Demonstration of Epstein-Barr Virus Replication in Reed-Sternberg Cells of Hodgkin’s Disease

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Epstein-Barr virus (EBV) is detectable in approximately 40% of cases of Hodgkin’s disease (HD). The viral genomes remain latent but positive staining with anti-ZEBRA antibody in a small fraction of Reed-Sternberg (RS) cells of some cases of HD would suggest possible activation of EBV replication within these cells. We report the investigation of 40 cases of EBV-associated HD (including 5 human immunodeficiency virus [HIV]–positive cases) using anti-ZEBRA antibodies. Positive staining was found in only three (HIV-negative) cases. One of these three cases showed approximately 1% of ZEBRA-positive tumor cells, whereas the other two cases showed rare positive cells. In the case with 1% ZEBRA-positive cells, a strong signal was obtained with anti–EA-R antibody and BHLF1 oligoprobes, which indicated early gene expression. EBV replication could be shown in this case by nonisotopic in situ DNA–DNA hybridization, which showed markedly increased numbers of EBV genomes in a few RS cells. Viral replication was confirmed using reverse transcriptase and polymerase chain reaction that detected transcripts from the BHLF1 gene encoding for the membrane antigen gp350/220. EBV replication in RS cells seems to be an exceptional event but may provide clues to mechanisms of control of viral latency and assume clinical implications in the future.

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872

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anti--EA-R (DuPont, Billerica, MA), anti-MA (DuPont), and anti-VCA (DuPont) antibodies.

In situ hybridization and Southern analysis. ZEBRA-positive cases were also investigated by in situ hybridization using fluorescein isothiocyanate (FITC)-labeled antisense EBER1/2 and BHLF1 oligonucleotides on routinely processed sections. In situ hybridization was performed with the DAKO hybridization kit as described previously.25 Southern blotting was performed in ZEBRA-positive cases when frozen tissues were available. The details of this procedure have been described elsewhere.26 The total DNA was digested with BamHI (Bethesda Research Laboratories [BRL], Rockville, MD). After transfer and immobilization on nylon membrane, digested DNA was hybridized with BamHI W (3.1 kb) and Xho I (1.9 kb) EBV probes labeled by random priming with dCTP-alpha-32P.

RNA extraction and RT-PCR. RNA extraction was performed in 10 EBV-positive cases as previously described.28 After RT with an oligo dT primer, PCR amplification of the cDNA was performed as follows: precycle at 95°C for 2 minutes, and cycles 1 to 25 and 26 to 30 at 93°C for 1 minute (strand separation), 60°C for 30 seconds (annealing), and 72°C for 1 minute (primer extension). Between cycles 25 and 26 and after cycle 50, the reaction was held at 72°C for 5 minutes. The primers for the BHLF1 gene were 5'-TATCCA-CTGCGAGTGATTT-3' (downstream) and 5'-AGACCTTGAA-TCTGTAAGT-3' (upstream). They delineate a 335-bp sequence of the BHLF1 fragment (late gene) of the EBV genome encoding for membrane antigen (gp 350/220). The internal control probe consisted of 5'-TATTAGTCTGGAATGCAGCC-3' and 5'-AGTTAGGCTCACGCTC-3'. The details of the probe with specific controls have been described elsewhere.29 To confirm that a specific BHLF1 amplification product was obtained from cDNA and not from contaminating genomic DNA, a part of the LMP1 (latent membrane protein 1) gene including the first 87-bp long intron was amplified simultaneously using the same cDNA as a template. The primers were 5'-GACTGGACTGGAGGAGCCCTC-3' (upstream) and 5'-GAGACGTCCCTCCCATAG-3'.

