Rapid Communication

High Expression of Latent Membrane Protein 1 of Epstein-Barr Virus and BCL-2 Oncoprotein in Acquired Immunodeficiency Syndrome-Related Primary Brain Lymphomas

By S. Camilleri-Broët, F. Davi, J. Feuillard, C. Bourgeois, D. Seilhean, J.-J. Hauw, and M. Raphaël

Most acquired immunodeficiency syndrome (AIDS)-related lymphomas are characterized by a B-cell phenotype, high-grade malignancy, and an extranodal localization. The association with the Epstein-Barr virus (EBV), observed in 67% of cases in one study, varies significantly according to the primary localization and the histologic type. EBV has been detected in more than 90% of AIDS-related primary brain lymphomas (AR-PBL). These lymphomas provide a unique model of relationship between viral infection and tumour cells in patients. In infected cells the virus can exist either in a latent or a replicative form. Viral latency is associated with the presence of various latent proteins encoded by the viral genome. The expression of latent membrane protein 1 (LMP1) has been shown in AIDS-related lymphomas of different localizations and histologic types. LMP1 may play a major role as an oncprotein in immortalizing and transforming cells. In addition, genetic alterations vary according to histologic category, especially c-myc rearrangement and P53 loss/mutation with small nucleated cell lymphoma (SNCCCL). Transactivation of the bcl-2 proto-oncogene by LMP1 has been reported in vitro. The Bcl-2 expression induced by LMP1 may protect infected B cells from apoptosis and result in a higher proliferative rate. This finding points to new perspectives in EBV-related oncogenesis.

To search for a correlation between LMP1 and BCL-2 expression, we have analyzed 11 AR-PBL and 57 AIDS-related systemic lymphomas (AR-SL). We show here a high expression of both LMP1 and BCL-2 in the AIDS-related primary brain lymphomas.

Materials and Methods

Tumor specimens and histology. Sixty-eight cases of AIDS-related non-Hodgkin lymphomas were analyzed. Eleven were AR-PBL from a series of autopsies from the neuropathology laboratory (Pitié-Salpêtrière Hospital) and 57 others cases were AR-SL from the French Study Group of HIV-Associated Tumors. Cases of disseminated lymphomas with secondary brain involvement were excluded. In all cases, part of the sample was snap-frozen in liquid nitrogen and kept at −80°C until it was used for immunophenotypic and genotypic studies. The rest was routinely processed after formalin fixation and paraffin embedding. Conventional histology was performed on paraffin-embedded material stained with haematoxylin-eosin and Giemsa’s method. All cases were classified according to the updated Kiel and Working Formulation criteria.

Immunophenotypic study. Immunophenotypic studies were performed on paraffin-embedded or frozen sections using either the avidin biotin peroxidase or alkaline phosphatase techniques (APAAP). The immunoperoxidase method using a commercially available kit (Vectastain; ABC Vector Laboratories, Burlingame, CA) was performed on paraffin-embedded material with monoclonal antibodies (MoAbs) directed against CD20 (L26), CD45RO (UCHL1) antibodies and on frozen section with CD3 and CD20 antibodies, kindly obtained from E. Kremmer (Institut für Immunologie, München, Germany) and F. Grässer (Department of Microbiology, University of Hamburg, Saar) was tested using the APAAP method. The tumor was considered positive when more than 10% of tumors cells expressed the differentiation antigens. EBV gene expression was estimated using a semiquantitative grading as previously described. 0, no positive cells; 1, scattered positive cells in a low-power field using 10 × ocular and 10 × objective; 2, few positive cells per medium-power field using 10 × ocular and 25 × objective, 3, positive cells easily seen in each medium-power field; 4, many positive cells in each medium-power field.

