RAPID COMMUNICATION

Persistence of the Same Viral Strain in Early and Late Relapses of Epstein-Barr Virus–Associated Hodgkin’s Disease

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Twelve cases of relapsing Hodgkin’s disease were investigated for the presence of Epstein-Barr virus (EBV). Of these, 7 cases contained EBV gene products (LMP1, EBER RNA) in the diagnostic Reed-Sternberg cells and variants at first presentation and at relapse(s), whereas 5 cases were negative at both first diagnosis and relapse. Among the 7 EBV-positive cases, material for DNA extraction was available in 2 cases at both diagnosis and relapse(s). Ig and T-cell receptor gene rearrangements displayed a germ-line configuration in the 2 cases. However, Southern blot analysis of the terminal repeats (TR) of EBV genome showed that, in 1 of the 2 cases, the fragment was of the same size at diagnosis and in the subsequent two relapses (1 early and 1 late). The second case contained monoclonal EBV genome at diagnosis, but the Southern analysis of the TR was negative at relapse. The latent membrane protein (LMP1) sequence analysis confirmed the persistence of a distinctive viral strain in each of the 2 cases with individual abnormalities within the carboxy-terminal region (5 point mutations and a 30-bp deletion for the first case and 6 point mutations for the second case). The persistence of a given strain in early and late relapses is evidence towards the view that in Hodgkin’s disease such relapses are related to a single residual tumor cell clone.

Hodgkin’s Disease (HD), particularly of mixed cellularity subtype, shows a strong association with the Epstein-Barr virus (EBV). Recent studies have shown the presence of EBV genomes and their latent gene products in tumor cells of HD. However, the clinical course and the therapeutic response appear not to be influenced by the presence of EBV and conventional chemotherapy and radiotherapy yield good results in about 75% of the cases. But, even after complete remissions, early and late unpredictable relapses may occur and the question remains open whether such relapses are caused by persistent tumor cells or by the emergence of another cell clone. The nature of Hodgkin’s and Reed-Sternberg cells of HD is still a subject of controversy. Ig and T-cell receptor (TCR) genes are found to be only infrequently rearranged and, thus, except for a few cases, reliable clonal markers are missing. An alternative method to assess clonality of the Reed-Sternberg cells consists in analysis of the terminal repeats (TR) of EBV by Southern blotting. This method provides molecular evidence that the viral genome, localized in the nucleus of Reed-Sternberg cells, is monoclonal in an episomal and circular form, similar to nasopharyngeal carcinoma. Additionally, we showed that virion genomes persist in Reed-Sternberg cells throughout the course of HD and, conversely, when absent at the onset of the disease, EBV has never been found at relapse. In EBV-positive relapsing cases of HD, it has never been shown whether the virus sustained changes at the molecular level. EBV genome appears to be very polymorphous because of the various numbers of iterated sequences within the 2 TR and within each of the 4 internal repeats. Moreover, peculiar deletions within the BNLF1 gene encoding the latent membrane protein 1 (LMP1) of EBV have been clustered in a few cases of HD and have been associated with an aggressive clinical behavior. Taking advantage of EBV polymorphism, we performed retrospective molecular analysis of the TR and the LMP1 sequences in 2 relapsing cases of EBV-associated HD. In each of the 2 cases, the same viral strain was identified at diagnosis and at relapses.

MATERIALS AND METHODS

Patient Selection and Tissue Samples

So far, we have investigated 12 relapsing cases of HD (including patients no. 1 and 2, see below) by combined in situ hybridization with EBER-1 oligoprobes and immunohistochemistry with anti-LMP1 (CS1-4; DAKO, Copenhagen, Denmark) and anti-ZEBRA (AZ130) monoclonal antibodies (MoAbs).

