Demonstration of Monoclonal EBV Genomes in Hodgkin’s Disease and Ki-1–Positive Anaplastic Large Cell Lymphoma by Combined Southern Blot and In Situ Hybridization

By Ioannis Anagnostopoulos, Hermann Herbst, Gerald Niedobitek, and Harald Stein

Forty-two cases of Hodgkin’s disease (HD) and 22 cases of Ki-1–positive anaplastic large cell (Ki-1 + ALC) lymphoma were examined by Southern blotting for the presence of Epstein-Barr virus (EBV) DNA. Seven cases of HD and one case of Ki-1 + ALC lymphoma scored positive with a probe specific for the internal repetitive region of the EBV genome. Subsequently, these viral genomes could be demonstrated to be monoclonal in origin using an EBV terminal region DNA probe. In situ hybridization revealed that in two HD cases, the EBV infected cells had the distinct morphology of Hodgkin and Reed-Sternberg cells, thus suggesting a direct pathoetiologic relationship between EBV and some cases of HD.

THE EPSTEIN-BARR VIRUS (EBV) is associated with three lymphoproliferative disorders of B cell origin, infectious mononucleosis, endemic Burkitt’s lymphoma, and many B cell lymphomas in immunosuppressed individuals. EBV has additionally been demonstrated in nasopharyngeal carcinoma, in thymic lymphoepithelial carcinoma, in parotid gland ducts, and in hairy leukoplakia of the tongue. Recently, EBV genomes were also detected in T lymphocytes of patients with chronic EBV infection. The observation of monoclonal EBV genomes in three cases of Hodgkin’s disease (HD) prompted us to screen our cases of HD for the presence and clonality of EBV genomes in relation to the genotype and phenotype of the neoplastic cells in those tumors. In addition, we included in our study Ki-1–positive anaplastic large cell (Ki-1 + ALC) lymphomas, which are a distinct entity of non-Hodgkin’s lymphomas composed of tumor cells with striking morphological and immunophenotypical similarities to Hodgkin and Reed-Sternberg cells, the tumor cells in HD. It had been proposed that both entities, HD and Ki-1–ALC lymphoma, may represent closely related malignancies differing mainly in the number of tumor cells and the abundance of the reactive cellular infiltrate. Since it could not be excluded that the EBV genomes were present in cells not belonging to the malignant cell population, which in most cases of HD accounts for only a small percentage of the total cell number in the affected tissues, we also investigated these cases for the presence of EBV DNA at the single cell level by in situ hybridization.

MATERIALS AND METHODS

Biopsy specimens. Forty-two cases of HD and 22 cases of Ki-1 + ALC lymphoma were randomly selected from the files of the Institute of Pathology at the Steglitz Medical Center of the Free University of Berlin. All biopsies had been obtained before initiation of therapy. Some of these cases had previously been included in a study correlating the immunophenotype and the immunoglobulin (Ig) and T cell antigen receptor (TCR) gene rearrangement status. Serological data as to previous or current EBV infection were not available for any of the cases except for no. 2 (Table 1), which was positive for the nuclear antigen of EBV (EBNA), positive for IgG but negative for IgM antibodies against the viral capsid antigen (VCA). The Paul-Bunnel test was negative.

DNA probes. The plasmids pBa-W, containing a 3.1 kb fragment of the EBV internal repetitive region (IR) subcloned in the BamHI site of pBR322, and pM961-20, consisting of a 6.4 kb HindIII/SalI fragment of the terminal EBV region in pACYC184, as well as the 3.1 kb EcoRI-HindIII Ig heavy chain (IgHC) J probe were kind gifts of Dr G.W. Bornkamm, Freiburg. The 1.9 kb XhoI fragment of pM961-20 containing unique sequences from the right terminal region adjacent to the 500 bp terminal repetitive sequence (TR) was used for the clonality analyses. pCM5018 harboring the EcoRI J-fragment of human cytomegalovirus (CMV) type AD169 subcloned in pACYC184 served as a control for in situ hybridizations and was kindly provided by Dr B. Fleckenstein, Erlangen. The TcRJ2 gene probe was obtained courtesy of Dr T.W. Mak, Toronto. The inserts excised from the plasmids with the appropriate restriction endonucleases were labeled by random primer extension with [32P]-dCTP or [35S]-dCTP for Southern blot or in situ hybridization, respectively. The specific activity obtained was 1 x 10^6 dpm/μg DNA for [32P] and 5 to 8 x 10^6 dpm/μg DNA for [35S].

