**BCL-2 But Not Its Epstein-Barr Virus-Encoded Homologue, BHRF1, Is Commonly Expressed in Posttransplantation Lymphoproliferative Disorders**


Posttransplantation lymphoproliferative disease (PTLD) is virtually always associated with Epstein-Barr virus (EBV) infection. BCL-2 and other proteins that confer resistance to apoptosis have been implicated in the pathogenesis of a variety of malignancies including lymphomas. One EBV protein, BHRF1, is a homologue of BCL-2, whereas another, the latency membrane protein 1 (LMP-1), upregulates BCL-2 expression in vitro. In the present study, we used immunohistochemistry to study the expression of these viral and cellular proteins as well as a variety of other EBV-encoded proteins in PTLD. BHRF1 was not detected in any PTLD specimen, whereas BCL-2 was shown in 12 of 17 lesions examined. With one exception, all LMP1-positive cases also expressed BCL-2 and the absence of LMP1 was always associated with a lack of BCL-2 expression. The results do not support a role for the EBV homologue of BCL-2 in PTLD, but they do support a role for viral induction of BCL-2 expression.

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**MATERIALS AND METHODS**

**Patients.** Lymphoproliferations from a total of 20 organ transplant recipients with a diagnosis of PTLD were studied. All but four patients (patients no. 3, 13, 14, and 15) received transplants and were diagnosed at a single institution (Loyola University, Chicago). Fourteen patients were cardiac recipients, 2 were renal recipients, 3 were single lung recipients, and 1 was a heart-lung recipient. Clinical data and flow cytometric analyses on a subset of these patients have been communicated elsewhere.

**Histopathology.** Diagnosis was based on examination of histologic material obtained by open biopsy or by core needle biopsy in all cases. Lesions were classified according to the PTLD classification system recently described by Knowles et al. and by the Working Formulation scheme whenever possible. Oral hairy leukoplaikia specimens from 5 patients with human immunodeficiency virus (HIV) infection that have been previously reported were included as positive controls for the immunohistochemical detection of BHRF1 expression. A histologically confirmed hairy leukoplaikia lesion obtained from PTLD patient no. 2, 4 months before a PTLD diagnosis was similarly included as a positive control. Previously reported specimens from patients with EBV-associated Hodgkin's disease were used as positive controls for EBER1 in situ hybridization and LMP1 immunohistochemical detection.

**BHRF1 antisera.** Polyclonal rabbit antiserum against BHRF1 was generated using a peptide corresponding to BHRF1 amino acids 151 through 165 (STLIDNPGSRRFS). A cysteine residue was added to the C-terminus for linkage and D-tyrosine was added to the aminoterminus to prevent degradation. The peptide was linked to keyhole limpet hemocyanin and used for immunization as previously described.

**Section preparation.** Specimens were obtained either as paraffin wax blocks or as frozen tissue samples. Paraffin wax sections were cut at 5 μm and attached to glass slides by incubation overnight at 60°C. Frozen sections were prepared at 6 μm on a cryostat and then fixed for 20 minutes in acetone at 4°C. Frozen sections were stored at -40°C before immunostaining. All slides were pretreated with an adhesive (aminopropylethoxysilane) before sectioning.

**In situ hybridization.** The EBV status of all specimens was determined before immunohistochemistry using a standard digoxigenin-based in situ hybridization protocol for the detection of EBER-1, as previously described. Positive controls consisted of known EBV-positive Hodgkin's disease specimens and B95-8 cells grown as a tumor in SCID mice. Negative controls consisted of the use of an EBER-1 sense probe.

**Immunohistochemistry.** Indirect immunofluorescence was performed to evaluate the specificity of the BHRF1 antisera. Slides were fixed with acetone and methanol, incubated in preincubation rabbit anti-serum or in postincubation serum for 20 minutes at 1:400, washed again, and incubated with fluorescein isothiocyanate (FITC)-conjugated antirabbit antibody.