Case history. Patient no. 1 is a 58-year-old man referred 9 years previously for fever, hepatomegaly, splenomegaly, and pancytopenia. Bone marrow, lymph node, and hepatic biopsies were involved by HD of mixed cellularity subtype. Standard EBV serology showed a past infection profile. The patient was treated with alternating combination chemotherapy of nitrogen mustard, vincristine, procarbazine, prednisone (MOPP) and doxorubicin, bleomycin, vinblastine, dacarbazine (ABVD). The patient achieved a complete remission after 12 months and the treatment was stopped. He relapsed 14 months after with cervical and para-aortic lymph nodes. Complete remission was obtained after 12 cycles of a salvage combination chemotherapy regimen including mitoguanazole, ifosfamide, methotrexate, and etoposide (MIME). He relapsed again 4 years after the end of treatment with isolated cervical lymph node. A computer tomography (CT) scan of the thorax, abdomen, and pelvis was normal. At that time, EBV serology showed reactivation profile with IgG anti-VCA greater than 320, IgM anti-VCA less than 10, IgG anti-EA greater than 40, and IgG anti-EBNA less than 5. A test for antibodies to human immunodeficiency virus (HIV) was negative. The patient was treated with radiotherapy. He is currently in complete remission, with a follow up of 14 months.

Patient no. 2 is a 38-year-old man, seronegative for HIV, who was admitted to the hospital 8 years previously with axillary, mediastinal, paraatrahecobic, and infradiaphragmatic lymph nodes. Complete response was obtained after 6 months of combination chemotherapy with doxorubicin, bleomycin, vinblastine, dacarbazine (ABVD). At the end of treatment, a cervical lymph node was still present. A computerized tomography (CT) scan of the thorax and abdomen was normal. A bone marrow biopsy was performed and showed persistence of a given clone of Reed-Sternberg cells. An isolated cervical lymph node was removed. No further relapse was observed after 5 years of follow up.

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and para-aortic lymph nodes and hepatosplenomegaly. Lymph node, liver, and pulmonary biopsies were typical of mixed cellularity HD. Alternating MOPP and ABVD treatments were administered for 12 cycles, resulting in complete remission. He relapsed 5 years later with cervical and iliac lymph nodes. The cervical lymph node was removed and histologic examination confirmed the relapse. After treatment with 6 cycles of the MIME protocol, the patient is now in continuous complete remission, with a follow up of 1 year.

Tissue samples. Routinely processed paraffin sections were available in all 12 cases tested at first diagnosis and at relapses. However, DNA extraction at first diagnosis and at relapses was possible only for 2 patients (patients no. 1 and 2). In patient no. 1, fresh frozen material was available at first diagnosis (sample 1.1) and at the 2 relapses (samples 1.2 and 1.3), whereas in patient no. 2, DNA was extracted from fresh frozen material at the onset of the disease (sample 2.1) and from formalin-fixed and paraffin-embedded material at the relapse (sample 2.2).

In Situ Hybridization and Immunohistochemistry

In situ hybridization was performed as previously described with the DAKO hybridization kit (DAKO) using a cocktail of EBER oligonucleotides (1 oligonucleotide corresponding to EBER1 and 1 corresponding to EBER2, both of 30 base length; ref AS Y.017) labeled with fluorescein isothiocyanate (FITC).

Immunohistochemistry was performed with anti-LMP1 (CS 1-4) and anti-ZEBRA (AZ130) antibodies on routinely processed and frozen material, respectively. The three-stage immunoperoxidase method was applied to the paraffin section and the APAAP method to the frozen sections. The standard immunophenotype of Reed-Sternberg cells was performed on paraffin sections with the following antibodies: anti-CD45 (pan leuco; DAKO), anti-CD30 (BerH2; DAKO), anti-CD15 (IONI; Immunotech, Marseille, France), antiepithelial membrane antigen (EMA; DAKO-EMA), anti-CD20 (L26; DAKO), anti-mbVCD79a (a generous gift from Dr D.Y. Mason, Oxford, UK), and anti-CD3 (DAKO-T3).

DNA Isolation and Southern Blotting

Total cellular DNA was extracted from frozen tissues with standard procedures. DNA extraction from paraffin blocks was also performed in the 12 cases, as described elsewhere. However, DNA was intact in only 2 cases, including the relapse material of case 2 that was fixed in formalin. DNA extracted from Boein or ethanol-based Boein's fluid fixed material was not intact.

The EBV probe used consisted of a 1.9-kb Xho I probe subcloned in Xho I site of pACYC 184 (Dr G.W. Bornkamm, Munchen, Germany). The insert was labeled by random priming procedure with d-CTP-32P.