Biot hybridization analyses. DNA was extracted from tissues, digested with the restriction endonucleases BamHI, BglII, EcoRI, and HindIII, separated by electrophoresis in a 0.7% agarose gel, and transferred to nylon membrane filters. Hybridization and washing were performed as described. DNA from the cell line B95-8 served as a positive control for EBV specific probes. DNA samples from 11 T and B cell-type non-Hodgkin’s lymphomas not derived from immunosuppressed individuals processed on the same blots as HD samples as well as the placenta DNA (germline control) lane on every blot provided negative controls for the EBV probes.

Immunohistology. Cryostat sections of snap-frozen tissue and sections of paraffin-embedded tissue were stained with a panel of monoclonal antibodies. For the assessment of the tumor cell number in tissue blocks, sections from the center of the blocks subsequently used for DNA extraction were stained with the CD30-specific monoclonal antibodies Ki-1 and Ber-H2, kindly provided by Dr R. Schwarting. In order to co-localize CD30 antigen and EBV genomes, paraffin sections were stained with the Ber-H2 antibody and subsequently processed as described below. Immobilized primary antibody was detected with the immunoperoxidase phosphate method and new fuchsin as chromogenic substrate.

In situ hybridization. In situ hybridization was performed according to established methods with minor variations. Briefly,
Table 1. Summary of Patient Data, Immunophenotypes, and Hybridization Results

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age, Sex</th>
<th>Diagnosis</th>
<th>CD30 (%)</th>
<th>Phenotype</th>
<th>Southern Blot Hybridization</th>
<th>In Situ Hybridization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IgH IgL TcRβ EBV-IR EBV-TRβ</td>
<td>EBV CMV</td>
</tr>
<tr>
<td>1</td>
<td>20,F</td>
<td>HD,ns</td>
<td>3</td>
<td>O</td>
<td>R   G   +    9.0 kb</td>
<td>+    -</td>
</tr>
<tr>
<td>2</td>
<td>14,F</td>
<td>HD,ns</td>
<td>5</td>
<td>B</td>
<td>R   R   +    9.5 kb</td>
<td>+    -</td>
</tr>
<tr>
<td>3</td>
<td>29,M</td>
<td>HD,ns</td>
<td>5</td>
<td>T</td>
<td>G   G   +    14.0 kb</td>
<td>-    -</td>
</tr>
<tr>
<td>4</td>
<td>52,M</td>
<td>HD,ns</td>
<td>1</td>
<td>O</td>
<td>G   R   +    16.0 kb</td>
<td>-    -</td>
</tr>
<tr>
<td>5</td>
<td>67,F</td>
<td>HD,ns</td>
<td>3</td>
<td>ND</td>
<td>G   R   +    9.5 kb</td>
<td>ND   ND</td>
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<tr>
<td>6</td>
<td>10,F</td>
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<td>ND</td>
<td>G   G   +    10.0 kb</td>
<td>ND   ND</td>
</tr>
<tr>
<td>7</td>
<td>62,M</td>
<td>HD,ns</td>
<td>1</td>
<td>ND</td>
<td>G   G   +    9.5 kb</td>
<td>ND   ND</td>
</tr>
<tr>
<td>8</td>
<td>16,M</td>
<td>Ki-1+</td>
<td>20</td>
<td>0</td>
<td>G   G   +    9.0 kb</td>
<td>-    -</td>
</tr>
</tbody>
</table>

ALC lymphoma

| Abbreviations: HD,ns, Hodgkin's disease, nodular sclerosing type; R, rearranged configuration; G, germline configuration; ND, not determined. |
| Percentage of CD30 positive cells in the center of tissue blocks used for DNA extraction. |
| The immunophenotypes were determined for case no. 1 as CD3-, CD4-, CD5-, CD8-, CD10-, CD19-, CD22-, CD23-, CD25+, CD30+, Ki-24+, Ki-27+, HLA-DR+, IgHC-, IgLC-, and for case no. 2 as CD1-, CD2-, CD3-, CD4-, CD5-, CD8-, CD10-, CD11c-, CD19+, CD22+, CD23-, CD25+, CD30+, Ki-24+, Ki-27+, HLA-DR+, IgHC-, IgLC-, L26+. The antibody panel and the immunophenotypes and genotypes of cases 3-8 were described in detail previously. For cases 5-7 some immunophenotyping and in situ hybridizations could not be carried out due to the lack of material. |
| The probe for the internal repeat of EBV (EBV-IR) exclusively hybridized to a 3.1 kb band in BamHI-digested DNA. |
| Sizes of single bands representing episomal fused termini as detected by the 1.9 kb XhoI EBV probe in BamHI-digested DNA. |

