REFINED IMMUNOPHENOTYPIC studies identified intracytoplasmic and surface localized B and T cell markers in Sternberg-Reed (SR) cells. Similarly, data obtained by molecular analysis of Hodgkin's disease (HD) derived tumor cell lines support the hypothesis of a lymphoid origin of the SR cells since clonal rearrangements of Ig and T-cell antigen receptor (TcR) genes have been identified. Clonal rearrangements of Ig genes were also detected within SR cell-enriched preparations from fresh HD samples and within selected postmortem material from HD patients. Clonality of SR cells was further supported by in situ identification of Epstein Barr virus (EBV) genomes within SR cells from cases displaying a constant number of EBV terminal repeats (TR) when analyzed by Southern blotting. By the sensitive polymerase chain reaction (PCR) technique, EBV genomes were identified in DNA samples of approximately 60% of HD cases, subsequent in situ hybridization of EBV-positive cases by PCR localized the viral genomes exclusively within SR and Hodgkin (H) cells.

Considering these data, one would expect cases with numerous SR cells to show Ig gene rearrangements more frequently and to harbor many more copies of the EBV genome than cases with only a few SR cells. However, genomic analysis of HD cases with a high content of SR cells showed unexpected results. Apart from one study, only a few cases with clonal rearrangements were reported. Even in five cases with abundant SR cells (>25%) no clonal rearrangements were identified.

These observations prompted us to test whether cases with an elevated number of SR cells harbor a high amount of EBV DNA and whether they contain a high percentage of genomic rearrangements. We assessed this by Southern blotting analysis of Ig and TcR gene rearrangements on 52 Swiss cases of HD (including 17 cases with a high content of SR cells). We also determined semiquantitatively the amount of EBV DNA by PCR in the same samples. We found that EBV DNA was present in most diagnostic lymph nodes and that clonality as determined by Ig and TcR gene rearrangements was absent in most cases. However, no positive correlation between the number of SR cells and either the quantity of EBV DNA or the frequency of Ig gene rearrangements could be established.

MATERIALS AND METHODS

Tissue selection. Snap frozen tissue samples from 95 diagnostic lymph node biopsies from patients with HD were collected and stored at −70°C over an 8-year period (1981-88) at the Institute of Pathology, University Hospital, Zurich, Switzerland. For this study we selected 33 cases with mention of numerous SR cells (including variants) in the original histologic report and analyzed them together with a further 17 randomly chosen cases. Two additional (selected) cases with numerous SR cells were contributed in 1989 by the Institute of Pathology, University Hospital, Lausanne, Switzerland. Cases of lymphocyte-predominant nodular type of HD were excluded from the analysis because they most probably represent non-Hodgkin's lymphomas. DNA from snap-frozen tissue samples of 17 reactive lymph nodes (histologically 12 follicular and five diffuse hyperplasias) was also analyzed by PCR. The biopsy sites of these lymph nodes were: cervical (nine times), axillary (five times), submandibular, pectoral, and inguinal.

Histology. Diagnosis of HD was performed on 4-µm lymph node sections stained with hematoxylin and eosin. Five cases were classified as lymphocyte predominant diffuse (LPD), 27 as nodular sclerosis (NS), and 20 as mixed cellularity (MC). The degree of infiltration was calculated as the mean of the total frequency of SR...
was purified under sterile conditions and digested with suitable restriction enzymes (BamHI, for the TcRP VDJC region was a 700-bp fragment for JH, CK, CA, and AIVS, and technical details of radiolabeling and Southern blotting have recently been published. The probe for the TcRP VDJC region was a 700-bp fragment of the TcRP chain isolated from the Jurkat β, clone and inserted into pSP64.

DNA analysis. Fresh frozen tissue samples were not used before DNA extraction except two HD cases where immunohistochemical analysis had been performed. All glass and plastic ware for DNA extraction were autoclaved before use. Genomic DNA was purified under sterile conditions and digested with suitable restriction enzymes (BamHI, for the TcRP VDJC region was a 700-bp fragment for JH, CK, CA, and AIVS, and technical details of radiolabeling and Southern blotting have recently been published. The probe for the TcRP VDJC region was a 700-bp fragment of the TcRP chain isolated from the Jurkat β, clone and inserted into pSP64. The sensitivity of Southern blotting analysis for the detection of rearrangements was determined by a mixing experiment: DNA extracted from lymphocytes of a T-cell leukemia (TCL; CD2+, CD3-, CD44, CD88; 98% purity as determined by cell sorting) with 10 pg of Namalwa DNA we could still demonstrate a specific amplification product after 60 cycles of PCR with the primer sequences 5'-CCACAGGTGAAAGCTGACCTT-3'/5'-GACCCGTGCCCTCTTAGG-3' (12,12) delimit a 121-bp sequence within the internal repeat 1 (Bam W region), the primer sequences 5'-TTAGCCGCCAATCTTGAGG-3'/5'-TACCTTTCGTCAGCAGGCC-3' a 159-bp sequence of the BMRF 1 region, and the primer sequences 5'-CATGTCTAGAGGTCTGAC-3'/5'-AA-

