Detection of Epstein-Barr Virus Messenger RNA in Reed-Sternberg Cells of Hodgkin’s Disease by In Situ Hybridization With Biotinylated Probes on Specially Processed Modified Acetone Methyl Benzoate Xylene (ModAMeX) Sections

By Pierre Brousset, Shashikant Chittal, Daniel Schlaifer, Josette Icart, Catherine Payen, Françoise Rigal-Huguet, Jean-Jacques Voigt, and Georges Delsol

Recent studies have demonstrated the presence of Epstein-Barr virus (EBV) DNA in tissues involved by Hodgkin’s disease. Monoclonal EBV genome has been identified using Southern blotting in approximately 20% to 30% of cases of Hodgkin’s disease (HD). Furthermore, using DNA extraction and polymerase chain reaction (PCR), EBV DNA was demonstrated in 56% of the cases of HD. EBV genome has also been localized to Hodgkin (H) and Reed-Sternberg (RS) cells by in situ hybridization with isotopic probes, in routinely fixed and embedded tissues.

The detection of EBV messenger (m)RNA in H-RS cells using nonisotopic biotinylated DNA probes in studying 55 cases of HD of different subtypes, of which one was seropositive for human immunodeficiency virus (HIV). Cases of non-Hodgkin’s lymphomas seronegative for HIV, B-cell lymphomas in acquired immunodeficiency syndrome (AIDS), lymphadenitis of infectious mononucleosis, and nonspecific reactive lymphadenitis were used for comparison. In all cases, the tissues were processed routinely as well as fixed and embedded by modification of the acetone, methyl benzoate, xylene (AMeX) method. The sections prepared by the modified AMeX (ModAMeX) method were technically suitable only for the detection of EBV mRNA. EBV mRNA localization to H-RS cells was confirmed owing to their simultaneous expression of CD30 and CD15 antigens. Results of positive and negative hybridization were retrospectively correlated with EBV serology and clinical data over 3 years.

Materials and Methods

Tissues and Control Specimens

Among the lymph node biopsy specimens acquired by the lymphoma study group at CHU-Purpan in Toulouse during the preceding 3 years, tissues in 55 cases of HD were processed by both routine (fixed in ethanol-based Bouin’s fluid and embedded in paraffin) and the ModAMeX method. All biopsies were performed for diagnosis with full consent of the patients. The cases of HD belonged to the following immunomorphologic groups: nodular, lymphocyte predominance (nLP); nodular sclerosis (NS), mixed cellularity (MC), 26; unclassifiable (UN), 1. One additional case of HD (MC) was associated with HIV+ serology. In 53 of 54 cases of HD, in situ hybridization was performed on first lymph node biopsies at the time of diagnosis, and in one case on a second lymph node biopsy at relapse. Of the cases of non-Hodgkin’s lymphomas (NHL) acquired during the same period, lymph node biopsies from 45 cases processed with the ModAMeX method were selected randomly and used for comparison. The NHL cases by immunophenotype were as follows: B-cell lymphomas, 26; T-cell lymphomas, 10; anaplastic large cell lymphomas (CD30+), 5; B-cell lymphomas occurring in patients with AIDS, 4 (Table 1).

Five cases of EBV-associated nasopharyngeal carcinoma, one case of lymphadenitis of infectious mononucleosis, two lymphoblastoid cell lines immortalized by EBV (cell lines from our laboratory), were used as putative positive controls. Three nonspecific reactive lymph node specimens and one EBV-negative T-lymphoma cell line (CEM) were used as negative controls.