paraffin sections were cut onto 3-aminopropyltriethoxysilane (APES) coated slides. Dewaxed and rehydrated sections were treated with 0.2 mol/L HCl for ten minutes at room temperature, washed in 2 x SSC (0.3 mol/L NaCl/30 mmol/L sodium citrate), incubated in 0.1% Triton X-100 for 90 seconds, rinsed in 2 x SSC, and incubated with pronase (1 mg/mL) for ten minutes at 37°C followed by acetylation with 0.1 mol/L triethanolamine/0.25% acetic anhydride for ten minutes and extensive washes in 2 x SSC.

Fig 1. Autoradiograms of Southern blots obtained from DNA of lymph nodes affected by Hodgkin's disease (cases 1-7) or Ki-1-positive anaplastic large cell lymphoma (case 8) digested with BamHI, subjected to electrophoresis through a 0.7% agarose gel, transferred to nylon filters, and hybridized to [32P]-labeled Epstein-Barr virus DNA probes (A) specific for the internal repetitive sequence (3.1 kb BamHI W-fragment), and (B) specific for unique DNA adjacent to the right terminal repeats (1.9 kb XhoI fragment). Numbers at the top correspond to case numbers in Table 1. HindIII-digested phage lambda DNA served as size marker (position and size in kilobases shown on the left side). All specimens show a band of variable intensity (A) in the range of 3.1 kb indicating the presence of EBV DNA in the tissues and (B) in the range between 8 kb and 16 kb, strongly suggesting monoclonality of the detected EBV episomes. Case 2 shows an additional band at 4 kb, most likely representing the right terminal BamHI-fragment of linear viral DNA.
Twenty-five microliters of the hybridization mixture (50% deionized formamide, 2 x SSC, 10% dextran sulphate, 0.1 mg/mL herring sperm DNA, and 5 x 10³ dpm of [³⁵S]-labeled probe) were applied to the dehydrated sections which were then covered with siliconized coverslips. Probe and cellular DNA were denatured simultaneously by placing the slides on a 90°C heat block for three minutes. Subsequently, slides were incubated overnight at 37°C. Cover slips were removed in 50% formamide/0.1 x SSC at 37°C, followed by extensive washing for four hours in several changes of the same solution at 37°C, and rinsing for 30 minutes in 0.1 x SSC at room temperature. After dehydration in graded ethanol baths, slides were dipped in Ilford-G5 emulsion, exposed at 4°C for three to ten days, developed with Kodak D19 Developer and Kodak Rapid Fixer, and finally counterstained with hematoxylin and eosin.

H and RS cells were identified by applying simultaneously histomorphological and immunophenotypical criteria as CD30-positive, large atypical cells with one or multiple nuclei, respectively, and prominent eosinophilic nucleoli. H and RS cells were considered to show a positive autoradiographic signal when the number of grains exceeded that over typical lymphoid cells by the factor of three. Reactive hyperplastic and infectious mononucleosis tonsils, hairy leukoplakia of the tongue, and various tissues from AIDS patients that are subjects of other reports were processed in parallel to the cases reported here providing positive and negative controls for EBV and CMV in situ hybridizations.

