Rapid Communication

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

By Pierre Brousset, Daniel Schlaifer, Fabienne Meggetto, Edith Bachmann, Sylvia Rothenberger, Jacques Pris, Georges Desol, and Hans KNecht

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 germline 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 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.

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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 (ION1; 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 only in 2 cases, including the relapse material of case 2 that was fixed in formalin. DNA extracted from Boain or ethanol-based Boain’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-a-32P*.

Gene rearrangement study was performed as described elsewhere with *JH* locus-specific, *Ck* and *Cg* probes and TCR-ß probe (specific for human ß 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 35S-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 JH, CK, CL, and TCR-ß 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 probable explanation for the negative results in case virus and/or the low copy number of viral genomes in these that there were small numbers of reported in metachronous localizations in two cases of EBV- and at relapse) were related to the persistence of the same viral strain. The most fragments was the same at diagnosis and at both relapses, suggesting the persistence of the same viral strain. The identical point mutations were in sample 2.1, 6 years later.

Table 1. Point Mutations, Deletions and Amino Acid Substitutions Observed in the LMP1 Gene of Isolates 1.1, 1.2, and 1.3 Compared With the Nasopharyngeal Carcinoma CAO and Another Case of HD (HD3)

<table>
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<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>
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<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>
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<tr>
<td>N-Nb</td>
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<td>168339</td>
<td>168321</td>
<td>168312</td>
<td>168309</td>
<td>168297</td>
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Nucleotide numbering of EBV sequences was performed as for strain 895-8 (wild type) according to Baer et al.39 Point mutations are underlined and amino acid substitutions are in bold. All isolates of patient no. 1 show identical point mutations at positions 16831, 16835, 168320, 168308, 168295, 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 168266 also described in CAO and HD3,4 suggesting that these isolates derive from a common viral strain.

Abbreviations: AA, amino acid; AA-Nb, amino acid number; N-Nb, nucleotide number.

reported cases of HD (Table 1 and Fig 2).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).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.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,4,5,11 their clonality still remains to be established. Indeed, molecular analyses of Ig or TCR gene rearrangement usually yield negative results.10 In this context, the demonstration of EBV clonality, as reported in nasopharyngeal carcinoma,12 appears to be a reliable method in assessing the clonal origin of Reed-Sternberg cells.1,6,14 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.19 In the present study, EBV clonality was investigated using Southern blotting and LMP1 sequencing to determine whether the viral genomes 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.4 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.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 LMP1 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)15 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 infection21 or increased oropharyngeal shedding,22 although anti-EBV approaches that may eradicate EBV from its latent sites are not yet available.23 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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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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