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Table 1. Patients in This Study Also Cited in Other Reports

<table>
<thead>
<tr>
<th>This Report</th>
<th>Swinnen et al⁹</th>
<th>Kowal-Vern et al¹⁰</th>
<th>Swinnen et al¹¹</th>
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Table 2. Antibodies Used in the Study With Optimum Dilutions and Microwave Pretreatment Times

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody Species and Clone</th>
<th>Optimum Dilution of Antibody</th>
<th>Microwave Pretreatment Time (min)</th>
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<tr>
<td>BCL-2</td>
<td>Mouse, 124*</td>
<td>1:50</td>
<td>40</td>
</tr>
<tr>
<td>BHRF1</td>
<td>Rabbit antipeptide</td>
<td>1:5,000</td>
<td>0</td>
</tr>
<tr>
<td>BZLF1</td>
<td>Mouse, BZ-1*</td>
<td>1:50</td>
<td>40</td>
</tr>
<tr>
<td>LMP1</td>
<td>Mouse, CS1-4*</td>
<td>1:500</td>
<td>20</td>
</tr>
<tr>
<td>EBNA1</td>
<td>Rat, IH4-1*</td>
<td>1:100</td>
<td>50</td>
</tr>
<tr>
<td>EBNA2</td>
<td>Mouse, PE2*</td>
<td>1:50</td>
<td>30</td>
</tr>
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</table>

* Dako Ltd.
† Grasser et al.⁴⁴

Immunohistochemistry was performed for the detection of the cellular protein BCL-2 and a variety of EBV-encoded proteins including BHRF1, LMP1, EBNA1, EBNA2, and BZLF1. Paraffin wax sections were deparaffinized and transferred to Tris-buffered saline, pH 7.6 (TBS). Frozen sections were transferred directly to TBS.

The demonstration of some antigens in paraffin material required microwave pretreatment in 0.1 mol/L citrate buffer, pH 6.0. The optimum microwave pretreatment times were determined for each antigen and are listed in Table 2. After pretreatment, endogenous peroxidase activity was blocked in 0.3% hydrogen peroxide in methanol and sections were transferred to TBS.

Sections were then incubated in primary antibodies at optimum dilutions previously determined for each antibody (Table 2). All primary antibodies were diluted in 10% normal sheep serum. Mouse monoclonal antibodies and the rabbit polyclonal serum to BHRF1 were detected using the peroxidase-based Dako Duet system (Dako Ltd catalog no. K492; Dako, Glostrup, Denmark). The rat monoclonal antibodies to EBNA1, IH4-1 and 2B4-1, were detected using rabbit antirat Iggs (Dako Ltd catalog no. Z455) at a dilution of 1:400, followed by the Dako Duet system. Peroxidase activity was visualized using the standard diaminobenzidine reaction.

Positive controls for EBV-encoded antigens consisted of paraffin sections of pellet B95-8, X50-7, and BIBJ cells; paraffin and frozen sections of an LCL grown as a SCID mouse tumor; and EBV-positive Hodgkin's disease specimens. Normal tonsil served as a control for BCL-2 expression. Negative controls consisted of the substitution of primary antibodies with appropriate nonimmune serum.

Ig gene rearrangement studies. DNA analysis for clonal Ig-gene rearrangements was performed on tumor specimens whenever technically feasible using standard techniques.²⁸,²⁹

Table 3. Cases of PTLD Examined Showing Correlation of EBER In Situ Hybridization and Immunohistochemistry With Histologic Type

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Transplanted Organ</th>
<th>Histology</th>
<th>Ig Gene Rearrangement</th>
<th>EBER</th>
<th>Bcl-2</th>
<th>BHRF1</th>
<th>BZLF1</th>
<th>LMP1</th>
<th>EBNA1</th>
<th>EBNA1</th>
<th>EBNA2</th>
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<td>1</td>
<td>Heart</td>
<td>GM/IBL</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Heart</td>
<td>DLC/PBL</td>
<td>ND</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Heart</td>
<td>IBL/IBL</td>
<td>M</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Heart</td>
<td>IBL/IBL</td>
<td>ND</td>
<td>+</td>
<td>+</td>
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<td></td>
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<td></td>
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<tr>
<td>5</td>
<td>Lung</td>
<td>IBL/IBL</td>
<td>M</td>
<td>+</td>
<td>+</td>
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<tr>
<td>6</td>
<td>Kidney</td>
<td>DM/PBL</td>
<td>MND</td>
<td>+</td>
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<tr>
<td>7</td>
<td>Heart</td>
<td>IBL/IBL</td>
<td>M</td>
<td>+</td>
<td>+</td>
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<tr>
<td>8</td>
<td>Heart</td>
<td>DCL/IBL</td>
<td>ND</td>
<td>+</td>
<td>+</td>
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<tr>
<td>9</td>
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<td>M</td>
<td>+</td>
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<td>10</td>
<td>Heart</td>
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<td>ND</td>
<td>+</td>
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<td>11</td>
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<td>M</td>
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<tr>
<td>12</td>
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<td>M</td>
<td>+</td>
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<tr>
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<td>ND</td>
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<td>14</td>
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<td>+</td>
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<td>15</td>
<td>Heart</td>
<td>IBL/IBL</td>
<td>ND</td>
<td>+</td>
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<td>+</td>
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<tr>
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<td>M</td>
<td>+</td>
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<tr>
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<td>a/PBL</td>
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<td>19</td>
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<td>+</td>
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<tr>
<td>20</td>
<td>Lung</td>
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<td>+</td>
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Abbreviations: WF, classified according to the Working Formulation; DM, diffuse mixed; DLC, diffuse large-cell; IBL, immunoblastic lymphoma; a, immunoblastic proliferation, not classifiable according to Working Formulation; b, atypical lymphoid infiltrate, necrosis too extensive to be classifiable by Working Formulation; Knowles, an alternate classification; PBL, polymorphic B-cell lymphoma; M, monoclonal; MND, monoclonal Ig gene rearrangement not detected; ND, not done.

