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Epstein-Barr Virus Latent Membrane Protein-1 Oncogene Deletion in Post-Transplantation Lymphoproliferative Disorders

Anke G. Scheinfeld, Roland G. Nador, Ethel Cesarman, Amy Chadburn, and Daniel M. Knowles

From the Department of Pathology, The New York Hospital-Cornell Medical Center, New York, New York

Epstein-Barr virus (EBV) latent membrane protein 1 (LMP1) is a multifunctional oncoprotein. A 30-bp deletion of the 3′ end of the LMP1 gene (del-LMP1) has been identified in some EBV isolates. This deleted LMP1 gene encodes a protein, altered on the carboxy terminus, which is thought to have greater oncogenic potential than the wild type. Recently, it was suggested that del-LMP1 plays a role in the development of malignant lymphomas occurring in immunocompromised patients. To further elucidate the role of del-LMP1 in post-transplantation lymphoproliferative disorders (PT-LPDs) we analyzed 58 PT-LPD lesions from 36 heart and kidney organ transplant recipients. Overall, del-LMP1 was detected in 44% of the cases. Four plasmacytic hyperplasias (36%), eight polymorphic B-cell hyperplasias/polymorphic B-cell lymphomas (38%), and five malignant lymphomas/multiple myelomas (71%) exhibited del-LMP1. Two of the three patients displaying disease progression showed wild-type LMP1 gene (w-LMP1) and one showed del-LMP1. LMP1 status remained the same in all three patients during disease progression. In patients undergoing biopsy of multiple separate PT-LPD lesions representing different clonal lymphoid proliferations, LMP1 status was the same in all of the lesions in each patient. Furthermore, although the polyclonal lesions harbor multiple EBV infectious events, they either showed w- or del-LMP1 but not both. Analysis of the tissues without an apparent PT-LPD (peripheral blood, bone marrow, or colon) revealed EBV and LMP1 type identical to that found in the lesions. In conclusion, the presence or absence of del-LMP1 in PT-LPDs does not correlate with the histopathological category or the malignant nature of the lymphoid proliferation. LMP1 status does not change during disease progression and is the same within multiple lesions occurring in the same patient regardless of their clonal relationship. These findings suggest that 1) EBV infection in patients with PT-LPDs occurs with a w- or del-LMP1-type EBV isolate and does not change once a patient acquires the virus and 2) the infection is an early event in the development of PT-LPDs and transformation is induced regardless of the type of LMP1. *(Am J Pathol 1997, 151:805–812)*

Post-transplantation lymphoproliferative disorders (PT-LPDs) represent a clinically and morphologically heterogeneous group of Epstein-Barr virus (EBV)-driven lymphoid proliferations.[1–4] Their incidence is highest after heart (1.8 to 9.8%) and heart/lung (4.6 to 9.4%) transplantation[5–7] and in patients who receive cyclosporin A[8] and monoclonal antibody OKT3.[9] PT-LPDs have a questionable malignant status in some cases because of spontaneous regression of these lesions after reduction of immunosuppressive therapy.[10] Recently, Knowles et al[11] suggested classifying these lesions into three categories based on morphological and molecular genetic criteria. The first category includes the plasmacytic hyperplasias (PHs), which are characterized morphologically by a retention of the architecture of the infiltrated tissue. The predominant cell population is plasmacytoid lymphocytes associated with plasma cells and rare immunoblasts. The lesions are usually polyclonal and contain multiple EBV infectious events. Polymorphic B-cell hyperplasias (PBCHs) and polymorphic B-cell lymphomas (PBCLs) are grouped together as a second category classified under the term polymorphic lymphoproliferative disorders (PLDs). They probably represent a continuous disease spectrum. Their morphology is characterized by disturbance of the underlying tissue architecture by a polymorphous lymphoid population exhibiting a variable degree of plasmacytic differentiation and cytological atypia and variable numbers of atypical immunoblasts and necrosis. These proliferations are monoclonal by immunoglobulin (Ig) heavy and light chain gene rearrangement or EBV terminal repeat analysis. The third category consists of malignant lymphomas (MLs) and multiple myelomas (MMs), which exhibit a morphology similar to those arising in immunocompetent individuals. These lesions are also mono-

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Address reprint requests to Dr. Daniel M. Knowles, The New York Hospital-Cornell Medical Center, Department of Pathology, 525 East 68th Street, New York, NY 10021.

clonal and contain a single form of EBV but in addition contain one or more oncogene or tumor suppressor gene alterations. LMP1 expression is seen in the majority of EBV-positive PT-LPDs.[12–15]