Briefly, the ModAMeX method consists of cutting the fresh tissue in fragments of 2 to 3 mm thickness for fixation in a mixture of acetone containing protease inhibitors (phenyl methyl sulfonyl fluoride and iodoacetamide) at 20°C for 6 to 24 hours, followed by clearance in methyl benzoate and xylene and subsequent embedding in low melting point paraffin (X-Tra 52-54°C; Momonjet Scientific Division, Sherwood Medical, St Louis, MO). Sections of 3.0 thickness, on glass slides (RNAse free) pretreated with 2% immunodeficiency virus (HIV)-related case of HD (mixed cellularity) and in 2 of 4 cases of B-cell lymphomas occurring in patients with acquired immunodeficiency syndrome (AIDS). In other non-Hodgkin’s lymphomas, EBV mRNA was detected in only 1 of 41 cases. Cases of HD positive for EBV mRNA were immunostained by CD30 and CD15 antibodies. The hybridization signals were exclusively restricted to Reed-Sternberg cells and variants. When analyzed retrospectively, no statistically significant correlation emerged between hybridization findings, EBV serology, or disease outcome over the 3 years of the availability of ModAMeX technique. The findings support the contention of a direct role of EBV in the pathogenesis of HD, at least in some cases.

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solution of 3-aminopropyl-triethoxysilane in acetone (Sigma, St Louis, MO) were used for hybridization studies.

DNA Probes

Two probes (3 kb and 3.1 kb), corresponding to the same BamHI-W region, are randomly repeated (7 to 12 times) in the viral genome. One probe (3 kb) was obtained in the biotinylated form (dUTP-11-biotin) from a commercial source (Enzo Diagnostics, New York, NY). The other probe (3.1 kb) was subcloned in pBR322 (kindly provided by Dr G W Bornkamm), purified, and labeled by nick translation using dATP-14-biotin (kit from BRL, Gaithersburg, MD). Biotinylated probes recognizing the sequences of human papillomavirus (HPV) 11 and 18 (Enzo Diagnostics) served as negative controls.

In Situ Hybridization

Both routinely paraffin embedded and ModAMeX sections were initially used for the detection of genomic DNA with the same protocol as for the detection of HPV in the uterine cervix. The denaturation conditions (heating to 100°C) and proteolysis required for DNA-DNA hybridization were followed by the routinely processed sections. However, the processing characteristics of the ModAMeX method proved unsuitable for the denaturation and usual proteolysis conditions of DNA hybridization because of disintegration of the sections. Detection of EBV DNA on ModAMeX sections, when further attempted by the gentler proteolysis as described below, was also unsuccessful. Therefore, only EBV mRNA was searched for in ModAMeX sections. In situ hybridization on ModAMeX was performed by adaptation of previously described methods with the object of increasing the sensitivity of detection. First, the sections were deparaffinized in two washes respectively of xylene (5 minutes each) and acetone (5 minutes each) and immersed in PBS. Gentler proteolysis was performed by 0.2N HC1 (10 minutes at room temperature, followed by two washes in PBS) and TE (10 mmol/L TRIS HCl, 1 mmol/L ethylenediaminetetraacetic acid [EDTA], pH 7.5) containing 1 µg/mL of proteinase K (BRL) (10 minutes, 37°C). Sections were then dehydrated in 70%, 90%, and 95% alcohol respectively (for 1 minute each) and vacuum dried for 1 hour. Probes were reconstituted in a mixture containing 43% deionized formamide, 3.4X SSC, 2% Denhardt’s solution (0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin, 0.02% Ficoll), 10% dextan sulfate and 100 ng/µL yeast tRNA. The DNA probes were denatured by immersion in boiling water for 5 minutes in Eppendorf tubes, which were subsequently plunged in ice cold water. Sections were then covered with 10 µL of the hybridization mixture containing 1 ng of the probe and incubated overnight at 42°C under DakoTisse sealed coverslips (Dako, Copenhagen, Denmark). After detaching the coverslips, sections were submitted to four washes of 1X SSC (0.15 mol/L NaCl, 0.015 mol/L sodium citrate) (two washes of 30 minutes each at room temperature and 2 washes of 30 minutes each at 40°C) and one wash of 0.1X SSC (30 minutes, 40°C). Following these steps, sections were incubated twice with 1X STM (1 mol/L sodium chloride, 0.1 mol/L TRIS, 2 mol/L MgCl2, 500 µL (Twee) polysorbate 20, pH 7.5) containing 1% skimmed milk (1 hour at 37°C followed by 15 minutes at room temperature) to inhibit endogenous biotin activity and initiate the application of streptavidine alkaline phosphatase (BRL). The latter, in a dilution of 1:100 in 1X STM (pH 7.5), was layered on the sections and left for 30 minutes at room temperature. Sections were then washed twice in 1X STM (pH 7.5), 1X STM (pH 9.5), and in 0.1X STM (pH 9.5) (5 minutes each). Sections were then incubated 2 to 3 hours in the dark in 0.1X STM (pH 9.5) containing 3.3 µg/mL of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 4.4 µg/mL of nitro blue tetrazolium (NBT) (both from BRL). Finally they were rinsed in running water, counterstained with 0.5% methyl green, and mounted in glycergel. A dark brown granular hybridization product was obtained with this procedure. Identical results were produced by the two EBV probes.