* Interpretation of Bcl-2 staining difficult because only scattered infiltrate was present. Surrounding small lymphocytes strongly positive for Bcl-2.

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RESULTS

*In situ hybridization.* The EBV association in this series of PTLD cases was confirmed by EBER in situ hybridization. The EBERs are abundant RNA polymerase III transcripts expressed in many EBV-associated tumors even when there is very limited viral protein expression.\(^\text{20}\) EBER-1 RNA was detected in 19 of 20 histologically confirmed cases of PTLD (Table 3). The single EBER-negative specimen (patient no. 20) was a necrotic needle biopsy of lymph node. Subsequently, immunohistochemical analysis showed the presence of latent gene products in the tumor cells of this lesion, thus implying poor preservation of RNA rather than a true absence of EBER expression. The presence of EBV in this tumor was also confirmed by Southern blot analysis for EBV-DNA.

*BHRF1.* A polyclonal rabbit antiserum was raised against a synthetic peptide from a region of the protein without homology to BCL-2 or members of the BCL-2 family. The specificity of the BHRF1 antisera was confirmed by indirect immunofluorescence of BHK and Vero cell lines transfected with a recombinant plasmid vector expressing BHRF1 or a control plasmid vector. Cells transfected with the BHRF1 expression vector and stained with postinnoculation antisera showed strong cytoplasmic staining for BHRF1.
toplasmic immunofluorescence, whereas no immunofluorescence was seen with cells incubated in preimmunization serum or in cells transfected with the control plasmid (data not shown). The sensitivity of the antiserum for detecting BHRF1 in conventionally fixed surgical specimens was evaluated by staining formalin-fixed paraffin-embedded EBV(−) and EBV(+) cell lines. In assays of a B-cell lymphoma line that does not contain EBV (BJAB), no signal was detected. In assays of an EBV(+) B-cell line with low-level lytic infection (B95-8), BHRF1 signal was detected in approximately 5% to 10% of cells (Fig 1A). This percentage is similar to that identified as lytically infected by immunohistochemical detection of the immediate early lytic protein BZLF1 (Fig 1B) and is consistent with the classification of BHRF1 as a lytic cycle protein. The suitability of the antisera to detect BHRF1 in fixed tissues was further confirmed by studying oral hairy leukoplakia specimens. In formalin-fixed paraffin-embedded specimens from 6 patients (patient no. 2 with PTLD and 5 patients with HIV infection and no lymphoproliferative disease), BHRF1 signal was detected in the upper layers of the epithelium. The signal was localized to the cytoplasm of positive cells and staining was characteristically granular in appearance (Fig 1C). The presence of EBV lytic gene expression in the upper layers of epithelium was confirmed in each case by the detection of BZLF1 (data not shown).

In PTLD lesions, immunohistochemistry with BHRF1 antiserum showed no staining of tumor cells. Nonspecific staining of macrophages seen in many cases was easily distinguished from the granular staining observed in control specimens.

**BCL-2 detection.** Immunohistochemistry for BCL-2 showed positive staining of tumor cells in 12 of 17 cases. In all positive cases staining was cytoplasmic and most tumor cells were stained (Fig 1D). In addition, all specimens showed strong BCL-2 staining of small noninvolved lymphocytes (Fig 1E) serving as a useful internal control in those cases in which BCL-2 could not be shown in the tumor cell population. In 1 case (patient no. 20) showing only a scattered tumor cell infiltrate, it was not possible to determine whether BCL-2 was being expressed in the tumor cell population or in surrounding noninvolved lymphocytes. This case was therefore not included in the analysis.