LMP1 is a multifunctional oncoprotein[16,17] capable of transforming rodent fibroblasts, rendering them tumorigenic in nude mice[18] and altering the growth of multipotent hematopoietic stem cells and epithelial cells.[19] It is also essential for primary B-lymphocyte growth transformation.[20–22] LMP1 is an integral membrane protein with a 200-amino-acid carboxy terminus necessary for the malignant transformation and also critical for the protein half-life.[23] A characteristic 30-bp deletion of the 3′ end of the LMP1 gene (del-LMP1), corresponding to amino acids 346 to 355 of the protein,[17,24,25] was first observed in isolates from the Chinese nasopharyngeal carcinoma (NPC) CAO[26] and the Taiwanese NPC 1510 cell lines.[27] Later, identical deletions were found in a subset of Hodgkin's disease (HD),[25,28] in B-immunoblastic lymphomas, in peripheral T-cell lymphomas,[25,29] and in Brazilian, Danish, and Malaysian Burkitt's lymphomas (BLs) as well as in benign, reactive EBV-positive lymphoid tissue from the same population.[30] Moreover, it is thought to be associated with histological signs of aggressive behavior in NPC CAO, NPC 1510, and HD.[24,31] A longer protein turnover[23,32] and prolongation of the protein half-life[33] was demonstrated in the case of the 30-bp deletion. Thus, an altered LMP1 protein may accentuate the transforming and oncogenic effects of the LMP1 protein. It has been shown recently that del-LMP1 is more tumorigenic than the wild-type protein when inoculated into severe immunodeficient (SCID) and nude mice.[19,27]

These findings led to the hypothesis that del-LMP1 may play a role in the development of EBV-driven malignant non-Hodgkin's lymphomas (NHLs) in immunocompromised hosts. The analysis of AIDS-related lymphoid malignancies in some but not all studies demonstrated a higher incidence of the deletion (100% in HD and 50 to 78% in NHLs) than in the matched group of malignant lymphomas arising within the HIV-negative population (24% in HD and 43 to 50% in NHL).[28,31] The significance of LMP1 deletion in PT-LPDs is even more controversial.[34–36] Kingma et al[35] reported a 100% occurrence of del-LMP1 within malignant lymphomas in a small number of cases but not in a reactive lesion analyzed in solid organ recipients. Recently, Smir et al[36] failed to find a correlation between the presence of del-LMP1 and the morphology or the clonal status of PT-LPDs or the survival of patients with PT-LPDs.

To further elucidate the role of del-LMP1 in PT-LPDs and to resolve this controversy, we studied the LMP1 gene in 58 PT-LPDs from 36 solid organ recipients. Furthermore, we analyzed the EBV and LMP status in multiple separate clonally unrelated PT-LPD lesions occurring in the same patient and in cases displaying disease progression as well as in uninvolved tissues from patients with PT-LPDs.

Materials and Methods

Pathological Samples

One lung, nine kidney, and twenty-six heart transplant recipients who developed one or more PT-LPDs after organ transplantation were included in this study. The clinical features of these patients as well as the morphological and molecular genetic features of their PT-LPDs have been reported previously for 23 patients (cases 1 to 22 (1 lung, 2 kidney, and 19 heart recipients), published by Knowles et al[11] and Frank et al,[37] and case 23 (1 heart recipient), published by Chadburn et al.[38]) Fifty-eight PT-LPD lesions were sampled from 36 patients during the course of clinical evaluation using standard diagnostic procedures or at the time of autopsy from different sites as follows: lymph node, n = 15; tonsil and/or adenoid, n = 11; colon, n = 10; lung, n = 7; skin, n = 5; bone marrow, n = 2; gingiva, n = 2; stomach, n = 1; liver, n = 1; kidney, n = 1; breast, n = 1; brain, n = 1; and soft tissue, n = 1. Eight patients had multiple (2 to 10) PT-LPD lesions that were evaluated in this study. Tissues from sites not involved by PT-LPD were also available from 10 patients as follows: peripheral blood, n = 7; bone marrow, n = 2; and colon, n = 1. Representative portions of these specimens were snap-frozen in a cryopreservative solution (OCT compound; Miles, Elkhart, IN) and stored at −70°C. Mononuclear cells from peripheral blood and bone marrow specimens were isolated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient centrifugation, and the residual cells were cryopreserved in the presence of fetal calf serum in dimethylsulfoxide at −170°C in liquid nitrogen. Portions of each specimen were also routinely fixed in formalin, B5, and/or Bouin's and embedded in paraffin from which hematoxylin-and-eosin-stained sections were prepared.