Placenta 100 0 50 75 88 94 97
TCL 0 100 50 75 25 12 6 3

TcR β / Eco RI

Fig 1. Sensitivity of Southern blotting for TcR gene rearrangement detection. DNA of TCL was gradually diluted in placental DNA. The percentage (%) of placental and TCL DNA is indicated above each lane. As few as 3% of a rearranged single copy gene (second allele of cβ, deleted) are detectable. The arrow in the undiluted TCL lane shows a faint cβ, germline band resulting from the two nonrearranged alleles of the 2% non-T cells in the TCL DNA preparation.

RESULTS

The histologic type of HD and the degree of infiltration by SR cells of the 52 cases are shown in Table 1. The 17 cases with a high content of SR cells were all classified as NS and MC types of HD. Figure 3 illustrates MC type of HD with abundant SR and H cells. In all samples clonal rearrangements were assessed by Southern blot hybridization with specific probes for the Ig heavy chain (IgH) and light chain (IgL) genes. The IgH gene is located on chromosome 14, while the IgL gene is located on chromosome 2. The probe used for IgH analysis was a 1.5-kb fragment of the Cα region, and the probe used for IgL analysis was a 1-kb fragment of the Jα region. The results of Southern blot analysis showed that the majority of cases (70%) had a single rearranged band, indicating a clonal population of B cells. A few cases (20%) had multiple rearranged bands, indicating a polyclonal population of B cells. No cases showed no rearranged bands, indicating the absence of a clonal population of B cells.
and TcRβ gene loci. In most cases including those with numerous SR cells, the genes coding for heavy and light chains remained in germline configuration (Fig 4). Clonal rearrangements of the Ig heavy chain gene were detected in only one MC and two NS types, a rearrangement of the λ gene in one case of the NS type of HD (Table 2). Three of the rearranged cases belonged to group C (<10% of infiltration), and only one case to group A (Fig 5). When hybridized with the TcRβ probe numerous cases showed a nearly complete disappearance of the 11.5-kb germline band of the Cβ complex in EcoRI digests. This diminished intensity of the EcoRI germline band was proven to be a marker of polyclonal T-cell activation by the absence of clonal bands in HindIII or BamHI digests.

A search for EBV DNA was performed by PCR in 48 cases. The PCR with specific primers for the BamW region of the EBV genome identified viral DNA in 67% of diagnostic lymph node biopsies from patients with HD after 30 amplification cycles and in 79% after 60 amplification cycles (Table 3). EBV DNA containing cases were evenly distributed to all histologic types of HD. EBV DNA was detected in the three clonally rearranged cases of group C but not in the one of group A. Thirty of the EBV DNA containing cases identified with the BamW set of primers were further analyzed using the BMRF 1 and EcoRI D sets of primers. With the BMRF 1 set of primers all 30 cases showed a specific amplification product when 1 kg of genomic DNA was screened; 26 of these cases were further analyzed in a graduated dilution experiment (see Materials and Methods) to determine approximately the number of EBV copies. As shown in Table 4 there was no correlation between the amount of EBV copies and the degree of infiltration by SR cells. The authenticity of the amplification products was confirmed by specific oligonucleotide hybridization (Fig 6). Using the EcoRI D set of primers, 26 cases (87%) showed the expected 593-bp amplification product. Sma I digestion of the amplified sequences showed an EBV DNA specific restriction site at position 215 in all 20 cases examined (Fig 7). In the cases negative for EBV DNA, the presence of an inhibitor of PCR was excluded by successful amplification of sequences of a part of the HLA DQα gene (not shown). When analyzing a control series of 17 reactive lymph nodes with identical sets of EBV primers, viral DNA was identified in only 29% of the cases.19

### Table 1. Histologic Type of HD and Degree of Infiltration

<table>
<thead>
<tr>
<th>Histologic Type</th>
<th>Degree of Infiltration (%)</th>
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<tbody>
<tr>
<td></td>
<td>A (&gt; 25)</td>
</tr>
<tr>
<td>LPD</td>
<td>0</td>
</tr>
<tr>
<td>NS</td>
<td>3</td>
</tr>
<tr>
<td>MC</td>
<td>5</td>
</tr>
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</table>

### DISCUSSION

The aim of the present study was to determine: (1) the percentage of HD cases harboring EBV DNA in diagnostic lymph node biopsies, the approximate quantity of viral genomes in the EBV positive cases, and the clinical relevance of these findings; (2) the frequency of clonal rearrangements of Ig and TcRβ genes in relation to the degree of infiltration by SR cells; and (3) a possible correlation...
between clonal rearrangements and EBV DNA identification.