Immunohistochemistry

After in situ hybridization, sections of EBV mRNA positive cases were immunostained with the APAAP technique of Cordell et al, with HSR4/CD30 and IONI/CD15 monoclonal antibodies (both from Immunotech, Marseille, France). The antigen-antibody reaction product at cell membranes was red, in clear contrast to the dark brown nucleic acid hybridization product.

Clinical Data and EBV Serology for HD

Clinical details were available for 51 patients of HD (Table 2). There were 36 men and 15 women in this group with a median age of 34 years (range, 10 through 81 years). EBV serologic data were available for 36 cases of HD. The anti-EBV antibodies were detected by standard immunofluorescence techniques. Serologic profiles by titers were defined as follows: (1) negative: capsid antigen (anti-VCA), IgM <10, IgG <5; early antigen (anti-EA) <5, nuclear antigen (anti-EBNA) <5; (2) latent infection: anti-VCA: IgM <10, IgG >20 or <160; anti-EA >5 or <40; anti-EBNA >10 or <80; (B) reactivation profile: anti-VCA: IgG >640; anti-EA >40; anti-EBNA >160. The hybridization results were statistically analyzed with respect to stage of HD, response to treatment and resolution of the signal.

Table 1. Detection of EBV mRNA in ModAMeX Sections and EBV DNA in Routine Paraffin Sections by Nonisotopic Biotinylated DNA Probes

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>EBV mRNA (+/tested)</th>
<th>EBV DNA* (+/tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hodgkin’s disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphocyte predominance</td>
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<td>0/5</td>
</tr>
<tr>
<td>Nodular sclerosis</td>
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<td>1/22</td>
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<tr>
<td>Mixed cellularity</td>
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<td>1/26</td>
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<td>Unclassified</td>
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<td>0/1</td>
</tr>
<tr>
<td>Total</td>
<td>18/54</td>
<td>2/54</td>
</tr>
<tr>
<td>Non-Hodgkin’s lymphomas</td>
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<td></td>
</tr>
<tr>
<td>B-cell phenotype</td>
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<td>1/10</td>
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<tr>
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<tr>
<td>Anaplastic LCL (CD30+)</td>
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</tr>
<tr>
<td>Total</td>
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<td>1/41</td>
</tr>
<tr>
<td>Lymphoid tumors in HIV+ patients</td>
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</tr>
<tr>
<td>Total</td>
<td>3/5</td>
<td>2/5</td>
</tr>
</tbody>
</table>

Table 1. Detection of EBV mRNA in ModAMeX Sections and EBV DNA in Routine Paraffin Sections by Nonisotopic Biotinylated DNA Probes

*EBV DNA was detectable only in those cases positively hybridized for EBV mRNA.
Fig 1. (A) Range and nuclear localization of EBV mRNA hybridization signals in Reed-Sternberg cells and variants (methyl green counterstain). At left, all large atypical cells show dark nuclear grains with complete absence of the signal in surrounding small lymphocytes (×500). At right is a typical Reed-Sternberg cell with grains clearly restricted to the nucleus (×800). (B) Same case but the section was pretreated with ribonuclease A (100 μg/mL) before in situ hybridization with BamHI-W probes. This treatment abolishes the signal in all Reed-Sternberg cells and variants (methyl green counterstain) (×500).