Gene rearrangement study was performed as described elsewhere with JH locus-specific, Cκ and Cλ probes and TCR-β probe (specific for human β J and C fragments).

LMP1 Polymorphism Study by Polymerase Chain Reaction (PCR) and DNA Sequencing

Genomic DNA was studied in each case with different primer sets, specific for the amino terminal, transmembrane, and carboxyterminal regions of the LMP1 gene, as described elsewhere. DNA sequencing was performed with a strategy recently published. Products were purified with a GeneClean II kit (Lucerna-Chem AG, Lucerne, Switzerland) and directly sequenced with 32P-d-ATP using a Sequenase kit (US Biochemicals Corp, Cleveland, OH).

RESULTS

Immunohistochemistry and In Situ Hybridization

Among the 12 cases tested, 7 cases (including case 1/patient no. 1 and case 2/patient no. 2) expressed EBER transcripts in the nucleus and LMP1 protein at the membrane and in the cytoplasm of Reed-Sternberg cells at the onset of the disease and at relapses. Immunostaining performed with anti-ZEBRA antibody remained negative in all cases tested. All cases negative for EBV at first diagnosis remained negative at relapses. In each of the 2 cases (patients no. 1 and 2), Reed-Sternberg cells showed the following phenotype (on paraffin sections): EMA−, CD15+, CD30+, CD45−, CD20−, mbVCD79a−, CD3−.

Ig and TCR Gene Rearrangement Study

The 3 samples from case 1 (1.1, 1.2, and 1.3) and the samples from case 2 (2.1 and 2.2) displayed germline configuration by Southern blotting with Jκ, Cκ, CL, and TCR-β probes.

Terminal Repeat Polymorphism Analysis by Southern Blotting

Southern blot analysis with Xho I probe showed that TR fragments were approximately 13 kb long and of the same size for the 3 DNA samples of case 1 (1.1, 1.2, and 1.3; Fig 1). Monoclonal episome was detected for the first biopsy of case 2 (2.1) but not at relapse (2.2).

Analysis of LMP1 Polymorphism

Sequencing of PCR-amplified DNA in cases 1 and 2 (samples 1.1, 1.2, 1.3, 2.1, and 2.2) confirmed that in each case the viral strain was the same at diagnosis and at relapses. In particular, in case 1, the virus showed 5 point mutations and a 30-bp deletion identical to that observed in previously
The persistence of the same viral strain was also reported in cases of HD (Table 1 and Fig 2).\textsuperscript{14} Additionally, a point mutation was identified at position 168311 in all of the 3 samples of case 1, excluding a contamination with DNA samples from cases previously investigated (Table 1 and Fig 2).\textsuperscript{14} In case 2, the sample 2.1 showed no deletion but 6 point mutations: 168357 (C → G; Gln → Ala), 168323 (A → T; Asp → Val), 168320 (T → C; Gln → Arg), 168308 (T → C; Leu → Ser), 168295 (A → T; silent), and 168266 (A → G; His → Arg). The identical point mutations were identified in sample 2.2, 6 years later.