Detection of EBV. In situ hybridization (ISH), polymerase chain
reaction (PCR) analysis, and Southern blot were used for the detection of EBV. EBV was first detected by PCR and the positive cases were confirmed by either ISH or Southern blot. ISH was performed on paraffin-embedded sections with a fluorescein isothiocyanate (FITC)-labeled oligonucleotide complementary to small nuclear EBV-encoded RNA1 (EBER-1; Dakopatts). An antifluorescein isothiocyanate antibody APAAP mouse system was used for the detection. EBV-positive control was the Raji cell line and D975 cell line was the negative control. For the PCR analysis, DNA was extracted from frozen tissues using a standard procedure and the EBNA1 gene was amplified according to previously published protocols. The specificity of the PCR product was assessed after Southern blot using an internal oligonucleotide (5'-CCATGCTCTCGTCCTGT 3') labeled with the digoxigenin tailing kit (Boehringer, Meylan, France). The EBV-positive B-cell line Raji was used as a control. Southern blot on genomic material was performed from frozen material using a random primed '*P-labeled probe specific for the BamH1 W internal repeats of the virus.

Detection of bcl-2 rearrangement by PCR analysis. The detection of bcl-2 rearrangements within the major breakpoint region (MBR) and minor cluster region (mcr) was performed according to a previously published protocol. Specificity of the PCR product was assessed after Southern blot using an internal oligonucleotide: 5' CCCTCCTCGTCCTGTGTCGGC 3' for the MBR and 5' GCCGCT- TCCTGTGTCGGC 3' for the mcr. These oligonucleotides were labeled with the digoxigenin tailing kit (Boehringer). Positive controls consisted of cases of follicular lymphoma and the negative control was placenta DNA.

Statistical analysis. A χ² test modified by the Yates test was used to assess the association between BCL-2 staining (positive versus negative) and primary localization (brain versus systemic) and LMP1 expression (positive versus negative staining).

RESULTS

Histopathology and immunophenotypic study (Table 1). Ten of eleven cases (91%) of AR-PBL were classified as immunoblastic (IB) with plasmacytic features, whereas only 1 case was classified as Burkitt's lymphoma (BL). Among the 57 AR-SL, 36 were nodal (63%) and 21 were extranodal (37%). Thirty-nine cases (68%) were large cells (LC) or IB (Figs 1B and 6) of 11 cases (54%) expressed EBNA2 (grade 1, 2 cases; grade 2, 1 cases; grade 3, 7 cases; Fig 1B) and 6 of 11 cases (54%) expressed EBNA2 (grade 1, 1 case; grade 2, 2 cases; grade 3, 2 cases; grade 4, 1 case).

For systemic cases, 11 of 57 AR-SL (19%) were negative using at least two methods previously described for EBV detection. EBV was detected in 46 cases (21 LC, 9 IB, and 16 BL). The frequency of EBV-positive BL cases in this series is higher than in previously published series, but this finding could be explained by the limited number of cases in this category. In EBV-positive cases (46 cases), LMP1 was expressed in 21 cases (4 cases of BL [22%] and 17 cases of IB/LC [44%]). EBNA2 was expressed in 4 AR-SL (7%).

Bcl-2 expression and gene rearrangement (Tables 2 and 3). Expression of BCL-2 protein was nearly constant in the cases of AR-PBL (10 of 11 [91%]) in more than 50% cells (Fig 1A). These 10 cases were IB lymphomas and also expressed LMP1. The BL case did not express either LMP1 or BCL-2. By contrast, only 3 of 57 AR-SL cases (5.4%) expressed BCL-2 (Fig 1C). These 3 cases were extranodal lymphomas (gum, lung, and nasopharynx). In all cases, BCL-2-positive reactive lymphocytes could also be observed in nodal lymphomas and were used as internal positive controls (Fig 1D). No rearrangement of the bcl-2 gene within the MBR and the mcr could be shown using PCR.

When BCL-2 expression was assessed in different localizations, the number of cases expressing BCL-2 in primary brain lymphomas and systemic cases differed significantly (P < .001). BCL-2 expression and LMP1 expression were also strongly linked (P < .001).

DISCUSSION

AR-PBL are quite different from systemic lymphoma. Nearly all cases are immunoblastic, with plasmacytic features, and their association with EBV is constant. Our data confirm the strong association between AR-PBL and EBV because the presence of the virus was demonstrated in all cases using at least two techniques for each case. ISH is a sensitive technique with the major advantage of precise localization of EBV-encoded RNAs on paraffin sections. We could show a good correlation with the results of PCR that was positive in all cases, in contrast to a previous study showing only 50% positive cases. The Southern blot analysis performed in our center confirmed these results in all 8 cases for which enough DNA was available. We also showed that the two latent proteins LMP1 and EBNA2 were more frequently expressed in AR-PBL than in systemic lymphomas: all immunoblastic cases expressed LMP1; half of them expressed EBNA2.