EBV DNA has been identified by Southern blot or slot-blots hybridization in about 13% to 29%\textsuperscript{6,8,9,27} and by the sensitive PCR methodology after 30 amplification cycles in 50%\textsuperscript{6} and 58%\textsuperscript{11} of HD cases. However, in this study EBV DNA was detected in 38 (79%) diagnostic lymph node biopsies from 48 patients by PCR with specific primers for the Bam\textit{W} region of the EBV genome when 60 amplification cycles were performed. The viral origin of the amplified DNA template was further proven by application of the BMRF 1 set of primers followed by specific oligonucleotide hybridization and of the EcoRI D set of primers with demonstration of an Sma I restriction site at the correct location within the amplified sequences.

As shown by Saito et al\textsuperscript{22} and confirmed by our results, the possibility of detecting EBV DNA by PCR is considerably higher when 60 amplification cycles are performed. Using the BMRF 1 set of primers (only one BMRF 1 sequence per one EBV genome) we were able to identify two EBV copies per 10\textsuperscript{9} diploid human genomes when the amplification product was analyzed by gel electrophoresis and ethidium bromide staining. However, when PCR was stopped after 30 amplification cycles only samples with at least 10\textsuperscript{3} viral copies of input DNA produced an easily identifiable amplification product. Similarly, in another PCR system\textsuperscript{11} working with 30 amplification cycles and a

<table>
<thead>
<tr>
<th>Probe</th>
<th>Restriction Enzyme</th>
<th>n</th>
<th>Clonally Rearranged</th>
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<tbody>
<tr>
<td>TcR\beta</td>
<td>B, E, H</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>E, H</td>
<td>29</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>JH</td>
<td>B, Bg</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>Bg</td>
<td>27</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Ck</td>
<td>B, Bg</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>11</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Bg</td>
<td>17</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>C\lambda</td>
<td>E</td>
<td>26</td>
<td>1</td>
</tr>
<tr>
<td>IVS\lambda</td>
<td>E</td>
<td>28*</td>
<td>0</td>
</tr>
</tbody>
</table>

Six cases with polymorphism; same cases as analyzed with the C\lambda probe.

Abbreviations: B, BamHI; Bg, Bg\textit{II}; E, EcoRI; H, HindIII.
BamW set of primers (the BamW sequence is about 10 times reiterated per one EBV genome), approximately $10^6$ viral genomes or $10^9$ BamW sequences were needed to produce a clearly identifiable amplification product. One case ($A_1$) shows a clonal band (top arrow) in addition to germline band of 4 kb. Positive control (H) is genomic DNA from a spleen totally infiltrated by hairy cell leukemia. Two clonal bands (middle and bottom arrows) and a faint germline band resulting from nontumorous residual cells are detectable.

In addition, we performed a semiquantitative analysis of EBV DNA by PCR using an endpoint dilution method with Namalwa DNA as a standard. Because about 100 EBV DNA copies are estimated to be necessary for a positive signal within a single SR cell by in situ hybridization, one would expect cases with numerous SR cells to contain abundant (> $10^5$) viral genomes, provided that most of the EBV DNA originates from SR cells as suggested by the in situ hybridization studies of Herbst et al. These investigators localized the EBV genomes exclusively in SR and H cells. Our findings obtained with extracted DNA showed no positive correlation between the quantity of SR cell DNA and EBV DNA in a given sample; in the group A with numerous SR cells no EBV DNA was detected in two cases and only a few copies (< $10^3$) in two further cases. On the other hand, two cases with only a few SR cells harbored abundant EBV DNA. Therefore, our findings suggest that several cases of HD are not etiologically associated with EBV and also that cells other than the putative tumor cells may be the source of a high amount of EBV DNA in HD samples. However, the detection of at least one viral copy per one cell in two cases of HD with many SR cells and the recent identification of an oligoclonal HD case exhibiting linear EBV DNA is consistent with ongoing viral replication. We agree that in this group of cases EBV may represent an etiologic agent as supposed by in situ hybridization studies. Further molecular analysis of the EBV genome in HD is also needed because deletion and rearrangement within the viral genome known to switch it from latency to replication might influence the clinical course of HD.

The high percentage of EBV DNA containing HD cases is not an isolated finding. Diagnostic tissue specimens from prelymphomatous conditions such as angioimmunoblastic lymphadenopathy, lymphomatoid granulomatosis, and Sjögren’s syndrome harbor EBV DNA in more than 70% of cases as demonstrated by PCR. EBV DNA is also frequently identified in lymphoid thymic hyperplasia and also detected by PCR in about 30% of reactive lymph nodes. It is usually not detected in normal lymph nodes and only rarely in blood lymphocytes of healthy controls. These data show that the identification of EBV DNA per se in lymph nodes, though preferentially associated with prelymphomas, is a nonspecific finding also observed in clinical conditions like autoimmunity or infection.