**LMP1.** LMP1 was detected in a total of 16 of 20 cases and in a varying proportion of the tumor cells (Fig 2A). In 1 case (patient no. 3) the absence of LMP1 expression in paraffin material was confirmed later on acetone-fixed frozen sections. LMP1 and BCL-2 were commonly expressed in the same cases. Thus, of the 16 cases in which LMP1 was...
shown, all but 1 expressed detectable levels of BCL-2 in the
tumor cell population and all 4 LMP1-negative cases were
also BCL-2 negative. EBNA2 was detected in the nuclei of
tumor cells in 15 of 20 cases (Fig 2B) and with 1 exception
(patient no. 5) was coexpressed with LMP1.

EBNA1. Nineteen of 20 PTLD cases expressed detect-
able levels of EBNA1. In most cases, the majority of tumor
cells were stained, although there was variation from case
to case and in general more cells were stained with the 2B4-
1 antibody than with the 1H4-1 reagent. Positive staining
was granular and confined to the nucleus of tumor cells (Fig
2C).

BZLF1 protein was detected in tumor specimens from 19
of 20 patients. In most cases, only occasional tumor cells
were positive; however, in several patients, much greater
numbers of the tumor cell population were stained (Fig
2D).

DISCUSSION

Proteins that inhibit apoptosis in tumors may enhance tu-
mor cell survival and facilitate tumor progression. The associ-
ation of EBV with a variety of malignancies and homology
of a viral protein with BCL-2 suggested the possibility that
BHRF1 might be expressed in PTLD lesions. However, in
the set of tumors examined here, we could find no evidence
of BHRF1 expression. The BHRF1 gene contains several
BZLF1 binding sites upstream of the coding sequence and
is transcriptionally upregulated by BZLF1 in vitro. However,
even in PTLD lesions showing large numbers of cells ex-
pressing BZLF1, we detected no BHRF1 expression. Failure
to detect BHRF1 expression in tumors expressing BZLF1
suggests that in vivo BZLF1 expression is insufficient to
bring about BHRF1 expression in PTLD lesions. This is
consistent with the recent report that BZLF1 alone is not
able to activate full lytic cycle in EBV lymphoblastoid cell
lines, in contrast with EBV Burkitt’s lymphoma cell lines. In
contrast, we did detect evidence of BHRF1 expression in
oral hairy leukoplaikia showing the ability to detect the anti-
gen in clinical specimens.

In contrast to its viral homologue, BCL-2 is commonly
expressed in PTLD. Whereas in follicular lymphomas BCL-
2 expression is driven by a chromosomal translocation, such
14-18 translocations are generally absent in PTLD. In
many other indolent B-cell tumors, BCL-2 expression is also
upregulated in the absence of the 14-18 chromosomal trans-
location. The data presented here showing a high concor-
dance between LMP1 expression and BCL-2 expression are
consistent with the hypothesis that LMP1 expression drives
BCL-2 expression in a subset of these lymphomas as it does
in B cells in vitro.

A possible relationship between LMP1 and EBV expres-
sion has also been investigated in other EBV-associated tu-
mors. As in PTLD, a consistent association has been reported
in acquired immunodeficiency syndrome (AIDS) primary
central nervous system lymphomas, but no association has
been shown in nasopharyngeal carcinoma, Hodgkin’s dis-
ease, or AIDS-associated systemic lymphomas. These
seemingly disparate results may be reconciled in the light of
in vitro evidence that the LMP1–BCL-2 relationship is
cell type specific and has only been shown in a subset of B
cells.

The results of this study confirm previous reports docu-
menting the heterogeneity of PTLD both with respect to its
histopathological appearance and the pattern of EBV gene
expression. Coexpression of EBNA2 and LMP1 in the
majority of cases in this series suggests an unrestricted
pattern of viral latency antigen expression similar to that
recognized in lymphoblastoid cell lines. In other cases, the
pattern resembled that seen in EBV-associated Burkitt’s
lymphoma with expression of EBNA1 alone. A single case
showed expression of LMP1 but not EBNA2, suggesting a
form of latency paralleling that in EBV-associated Hodg-
kin’s disease, some cases of nasopharyngeal carcinoma, and
some EBV lymphomas in AIDS patients. BZLF1 has been
detected in this and other series. The importance of
lytic cycle expression in the biology of these tumors remains
unclear.

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13. National Cancer Institute sponsored study of classifications
of non-Hodgkin’s lymphomas: Summary and description of working


BCL-2 but not its Epstein-Barr virus-encoded homologue, BHRF1, is commonly expressed in posttransplantation lymphoproliferative disorders [see comments]

PG Murray, LJ Swinnen, CM Constandinou, JM Pyle, TJ Carr, JM Hardwick and RF Ambinder