RESULTS

Initially, the DNA extracted from tissues of 42 cases of HD and 22 cases of Ki-1 + ALC lymphoma was probed with the internal repetitive EBV BamHI-W-fragment $^{10,11}$ In BamHI-digested DNA, a band of 3.1 kb size was detected in seven cases of HD and one case of Ki-1 + ALC lymphoma, indicating the presence of EBV DNA in the biopsy materials (Fig 1A, Table 1), whereas all other cases displayed only a background signal. The clonality of these EBV genomes was analyzed by hybridizing BamHI-digested cellular DNA to the 1.9 kb XhoI-EBV probe representing unique DNA adjacent to the right 500 bp terminal repeats. In BamHI-digested DNA, this probe detects a polymorphic restriction fragment that varies in size corresponding to the number of terminal repeats in a particular EBV episome. The detection of a single band by this probe indicates that the EBV-infected cell population is a clonal expansion of a progenitor cell infected by a single virion. $^{12}$ As shown in Fig 1B, the 1.9 kb XhoI probe hybridized to only one band in the range of 9.0 kb to 16 kb in all eight cases, therefore corroborating the results obtained with the EBV-IR probe and strongly suggesting the presence of clonally amplified episomal EBV population in each of the EBV-IR-positive tissues. One HD specimen, case no. 2, had an additional 4.0 kb fragment. This fragment is too small to be derived from a fused terminal fragment and most likely represents a linear right terminal fragment, indicating the presence of virion DNA. $^{12}$

All seven EBV-positive HD cases were of the nodular sclerosing type of HD, characterized by a small number of CD30-positive H and RS cells with an admixture of high numbers of reactive small T and B lymphocytes, macrophages, and epitheloid-type macrophages, as well as few neutrophilic and eosinophilic polymorphonuclear leukocytes in the presence of sclerosis (Fig 2). The B lymphocytes reacting with kappa and lambda Ig light chain (IgLC) specific antibodies displayed a mosaic-like distribution pattern. The lymph node architecture was largely preserved in most areas presenting with follicular hyperplasia and partial lymphoid depletion of the T-dependent area. The EBV-positive Ki-1 + ALC lymphoma showed a high tumor cell content with infiltration of the skin. The immunophenotypical and genotypical analysis of these cases is summarized in Table 1. In HD case 2, the B cell phenotype of the tumor cells was in agreement with the B cell genotype. Cases 1, 3, and 4 showed either a discrepancy between phenotype and genotype or did not express lymphocyte markers with the exception of CD30, CD25, Ki-24, and HLA-DR. $^{12}$ For cases 5, 6, and 7, detailed phenotypical studies could not be performed due to the lack of tissue. Only one of these cases showed a rearrangement for a TcRβ gene. The EBV-positive Ki-1 + ALC lymphoma case did not express any T or B lineage-specific markers and did not show any Ig and TcR gene rearrangements.

In situ hybridization was performed with a mixture of the BamHI-W-fragment and the terminal 6.4 kb HindIII/ SalI-fragment of EBV. Hybridizations with the CMV probe served as controls. HD case 1 displayed an EBV-specific autoradiographic signal over virtually all CD30-positive cells morphologically consistent with H and RS cells (average number of grains, 19; range, 7 to 53), while nonmalignant...
cells showed only background signal (average number of grains, 2.3; range, 0 to 5). The same result was obtained when both EBV probes were used separately (Fig 3). Hybridization with the control DNA resulted in background signal. In case 2, a slightly increased EBV-specific signal as compared with nonmalignant cells was consistently observed over H and RS cells (average number of grains, 13; range, 0 to 36) as compared with the controls (average number of grains, 2.1; range 0 to 5). The other tissues available for in situ hybridization studies showed no signal above background for both EBV and CMV probes.