RESULTS

All results are summarized in Table 1. Among the 40 cases of HD tested, three cases of the MC subtype, occurring in nonimmunocompromised patients, showed ZEBRA expression on ModAMeX sections. The staining was nuclear, strong, and exclusively localized to large cells (Fig 1A). The signals obtained were comparable with the four antibodies used. Approximately 1% of tumor cells were labeled in one case (case H1), and rare scattered cells were labeled in the other two. It was not possible to detect MA or VCA antigens in these three cases, but the results were difficult to interpret because of the background yielded by these antibodies on tissues sections, particularly by cross-reactivity with macrophages. However, a strong labeling was noted in few cells only in the case H1 containing numerous ZEBRA-positive cells, with anti--EA-R antibody (cytoplasmic) and BHLF1 oligoprobes (nuclear) (Fig 1B). The number of EA-R/BHLF1-positive cells was slightly lower than those with anti-ZEBRA antibodies. Surrounding small lymphocytes were distinctly negative with anti-ZEBRA and anti--EA-R antibodies and by in situ hybridization using BHLF1 oligoprobe in all investigated cases. The results of the in situ hybridization with BamHI W probe indicated weak but clear nuclear signals in a high percentage of cells in these 3 cases as well as in the 37 other cases (Fig 2, arrows). But the in situ hybridization signals with BamHI W probe were exceptionally strong in few tumor cells of the case H1 containing numerous ZEBRA/E-A-R/BHLF1+ cells (Fig 2) instead of the usual scant granular hybridization product in the nuclei of most infected cells (Fig 2, arrows). This was indicative of greater quantity of the hybridization targets. As expected, the greatest proportion of tumor cells and a few scattered small lymphocytes were stained with EBER1/2 oligoprobes in all cases. Double labeling obtained with biotinylated BamHI W probe and anti-ZEBRA antibodies clearly indicated that all cells with strong in situ hybridization signal were ZEBRA positive, but not all ZEBRA-positive cells contained abundant EBV DNA. Double labeling with anti-CD30/HSR4 or anti-CD15/ION1 antibodies and BamHI W probe confirmed that hybridization signal was restricted to CD15+/CD30+ cells. Last, a careful examination of the routine sections of the case H1 showed the presence of big inclusions with irregular outline (possibly related to high amount of viral genomes), different from the usual large nucleolus of RS cells, and frequent mitotic features (Fig 1C). Southern analysis confirmed the presence of EBV genomes in the case H1 and in another ZEBRA-positive case. The Southern analysis also demonstrated that the viral genomes were monoclonal and in a circular episomal configuration (with fused terminal repeats) with Xho I probe, which hybridized a unique band between 9 and 23 kb in size, but a linear component was not detectable. Nevertheless, EBV replication could be finally confirmed in case H1 by RT-PCR, which detected transcripts of the BHLF1 gene with a specific band 335 bases long positively hybridized with internal oligoprobe (Fig 3). No BHLF1 gene transcription was detectable in the other cases, including one case containing rare ZEBRA-positive cells. No RAG-1 gene expression (Fig 3) and no Ig and T-cell receptor gene rearrangements were found for these two cases (not shown).

DISCUSSION

This study confirms the activation of replication of EBV in few cells of a minority of EBV-associated HD as shown

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### Table 1. Results Obtained in the 40 EBV-Positive Cases of HD With the Different Methods Used to Detect EBV Replication

<table>
<thead>
<tr>
<th>Cases</th>
<th>Anti-ZEBRA</th>
<th>Anti--EA-R</th>
<th>BHLF1</th>
<th>EBV DNA+++</th>
<th>SB</th>
</tr>
</thead>
<tbody>
<tr>
<td>P/T</td>
<td>3/40</td>
<td>1/3</td>
<td>1/3</td>
<td>1/3</td>
<td>1/101/2/2</td>
</tr>
</tbody>
</table>

*Abbreviations: P/T, positive/tested; EBV DNA++++, strong signal obtained by in situ DNA-DNA hybridization; SB, Southern blotting.

* 10 EBV-positive cases have been tested.

† Absence of linear component of EBV genome including the case with BHLF1 transcripts (H1).
by Pallesen et al.\textsuperscript{11} This could be demonstrated by immunohistochemistry with anti-ZEBRA antibodies in 3 of 40 cases, with a higher proportion of cells in 1 of 3 cases (case H1). However, it was not possible to detect the expression of MA or VCA by the mean of immunohistochemistry. It would seem therefore that either EBV is involved in an abortive lytic cycle or that these different antigens are expressed at a low level in EBV-infected RS cells and thus are not detectable by our immunohistochemical methods with the antibodies tested. Using both anti-EA-R antibody and antisense oligonucleotides specific for BHLF1 transcripts (hallmark of the lytic cycle),\textsuperscript{16,18} it was possible to detect positive RS cells in one case only (case H1). In this case, numerous tumor cells were also positive with anti-ZEBRA antibodies. Thus, the pathways of activation of EBV replication involve an immediate early gene, BZLF1, and at least two of the early gene expression. This finding prompted us to analyze the results of the in situ hybridization with DNA probes.
EBV REPLICATION IN HODGKIN'S DISEASE

