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 an exceptional event but may provide clues to mechanisms of control of viral latency and assume clinical implications in the future.

HODGKIN’S DISEASE (HD), particularly of mixed cellularity (MC) subtype, is considered to be EBV associated in about half of the cases. Monoclonal Epstein-Barr virus (EBV) genomes have been found in the neoplastic cells of HD by different molecular methods,1,6 and EBV latent gene transcripts and proteins have been detected by in situ hybridization and immunohistochemistry.5,7-10 The viral gene expression of EBV-infected Reed-Sternberg (RS) cells is now better known (EBNA2+, LMP1+, EBER1/2 transcripts11) and confirms that the virus remains latent in this disease. Pallesen et al.11 have recently shown that activation of EBV replication could be suspected because of the detection of ZEBRA (BZLF1) protein in rare cells of a few cases of EBV (LMP1)–positive HD. This protein, ZEBRA, originates from the BZLF1 (BamHI Z Left Frame 1) sequence and is known to be involved in the switch of EBV from a latent to lytic (productive) cycle.12-14 The expression of ZEBRA in rare cells would suggest that in these cells, EBV replication is activated despite the yet unsuccessful detection of early (EA), membrane (MA), and viral capsid (VCA) antigens.10,11 Thus, it could be considered that this activation may induce an abortive instead of a full lytic cycle. In this study, we reexamined 40 cases of EBV (DNA and LMP1)–positive HD by using anti-ZEBRA antibodies. Additionally, anti–EA-R (early antigen restricted), anti-MA (membrane antigen), and anti-VCA (viral capsid antigen) antibodies were applied to ZEBRA-positive cases. Nonisotopic in situ hybridization was performed in the same cases using RNA probes to detect EBV genomes and antisense oligonucleotides to detect EBER1/2 gene transcripts (detected during latency)9,15-16 and BHLF1 gene transcripts (specific of lytic cycle).17,18 Combined reverse transcription and polymerase chain reaction (RT-PCR) were used to search for BHLF1 gene (gp 350/220) transcription19 when material for RNA extraction was available. Last, DNA extraction and Southern analysis with Xho I probe were performed to determine whether EBV genomes are monoclonal or polyclonal and in circular (with fused termini) or in linear form.20

MATERIALS AND METHODS

Cases selection. We selected 40 cases of HD (5 acquired immunodeficiency disease (AIDS)-related cases) in which EBV DNA and EBV latent membrane protein 1 (LMP1) were detected by in situ hybridization and immunohistochemistry, respectively.10 in tissues processed by the (ModAMeX) method.21 Details of the ModAMeX method have been already described.11,16 Immunohistochemistry was performed by the alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP) method.22 Dual labeling was also performed by combined in situ hybridization with BamHI W probe and immunohistochemistry with anti-CD30/HSR4, anti-CD15/ION1 (Innunotest, Marseille, France), anti-LMP1 (CS 1-4, Dako, Copenhagen, Denmark),23 or anti-ZEBRA antibodies (see below).

Immunohistochemistry. All 40 cases were investigated on ModAMeX sections by APAAP technique with four different anti-ZEBRA antibodies: clone BZ1 (Dako) and clones AZ594, AZ125, AZ130. These three AZ-antibodies were produced with recombinant ZEBRA protein obtained after transfecting b21 bacteria with the expression factor pRT3CZ25 as described elsewhere.24 The ZEBRA protein obtained was used to immunized BALB/c mice. The three MoAbs isolated consisted of clone AZ125 (IgG2a) against the region located between amino acids 59 and 93 (transcriptional activation domain) and the clones AZ94 and AZ130 (IgG1 and IgG2b) against ZEBRA basic region located between amino acids 145 and 200. ZEBRA-positive cases were immunostained with monoclonal...
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. Southern blotting was performed in ZEBRA-positive cases when frozen tissues were available. The details of this procedure have been described elsewhere. The total DNA was digested with BamHI (Bethesda Research Laboratories [BRL], Rockville, MD). After transfer and immobilization onto nylon membrane, digested DNA was hybridized with BamHI W probe 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. 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-CCTGCGAGGTAAGTT-3' (downstream) and 5'-AGGCGGTGAA-TCTGTAATGT-3' (upstream). They delineate a 335-bp sequence of the BHLF1 fragment (late gene) of the EBV genome encoding for membrane protein 1) gene including the first 87-bp long intron was searched for with the following primers: HK3 1, S-GCCATGAAG-TCTGTAATGT-3'. Amplification products from genomic DNA were 255 bp long. Last, expression of the BLLF1 fragment (late gene) of the EBV genome encoding for membrane antigen (gp 350/220). The internal control probe consisted of 5'-TTAGTTGCGAACTGAGC-3' and was 5' end labeled in gamma with 32P. The details of the protocol with specific controls have been described elsewhere. 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 intron was amplified simultaneously using the same cDNA as a template. The primers used were 5'-GACTGGAGACTGGAGGACCCCTC-3' (upstream) and 5'-TGCTGTGCTGCAATCCTTA-3' (downstream). The control of specificity was performed with the internal oligoprobe 5'-AGACCTTCTCTGTCCACTG-3'. Amplification products from cDNA templates were 177 bp long, whereas amplification products from genomic DNA were 255 bp long. Last, expression of human recombination activating gene 1 (RAG1) was searched for with the following primers: HK3 1, S-GCCATGAAG-AAGGAATGAAATT-3' (upstream), HK3 2, S-AGAGAATTAACCTACAAACTGC-3' (downstream), and the internal oligoprobe 5'-GAGACGTTCCCTCCATGAT-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 oligo-probe 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+/EA-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 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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<th>CASE</th>
<th>H1</th>
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<th>EBV DNA+++</th>
<th>BHLF1 SB</th>
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<td>P/T</td>
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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 with BHLF1 transcripts (H1).
by Pallesen et al. 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), 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.

Fig 1. (A) Positive nuclear staining with anti-ZEBRA antibody in a RS cell in case H1. ModAMex sections X 800, APAAP method, and new fuscin. Hematoxylin counterstaining. (B) Same case showing strong nuclear labeling of rare tumor cells by in situ hybridization with FITC-labeled BHLF1 oligonucleotides. Routinely processed sections X 250, new fuscin. Hematoxylin counterstaining. (C) Same case showing the presence of RS cells with big nucleus inclusions (arrow). Routinely processed section X 310. Giemsa counterstaining.

Fig 2. In situ hybridization findings with biotinylated BamHI W probe in the case H1 containing numerous ZEBRA-positive cells. Exceptionally strong nuclear signal in the form of sizable dots in 1% of cells (bottom left and insets) instead of the usual scant granular hybridization product in the nuclei of most infected cells (arrows). ModAMex sections, X 310, BCIP-NBT. Methyl green or no counterstaining.
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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 long 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. This strong signal seems to indicate that, in rare cells (ZEBRA+, EA-R+, BHLF1+), EBV replication does occur and that, in these cells, EBV genomes are probably in high copy number, similar to the demonstration of Tagaki et al28 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-fos13,29 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 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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