aged 14; case 74: HDNS aged 82; case 80: HDMS aged 54; case 85: HDMC aged 75; case 86: HDMC aged 72; case 88: HDMS aged 53. Additional bands visualised in B95-8, the positive control, represent the fragments flanking the BamHI-W repeat sequence.

**Figure 2**  Panel A: Southern blot analysis of representative EBV genome positive Hodgkin’s disease samples using the EcoRI-D probe. Case 37: HDMC aged 14; case 40: HDMC aged 8; case 45: HDNS aged 24; case 54: HDMC aged 31; case 74: HDNS aged 82; case 82: HDMC aged 78; case 87: HD lymphocyte predominance aged 65. In EcoRI and BamHI double-digested samples containing EBV DNA an invariant band of 6.5 kilobase pairs is seen (just visible in case 54). In the Hodgkin’s disease samples there is a single additional band of variable size (arrowed). Panel B: Southern blot analysis of case 60, HDNS aged 59, using the EcoRI-D probe. In this case only the 6.5 kilobase pair fragment is detected. The ladder of fragments seen in the positive control lanes (B95-8) indicates the presence of linear EBV genomes and episomal forms with different numbers of terminal repeats.\textsuperscript{9}
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Discussion
In this study we detected EBV genomes in tumour samples from most of the paediatric and older patients with Hodgkin's disease. This contrasted with the case in young adults in which EBV genomes were detected infrequently. Statistical analysis showed that the association between age and EBV was highly significant. Analysis of control samples did not show an excess of EBV positivity in lymph nodes from paediatric or older cases. No additional significant association between EBV positivity and Hodgkin's disease subtype emerged.

Most groups who have studied the association between EBV and Hodgkin's disease using Southern blot analysis have not examined the association between age and EBV positivity. Two exceptions to this are Boiocchi et al. and Libetta et al. Boiocchi et al did not find an association between age and EBV positivity, but only five older cases were examined out of a total of 17. In this study we detected a difference in the number of EBV positive cases in older patients compared with children and young adults, but this was not significant. The results of this latter study are difficult to interpret as in over half of the cases which they considered EBV positive the EBV BamHI-W probe hybridised to a DNA fragment of unexpected size.

The possibility of an association between EBV and Hodgkin's disease subtype has been addressed by most studies. In keeping with our results three other groups have reported a slight excess of positivity among the HDMC or HDAOS cases, but this did not reach significance. Pallesen et al, using immunohistochemistry, did find a significant association between expression of EBV and HDMC. Our results suggest that subtype should not be analysed separately from age as age is likely to be an important confounding variable. This is of particular relevance here as the relative incidence of HDMC is greater in older patients with Hodgkin's disease.

In the present study Southern blot analysis was used to detect EBV genomes. Recently the PCR has been used to look for EBV in Hodgkin's disease tumours and this has resulted in the detection of larger proportions (50% and 58%) of positive samples. Using Southern blot hybridisation and the BamHI-W probe we detected a single EBV genome in about 0-45% of the sample; this technique should therefore be sufficiently sensitive to detect EBV in most cases of Hodgkin's disease if the EBV genomes are present in Reed-Sternberg cells and their mononuclear equivalents. Indirect support from this comes from the results of an earlier study in which we did not find a correlation between Reed-Sternberg cell numbers and EBV positivity. Our assay might not have been sensitive enough to detect EBV in samples with low numbers of Reed-Sternberg cells, but the method used avoids the possibility of detecting low levels of EBV DNA present in residual B cells in the biopsy tissue.

We have shown that in 25 of the 26 cases examined the EBV genomes, in each case, contained terminal fragments of identical size.

This finding correlates with clonality and therefore suggests that the infected population has arisen from a single infected cell. This, in turn, supports the idea that EBV is involved in the pathogenesis of Hodgkin's disease in these cases.

In one case we could not detect a single discrete fragment containing the terminal repeat sequences. The most likely explanation for this is that the infected cells (either B cells or Reed-Sternberg cells) are not clonal with respect to EBV.

The EBV DNA sequences from 30 of the positive cases were subtyped into type A or type B viruses using the PCR. All were type A viruses. This result indicates that type B viruses, which have been detected more frequently in patients with immunosuppression than in normal controls, are not overrepresented in Hodgkin's disease tumours.

The results of this study support the hypothesis that Hodgkin's disease is a heterogeneous disease with different genetic etiologies in different age groups. The finding that EBV is present in tumours from most paediatric and older cases strengthens the argument that EBV is involved in the pathogenesis of these cases. The pattern of EBV infection is not necessarily identical in these two age groups. We speculate that de novo infection is important in the paediatric group; reactivation of latent infection, possibly as a result of declining T cell immunity, would seem more likely in the older patients. The observation that most Hodgkin's disease tumours from patients infected with the human immunodeficiency virus (HIV) are also EBV positive provides some support for an association between decreased T cell immunity and EBV positivity, although it is possible that other interactions are involved in the HIV positive cases.

The low level of EBV positivity in young adults, particularly in those with HDNS, suggests that other factors are involved in these cases. This is particularly exciting as it is in this group that there is most epidemiological evidence for a transmissible agent. Risk factors suggestive of lack of early social contact have been found in the young adult peak incidence group, suggesting that delayed exposure to a common infectious agent may be involved. Clustering of Hodgkin's disease cases is also most likely to occur in cases aged under 35 years and in HDNS. We are currently investigating the possibility that other viruses are involved in this group.

The possibility that EBV genome positivity is associated with an unfavourable clinical outcome should also be evaluated further as age over 50 years, HIV positivity, and HDMC, rather than HDNS, are all indicators of a poor prognosis.

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