BCL-2 is an inner mitochondrial membrane protein that prevents programmed apoptotic cell death. The bcl-2 gene is located on chromosome 18 in the vicinity of the t(14;18) breakpoint. BCL-2 is overexpressed in follicular lymphomas and in a variety of normal hematopoietic cells during B- or T-cell development and activation. In vitro studies have shown that bcl-2 can be transactivated by LMP1, which results in protection of B cells from programmed apoptotic death. Expression of bcl-2 proto-oncogene enhances cell survival and increases the chances of further DNA alterations. This is a possible mechanism for EBV in the pathogenesis of AR-PBL. However, previous
studies have failed to show an in vivo correlation between BCL-2 and LMP1 in Hodgkin’s disease.

The present study shows that nearly all AR-PBL express BCL-2 without DNA rearrangement within the MBR and mcr. This is not found in AR-SL, few of which are positive for BCL-2; these BCL-2-positive cases also express LMP1 in numerous cells. All of the AR-PBL cases simultaneously expressing BCL-2 and LMP1 were IB in type. In contrast, the only BL case did not express either LMP1 or BCL-2. BCL-2 expression seemed to be different in EBV-associated tumors according to their histologic type and primary site. In this study, we have shown a strong association between the primary brain localization and the expression of both BCL-2 and LMP1. BCL-2 is highly expressed in AR-PBL. This finding could be related to LMP1 expression. It may also be due to the brain localization of lymphomas. This suggestion makes it difficult to evaluate the influence of each of these factors on BCL-2 expression. A preliminary study of embedded paraffin material in 23 HIV-negative primary brain lymphomas, not associated with EBV, has shown that BCL-2 protein was expressed in only 48% of cases. Similar results have been found in HIV-negative systemic lymphomas. Therefore, the overexpression of BCL-2 in nearly all AR-PBL may result from the presence of EBV and the expression of the latent protein LMP1.

Latent viral proteins LMP1 and EBNA2 are overexpressed in AR-PBL, in contrast to AR-SL. These latent viral proteins are known to constitute a target for cytotoxic T-cell-mediated rejection and to initiate a reaction from the host. Neural tissue is considered to be immunologically privileged, although this is not absolute. Conceivably, this peculiar immune response of the brain could allow LMP1 and EBNA2 overexpression within infected cells without elic-

Table 2. BCL-2 in EBV-Associated AIDS-Related Lymphomas

<table>
<thead>
<tr>
<th></th>
<th>AR-PBL (11)</th>
<th>Extranodal (21)</th>
<th>Nodal (36)</th>
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<tbody>
<tr>
<td>LMP1⁺ (31)</td>
<td>10</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>BCL-2⁺ (13)</td>
<td>10</td>
<td>3</td>
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Table 3. Statistical Analysis

<table>
<thead>
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<th>Localization</th>
<th>No.</th>
<th>BCL2⁺</th>
<th>P (x² test)</th>
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<tbody>
<tr>
<td>AR-PBL</td>
<td>11</td>
<td>10</td>
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</tr>
<tr>
<td>AR-SL</td>
<td>57</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>LMP1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LMP1⁺</td>
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</tr>
<tr>
<td>LMP1⁻</td>
<td>37</td>
<td>0</td>
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iting a cytotoxic rejection, therefore allowing bcl-2 transactivation by LMP1.

These findings confirm the originality of AR-PBL that are associated with EBV and overexpression of LMP1 and EBNA2. To our knowledge, this is the first report showing a correlation between BCL-2 and LMPl in vivo. BCL-2 overexpression could play a part in the development of AIDS- and EBV-related primary brain lymphomas.

ACKNOWLEDGMENT

The authors thank E. Kremmer (Institut für Immunologie, Munchen, Germany) and F. Grasser (Department of Microbiology, University of Homburg, Saar) for the anti-EBNA2 antibody; A. Homond, A. Fayon, and S. Laye for their expert technical assistance; V. Sadowsitich for providing us with autopsy material; P. Broet for statistical analysis; and E. Macintyre for reviewing the manuscript.

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