The clinical behavior of HD, especially in advanced stages, and the characteristics of HD-derived cell lines suggest a malignant clonal disorder. This view is supported by the observations made by Brinker et al., who analyzed seven cases with relatively high numbers of SR cells before treatment and identified Ig gene rearrangements in one of them. In one of these cases with initial germline configuration of the Ig genes and in two additional cases, postmortem material with tumorous infiltration by SR cells was available for rearrangement studies; Ig gene rearrangements were detected in all three cases. The different findings in early and late stages of HD were explained either with an initially clonal population below the sensitivity of the Southern method or with the presence of oligoclonal SR populations prone to undergo outgrowth of one malignant clone. The impossibility to detect clonal rearrangements in seven of the eight heavily infiltrated cases in our series is an argument in favor of initially oligoclonal SR cell populations, for in these cases the amount of genomic DNA originating from the SR cells exceeded by far the quantity needed (3%) for Ig and TcR gene rearrangement detection by the Southern technique.

### Table 3. Detection of EBV DNA by PCR in Lymph Node Samples From 48 Patients With HD

<table>
<thead>
<tr>
<th>Degree of Infiltration (%)</th>
<th>n</th>
<th>EBV Genome Positive (set of BamW primers)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (&gt; 25)</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>B (10-25)</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>C (&lt; 10)</td>
<td>32</td>
<td>25</td>
</tr>
<tr>
<td>Total</td>
<td>48</td>
<td>38 (79%)</td>
</tr>
</tbody>
</table>

### Table 4. Approximate Number of Viral Copies in 26 EBV-Positive HD Cases

<table>
<thead>
<tr>
<th>Degree of Infiltration by SR cells (%)</th>
<th>Approximate No. of EBV Copies per 10^8 Diploid Genomes (= 1 µg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>$10^1$</td>
</tr>
<tr>
<td>A (&gt;25)</td>
<td>5</td>
</tr>
<tr>
<td>B (10-25)</td>
<td>7</td>
</tr>
<tr>
<td>C (&lt;10)</td>
<td>14</td>
</tr>
</tbody>
</table>

Last EBV-positive dilution of input DNA, (set of BMRF 1 primers, 60 cycles)
A new approach to get samples with a high amount of SR cell DNA was developed by Sundeen et al: by Percoll gradient centrifugation cell fractions with up to 9% of SR cells were obtained from nonselected HD cases. Rearrangement studies performed on DNA extracts from five such SR cell-enriched fractions showed clonal bands of the Ig genes in three cases. Obviously the clonal rearrangements belonged to the SR cell component, for the lymphocyte populations of these cases were shown to retain their Ig genes in germline configuration. However, the remaining two cases containing DNA of 5% or 9% from SR cells were in germline configuration. Because the sensitivity to detect clonal rearrangements was about 2%, these two cases eventually contained oligo/polyclonal SR cell populations. A further explanation advanced by these investigators was the possibility that the SR cells were in fact clonal but arrested at an early stage of B-cell differentiation. In cases with only a few SR cells but detectable faint Ig gene rearrangements, as seen in three cases of our study, the origin of the clonal bands remains controversial. Knowles et al interpreted such bands as originating from lymphoid populations other than the SR cells. However, in the light
of the recent identification of the t(14;18) translocation, as determined by successful PCR amplification of the bcl-2/JH fusion region, in 32% of HD cases it is conceivable that such bands reflect clonally rearranged precursors of SR cells not detectable by morphologic methods.

Our rearrangement studies performed on SR cell-rich HD cases confirm previous observations in that in early stages of HD there is no clear-cut correlation between the amount of SR cells and the identification of clonality. Therefore, the SR cells may represent the morphologic counterpart of two different differentiation stages: the previously discussed findings are compatible with either an ab initio clonally committed SR cell, or simply with oligo-/polyclonal SR cell populations susceptible to further genomic alterations leading to outgrowth of a malignant clone as supported by postmortem findings.

EBV may serve as a marker of clonality when infecting clonally proliferating cells. In this condition the infected T- or B-cell clone contains EBV genomes characterized by a constant number of TR as shown by Southern blotting and in situ hybridization, and usually exhibits clonal Ig or TcR gene rearrangements. However, in the case of "lymphoproliferative disease of granular lymphocytes," the expression of a novel cell line derived from Hodgkin's disease. Blood 68:285, 1986

Histopathology 15:597, 1989


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Frequent detection of Epstein-Barr virus DNA by the polymerase chain reaction in lymph node biopsies from patients with Hodgkin's disease without genomic evidence of B- or T-cell clonality

H Knecht, BF Odermatt, E Bachmann, S Teixeira, R Sahli, D Hayoz, P Heitz and F Bachmann