**DISCUSSION**

During the past 20 years, data have been collected suggesting a relationship between EBV and HD. Several investigators described increased titers of EBV-specific antibodies in HD patients.34–36 Also, patients with infectious mononucleosis were shown to be exposed to an increased risk of developing HD.37 Poppema et al38 reported immunocytological detection of EBV nuclear antigen (EBNA) in cells resembling Reed-Sternberg cells in a patient with chronic EBV infection. These observations were recently substantiated by the demonstration in Southern blots of monoclonal EBV proliferations in three HD biopsies.39 Our investigations using the same technique led to similar results. We found monoclonal EBV proliferations in seven of 42 cases of HD, and one of 22 cases of Ki-1 + ALC lymphoma. Since Southern blot analysis does not identify the cellular source of the EBV-specific DNA sequences, and since it is conceivable that the clonal cell population containing the monoclonal EBV genomes is different from the tumor cell population, we applied in situ hybridization with two distinct EBV-specific probes for the detection of EBV at the single cell level. This approach was successful in two of four HD cases, thus permitting the direct attribution of EBV to the tumor cell population, ie, the H and RS cells. In the other cases, no specific signal for EBV was noted. A reason for this disagreement between Southern blot and in situ hybridization may be variations of the absolute EBV copy number in the infected cells being sufficient in the tissue blocks used for DNA extraction to permit detection after extraction and size-specific enrichment in the blot, while being below the threshold of detection by in situ hybridization in the paraffin sections. More likely, however, are technical problems such as storage, fixation, and embedding procedures possibly causing damage to the DNA so that in some cases specific sequences were removed during the washing steps. Thus the cellular source of the EBV specific sequences in cases 3 to 8 remains uncertain. It can be argued that the EBV infection may reflect an epiphenomenon of the underlying malignant disease, eg, similar to the clonal B lymphoid proliferations associated with EBV previously observed in immunosuppressed transplant patients.34–36 However, the absence of Ig rearrangements in five of seven HD cases and the absence of opportunistic CMV co-infections argue against this explanation. The monoclonal nature of the EBV proliferations in our series of HD and Ki-1 + ALC lymphoma cases points to monoclonality of the infected cell type. The mosaic-like immunohistological distribution of kappa and lambda IgLC-positive cells seen in HD tissue sections indicates a polyclonal origin of the B cells in the lymphoid cell admixture. Thus in HD, only the tumor cells are likely to be the cell population harboring the EBV episomes.

Many studies have shown the ability of the EBV to infect mature resting B cells by interaction with the receptor for the complement component C3d and to induce their proliferation in culture.39,40 In our HD cases 1 and 2, the immunophenotypical and gene rearrangement data are consistent with a derivation of the tumor cell population from B lymphoid cells. These cases may therefore represent examples of a new entity of EBV-associated B-lymphoid neoplasia. The additional finding of a TcRβ gene rearrangement in case 2 is not in conflict with a delineation from B cells, as approximately 10% of B cell non-Hodgkin's lymphomas carry such rearrangements.41

The rearranged TcRβ gene configurations in the absence of IgHC rearrangements in cases 4 and 5 as well as the T cell phenotype of the H and RS cells in case 3 also do not argue against an association of the monoclonal EBV proliferations with these cells. Recently, EBV genomes were demonstrated in isolated CD4-positive lymphocytes in one case of a child with Kawasaki-like disease42 and in T cell lymphomas in three patients with chronic EBV infection.43 However, it is interesting to note that HD cases with rearranged TcRβ gene configurations seem to be overrepresented (three of seven cases) as compared with the total series investigated by us (seven of 42).44

For those cases without detectable Ig and TcR gene rearrangements, two possibilities have to be discussed: (a) the tumor cell population actually does carry Ig and/or TcR rearrangements, but the size of the clonal population is too small to produce autoradiographic signals in Southern blots; the EBV DNA, however, does not escape detection because each infected cell contains multiple copies of the viral genome, or (b) the malignant cells do not carry Ig and TcR rearrangements, because they had been transformed at a differentiation stage before expression of T or B lymphoid markers and Ig or TcR rearrangements had occurred. The latter explanation applies at least to the Ki-1 + ALC lymphoma case, which contains a number of tumor cells sufficient for rearrangement analysis, and is supported by the establishment of lymphoid cell lines by EBV-induced immortalization before any Ig and TcR rearrangement.45,46

In conclusion, this study and the report of Weiss et al40 have shown monoclonal EBV episomes in HD and Ki-1 + ALC lymphoma. The findings suggest that the monoclonal cellular proliferation took place after EBV infection. In combination with the Southern blot analysis the direct demonstration of EBV DNA in H and RS cells in two cases by in situ hybridization provides strong evidence for the clonal origin of H and RS cells and for a pathoetiologic role of EBV in HD. The fact that only approximately 10% of the CD30-positive lymphomas in our series contained EBV DNA, however, implies that EBV is likely to be only one among several possible carcinogens involved in the development of these tumors.
NOTE ADDED IN PROOF

During the revision of this manuscript, similar observations were reported by Weiss et al, N Engl J Med 320:502, 1989.

REFERENCES


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Demonstration of monoclonal EBV genomes in Hodgkin's disease and Ki-1-positive anaplastic large cell lymphoma by combined Southern blot and in situ hybridization [see comments]

I Anagnostopoulos, H Herbst, G Niedobitek and H Stein

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