Fig 2. Double labeling: in situ hybridization with EBV BamHI-W probe followed by immunostaining with anti-CD30/HSR4 antibody. EBV mRNA hybridization signals are restricted to the nuclei of CD30-positive Reed-Sternberg cells and variants.
EBV serology, using the Irwin-Fisher exact test. The median follow-up at the time of writing was 13 months.

RESULTS

In Situ Hybridization

**DNA-mRNA hybridization.** The results of EBV mRNA detection on ModAMeX sections are shown in Table 1, along with the rate of EBV DNA detection in routinely embedded paraffin sections. The EBV mRNA hybridization signals were remarkably clear with negligible background, allowing an immediate recognition of a positive or a negative case, even on low power examination. The positive signals ranged from a few dark brown grains in a variable proportion of H-RS cells to a strong hybridization signal in most H-RS cells (Fig 1A). In the majority of cases and in most H-RS cells, the grains appeared to be in or overlapping the nuclei but sometimes distributed towards the periphery of the nuclei (Fig 1A). In some H-RS cells the hybridization product was seen to extend into the cytoplasmic area adjacent to the nucleus. Sixteen of 54 cases (29.7%) of HD exhibited the presence of EBV mRNA in ModAMeX sections. In one case of HD, EBV mRNA was found in two lymph node biopsy specimens—at first diagnosis and at relapse. The single HIV-positive case of HD also showed a positive hybridization signal for EBV mRNA (Table 1).

In all EBV mRNA positive cases, immunostained by H-RS cell-associated antibodies, a clear membrane staining was obtained for CD30 (Fig 2). These cells were also positive for CD15, but the staining was weaker compared with that of CD30. EBV mRNA was detected in a single case of B-cell lymphoma among 41 non-Hodgkin’s lymphomas occurring in HIV-negative patients. This CD30-negative large B-cell lymphoma, containing both centroblasts and immunoblasts, showed a strong EBV mRNA signal in virtually all neoplastic cells. Two high grade (Burkitt type) AIDS-related B-cell lymphomas were found to be positive for EBV mRNA (Table 1). In the one case of infectious mononucleosis, mRNA hybridization product was present in immunoblastic cells, again in the nuclear area. Both EBV-positive lymphoblastoid cell lines gave positive hybridization signals similar to those of HD tumor cells. The nonspecific reactive lymph node and the T-lymphoma cell line were negative.

**DNA-DNA hybridization.** As mentioned above (see Materials and Methods) we were unable to use the ModAMeX prepared tissues because of the poor preservation of the sections after obligatory heating-step of the DNA-DNA hybridization procedure.

In routinely processed paraffin sections, EBV DNA was detectable in H-RS cells in only 2 of 54 cases of HD and in only 1 of 26 cases of HIV-negative B-cell lymphomas. These three cases were also positive for EBV mRNA. EBV DNA was detectable in two of four cases of AIDS-related B-cell lymphomas, but the single HIV-related case of HD was
negative (Table 1). The EBV DNA signals in all of these cases were comparable with those seen in the five cases of nasopharyngeal carcinomas used as positive control. The two EBV-positive lymphoblastoid cell lines also gave strong positive nuclear signals with DNA-DNA hybridization. RNase pretreatment did not abolish the DNA-DNA signals. As expected, the T-lymphoma cell line (CEM) was negative.

Correlation of In Situ Hybridization Findings With EBV Serology and Clinical Data

No significant difference between EBV mRNA-positive and EBV mRNA-negative groups was observed in EBV serologic profiles. The majority of HD patients presented with serologic profiles that were consistent with a latent infection with EBV (Table 2). One patient with reactivation profile belonged to EBV mRNA-negative group. Moreover, no difference was found in the response to chemotherapy, relapse, or death in the two groups (Table 2). Median follow-up of 13 months is obviously short for a disease like HD, but a further follow-up could be envisaged.