**DISCUSSION**

As shown by a number of recent studies, EBV is now firmly linked to HD and appears to be not a simple, silent passenger.\textsuperscript{14} However, the detection of EBV within Reed-Sternberg cells does not shed any light on the origin of these cells and, except for rare reports,\textsuperscript{6,11} their clonality still remains to be established. Indeed, molecular analyses of Ig or TCR gene rearrangement usually yield negative results.\textsuperscript{10} In this context, the demonstration of EBV clonality, as reported in nasopharyngeal carcinoma,\textsuperscript{12} appears to be a reliable method in assessing the clonal origin of Reed-Sternberg cells.\textsuperscript{1,14} An alternative method, based on image analysis, consists in the determination of DNA content of the cells, which can be constant at different time periods for a given patient.\textsuperscript{19} In the present study, EBV clonality was investigated using Southern blotting and LMPI sequencing to determine whether the viral genomes detected in Reed-Sternberg cells throughout the course of HD (ie, at diagnosis and at relapse) were related to the persistence of the same viral strain or to an unrelated EBV strain. In case 1, Southern blot analysis with Xho I probe showed that the size of TR fragments was the same at diagnosis and at both relapses, suggesting the persistence of the same viral strain. The most probable explanation for the negative results in case 2 was that there were small numbers of tumor cells that carry the virus and/or the low copy number of viral genomes in these cells.\textsuperscript{8} The persistence of the same viral strain was also reported in metachronous localizations in two cases of EBV-associated HD occurring in patients with acquired immunodeficiency syndrome.\textsuperscript{20} However, in the latter study, the demonstration of the same viral strain (by Southern blot of the TR fragment) could be expected because EBV was detected in different sites at the same time or after a few months in patients with partial remission. In the present study, relapses occurred after a few years of complete remission of the disease and thus the implication of two different viral strains but with TR fragment of similar size could not be excluded. The hypothesis that relapses were related to the same viral strain, suspected on the basis of the TR fragment study in case 1, was further confirmed by the results of LMPI gene sequence analysis in both cases. The sequence abnormalities were found to be distinctive in each case and persisted throughout the course of the disease. Despite molecular evidence that relapses were caused by the same EBV isolate, it was difficult to know whether such relapses were related to the viral persistence in residual malignant cells. Alternatively, the virus could persist in nonneoplastic cells (eg, nonneoplastic lymphocytes and epithelial cells)\textsuperscript{13} with possible replicative cycle followed by infection and transformation of a new malignant cell clone emerging at relapses. This may be clinically relevant, because anti-EBV therapy was proved to be efficient in some patients with acute EBV infection\textsuperscript{21} or increased oropharyngeal shedding,\textsuperscript{22} although anti-EBV approaches that may eradicate EBV from its latent sites are not yet available.\textsuperscript{22} However, such a phenomenon appeared not to be relevant to explain the first relapse in case 1 because this relapse occurred early with no evidence of EBV reactivation. In addition, despite a serologic profile of EBV reactivation at the second relapse of the same case, Reed-Sternberg cells were latently infected without activation of replicative cycles (ZEBRA-). This finding made it unlikely that surrounding cells had been infected by virions released from the tumor cells. Thus, in our 2 cases, the demonstration of an identical EBV strain in Reed-Sternberg cells in all tissue samples strongly suggested that the same Reed-Sternberg cell clone was present at diagnosis and at relapses.

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**Table 1. Point Mutations, Deletions and Amino Acid Substitutions Observed in the LMPI Gene of Isolates 1.1, 1.2, and 1.3 Compared With the Nasopharyngeal Carcinoma CA0 and Another Case of HD (HD3)**

<table>
<thead>
<tr>
<th>AA</th>
<th>Gln</th>
<th>Glu</th>
<th>Gln</th>
<th>Pro</th>
<th>Leu</th>
<th>Gly</th>
<th>His→</th>
<th>→Gly</th>
<th>Asp</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA-Nb</td>
<td>322</td>
<td>328</td>
<td>334</td>
<td>337</td>
<td>338</td>
<td>342</td>
<td>346→</td>
<td>356</td>
<td></td>
</tr>
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</table>
| N-Nb  | 168357 | 168339 | 168321 | 168312 | 168309 | 168297 | 168285→ | 168256 | 168255

Nucleotide numbering of EBV sequences was performed as for strain B95-8 (wild type) according to Baer et al.\textsuperscript{25} Point mutations are underlined and amino acid substitutions are in bold. All isolates of patient no. 1 show identical point mutations at positions 168357, 168355, 168320, 168308, and 168295, resulting in identical amino acid substitutions at positions 322, 334, 337, and 338 as well as a 30-bp deletion including nucleotide positions 168285 to 168256 also described in CA0 and HD3,\textsuperscript{14} suggesting that these isolates derive from a common viral strain.

Abbreviations: AA, amino acid; AA-Nb, amino-acid number; N-Nb, nucleotide number.
The concept that HD relapses are related to one residual tumor cell clone, supported by our findings, has important clinical implications. It may indicate that treatment does not always eradicate the disease and that, in a few cases, tumor cells remain silent for a long period of time by escaping antineoplastic treatment and the immune response. Deletions within the LMP1 oncogene, in a region where the half life of the protein is regulated, may favor the progression of the disease, such as in case 1, who relapsed twice. Such deletions might also have some consequences on the immune T-cell surveillance because LMP1 is one possible target protein to elicit a cytotoxic T-cell reaction.

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