**Fig 3.** Detection of the BLLF1 mRNA in HD using RT-PCR. cDNA from HD case H1 (ZEBRA+) and H2 (ZEBRA+) were assessed for BLLF1 and LMP1 gene expression by RT-PCR. Genomic DNA from Jijoye (Ji) cell line served as positive control. (a) Gel electrophoresis profile of the amplification products. With the BLLF1 set of primers, specific bands (335 bp) are seen in the positive control Ji and also in H1 lanes. No amplification product is detectable for case H2. Amplification with the LMP1 set of primers results in a 255-bp band of genomic DNA–specific amplification product in lane Ji and in two 177-bp long bands of cDNA amplification products in lanes H1 and H2. Absence of additional bands at 255 bp indicates high purity of input cDNA in H1 and H2 and simultaneously proves that the BLLF1 amplification product detected in H1 originates from cDNA (mRNA). No RAG-1 gene expression was detected in both cases H1 and H2. (b) Southern blot of gel shown in (a). Internal oligonucleotide probe hybridization proves specificity of the BLLF1 amplification products. (c) Southern blot shown in (b) was washed and rehybridized with an LMP1–specific internal oligonucleotide. Specific signals are seen in the genomic DNA lane (Ji) and in both HD cases H1 and H2. Absence of additional band at 255 bp confirms the purity of cDNA in H1 and H2.

**BamHI** W probe to see whether the signal was accentuated in a few cells, as it would be expected after viral replication.12,28 The use of nonisotopic in situ hybridization was helpful because of the high resolution of the labeling and the absence of background.6,28 Moreover, this method allows semiquantification of the number of EBV genomes. Interestingly, in the case containing 1% of ZEBRA-positive cells (H1), it was possible to show a signal of unusual strength in few cells, similar to that commonly observed in EBV-infected epithelial cells of oral hairy leukoplakia.29 This strong signal seems to indicate that, in rare cells (ZEBRA+, EA-R+, BHLF1+), EBV replication occurs and that, in these cells, EBV genomes are probably in high copy number, similar to the demonstration of Tagaki et al.28 in EBV-infected cell lines.

The expression of BLLF1 gene (expressed after viral replication and encoding for membrane antigen gp 350/220) confirmed the replication of EBV in HD tissues as previously shown in one case by Joske et al.19 Together with the labeling with anti-ZEBRA and anti–EA-R antibodies and with BHLF1 oligoprobe, the demonstration of BLLF1 expression clearly indicates that EBV replication occurs within RS cells. The negative staining of non-neoplastic surrounding lymphocytes with anti-ZEBRA and anti–EA-R antibodies and with BHLF1 oligoprobe makes it unlikely that this replication could take place in these non-neoplastic cells. One would expect that viral replication yields linear EBV genomes.16,20 This finding could not be confirmed by Southern blot analysis and only the circular form of EBV genome was detected in this case as well as in the lymphoblastoid cell line used as control. The most probable explanation is that only few cells are involved by EBV replication, and thus linear EBV DNA cannot be detected by Southern blotting because of the extreme dilution in total cellular DNA. The activation of EBV replication in HD occurs in sporadic cases and seems to involve few cells in each case. Even though we have confirmed the evidence of EBV replication, it would be difficult to consider the occurrence of a full lytic cycle in all ZEBRA-positive cells but consideration of abortive lytic cycles is more appropriate. Instead of being responsible for the cell death, these abortive lytic cycles might augment the cell growth and thus the course of the disease. Indeed, ZEBRA protein is known to be partially homologous (ie, DNA-binding domains) to c-fos13 and, in addition, is capable of transactivating the promoter of c-fos.29 So it is conceivable that during abortive lytic cycles, ZEBRA and other early gene products such as BMLF1 (EA-D transactivator)13 might play a role in the proliferation of EBV-infected RS cells. This was further suggested by the high number of mitotic figures observed on conventional examination in the case H1 showing RS cells with replicative EBV genomes. EBV replication in RS cells is apparently an unusual event with as yet no clear etiologic and clinical implications. Spontaneous activation of replication is constantly observed, although in rare cells (10^-3 to 10^-6) in lymphoblastoid cell lines.30 The frequency of this phenomenon compared with its rarity in HD could be explained by the lack of host control in in vitro conditions. An immunologic mechanism could be therefore envisaged for the control of viral latency, as suggested by Pallesen et al,11 who found a high incidence of ZEBRA-positive cases in AIDS-related non-Hodgkin's lymphomas. Surprisingly, no ZEBRA expression could be detected in our five AIDS-related cases of HD.

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