DISCUSSION

EBV, the causative agent of infectious mononucleosis, has an established relation with Burkitt’s lymphoma, nasopharyngeal carcinoma, and B-cell neoplasms in immunosuppressed patients. However, the precise role of EBV in the initiation and development of malignancy remains undefined. The latency and the complexity of intracellular events following EBV infection add further to the inherent technical difficulties of identifying intracellular EBV nucleic acids. The possible role of EBV in Hodgkin’s disease has long been questioned on the basis of epidemiologic studies and is being currently reinforced by the detection of genomic EBV in the diagnostic cells of some cases of Hodgkin’s disease. EBV genome has been demonstrated with Southern blot analysis in 19%, 29%, and 58% (with PCR) of cases respectively, but in situ hybridization with radiolabeled probes was investigated in only a few cases. The morphologic demonstration of EBV nucleic acids in HD appears promising. In our study, the fewer positive cases of HD for EBV DNA in routinely fixed and embedded tissues, compared with the EBV mRNA positive cases, is probably related to the low number of viral DNA copies in HD as demonstrated by Staal et al. The differences are not attributable solely to the deproteinizing of routine sections, because all five control cases of nasopharyngeal carcinomas as well as two cases of AIDS-related lymphomas (both groups with probable high number of viral copies) were clearly hybridized for DNA. No formal comparison is yet available between isotopic and nonisotopic probes for EBV DNA detection in Hodgkin’s disease. The literature suggests a greater sensitivity of isotopic probes but the precise cellular localization of autoradiographic signal is often difficult. Biotinylated probes with ModAmex permit a sharp cellular localization of EBV mRNA and, in addition, the detection rate is higher compared with the EBV DNA rate. This finding suggests that EBV mRNA transcripts are in greater number than copies of EBV DNA and thus are detectable with nonisotopic biotinylated probes. As mentioned in Results, DNA-DNA hybridization was not possible on ModAmex sections; only EBV mRNA could be detected with this method.

The viral DNA in most infected cells remains as an episome. Our findings of EBV mRNA in a proportion (30%) of cases of HD means that the BamH1-W fragment is transcribed, which may occur in both latent as well as proliferative infections. Therefore, it is not possible to conclude on the state of viral activation in these patients. The dominant localization of the signals in nuclei is in agreement with studies by Lawrence et al and Bashir et al with nonisotopic probes and EBV-positive cell lines. The extinction of signals after ribonuclease A pretreatment suggests that the signals are truly related to intranuclear mRNA or primary mRNA transcripts (see Fig 1A). Importantly, the signals were exclusively restricted to the phenotypically characteristic H-RS cells, the surrounding lymphocytes being totally negative. We did not find EBV mRNA in the lymphocytic and/or histiocytic cells in any of the five cases of nLP HD, which may be relevant because of the current implications of nLP HD as a B-cell disorder different from HD. EBV mRNA was detected in H-RS cells of the single HIV-related case of HD and in two cases of B-cell lymphomas in AIDS. The latter finding is in concert with previous studies emphasizing the stronger association of EBV in AIDS-related lymphomas than in usual B-cell lymphomas as also suggested by our results (only 1 of 26 EBV mRNA-positive cases). Lack of correlation of hybridization results with disease outcome in HD may be attributable to the obligatory short follow-up because of availability of the ModAmex method. Likewise, no correlation was found with viral serolog and, in contrast to the report by Mueller et al, only one of our patients of HD showed elevated titers of antibodies against EBV.

This is the first demonstration of EBV mRNA in the diagnostic cells of Hodgkin’s disease, to our knowledge. Although, the clonality of the EBV genome was not investigated in this study, the findings support the possible etiologic role of EBV in at least some cases of HD. Whether EBV is responsible for the generation of the pathogenic cell of HD by conferring immortalization properties of B-lymphocytes to an as yet unidentified precursor cell or by fusion of an EBV-infected B-lymphocyte with an interacting cell remains hypothetical at this time.

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REFERENCES


Detection of Epstein-Barr virus messenger RNA in Reed-Sternberg cells of Hodgkin's disease by in situ hybridization with biotinylated probes on specially processed modified acetone methyl benzoate xylene (ModAMeX) sections [see comments]

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