DNA Extraction

Genomic DNA was extracted from cryopreserved tissue blocks using a salting out procedure.[39] Briefly, the frozen tissue sections were resuspended in 3 ml of nuclei lysis buffer containing 10 mmol/L Tris/HCl, 400 mmol/L NaCl, and 2 mmol/L EDTA, and 200 ul of 10% sodium dodecyl sulfate, (SDS) and 500 ul of proteinase K solution (1 mg of proteinase K in 1% SDS and 2 mmol/L EDTA) were subsequently added. After overnight digestion at 37°C, 1 ml of saturated NaCl was added. This mixture was centrifuged at 2500 rpm for 20 minutes, and 2 vol of ethanol was added to the supernatant to precipitate the DNA, which was washed several times in 70% ethanol.

Southern Blot Hybridization Analysis

Five-microgram aliquots of genomic DNA were digested with the appropriate endonucleases according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN), electrophoresed in 0.8% agarose gels, denatured with alkali, neutralized, and transferred to nitrocellulose filters according to Southern.[40] The filters were

Table 1. LMP1 Primer/Probe Sequences for PCR/Slot Blot Hybridization

Primer/probe	Sequence
5′	5′-CGGAAGAGGTGGAAAACAAA-3′
3′	5′-GTGGGGGTCGTCATCATCTC-3′
Flanking probe	5′-GGCGGGCCCTGGTCACCTCC-3′
Deleted probe	5′-GCCGCCATGGCCGGAATCAT-3′

hybridized as previously described[41] to probes that had been ^{32}P labeled by the random-primer extension method.[42]

The presence and the clonality of EBV infection was investigated by hybridization of *Bam*HI-digested DNAs to a probe specific for EBV genomic termini (5.2-kb *Bam*HI-*Eco*RI fragment isolated from the fused *Bam*HI-terminal fragment NJ-het).[43] The immunoglobulin heavy-chain (IgH) gene was investigated by hybridization of *Eco*RI- and *Hin*dIII-digested DNAs to an IgH gene joining region (J_H) probe.[44]

Oligonucleotide Primers and Probes

The oligonucleotides used in this study as primers for polymerase chain reaction (PCR) amplification and as probes for hybridization of PCR products were synthesized by the solid-phase triester method. Pairs of primers and probes derived from published sequences were used to analyze EBV regions EBNA-2 and EBNA-3c[45–47] to determine the EBV type. Two 20-base oligonucleotide primers flanking the site of the characteristic 30-bp deletion of the LMP1 gene (BNLF1 gene) were used as previously published[28] and are listed in Table 1. For the hybridization of PCR products, two internal probes were used as published by Chen et al.[30] One probe was specific for the deleted segment, which hybridized with wild-type but not deleted LMP1 gene, and the second probe was specific for a region flanking the deleted segment and thus hybridized with both the wild-type and deleted LMP gene. The sequences of these probes are also listed in Table 1.

PCR Amplifications and Hybridizations

The conditions for the PCR analysis and hybridizations for EBNA-2 and EBNA-3c have been previously described.[37,45–47] PCR analysis for LMP1 was performed with 100 ng of genomic DNA, 10 pmol of each primer, 200 μmol/L dNTPs, 10 mmol/L TrisHCl (pH 9.0), 50 mmol/L KCl, 0.1% Triton X-100, 1.5 mmol/L $MgCl_2$, and 2.5 U of *Taq* polymerase, in a final volume of 50 μl. Fifty cycles of denaturing (30 seconds at 94°C), annealing (30 seconds at 60°C), and extension (40 seconds at 72°C) were performed on an automated heat block (DNA thermal cycler; Perkin-Elmer Cetus, Norwalk, CT) after an initial denaturing step of 5 minutes at 94°C. Reaction products were fractionated by electrophoresis in 2% agarose and visualized under ultraviolet light by ethidium bromide fluorescence. They were then denatured with alkali, neutralized, and transferred to nitrocellulose filters according to Southern.[39] Filters were prehybridized for 2 hours at 37°C and hybridized with a ^{32}P-end-labeled probe overnight at 37°C in a solution consisting of 5X SSPE, 5X Denhardt's solution, 0.5% SDS, and 0.1 mg/ml salmon sperm DNA. Filters were washed twice for 15 minutes at room temperature in a solution consisting of 2X SSPE and 0.1% SDS and once for 10 minutes at 60°C in a solution consisting of 5X SSPE and 0.1% SDS and then rinsed briefly in 2X SSC. Autoradiography was performed at −70°C with an intensifying screen for 1 to 2 hours.

Results

The morphological and molecular findings for patients 1 to 23 have been previously published[11,37,38] and are included here. The study is completed using findings from 13 additional patients (cases 24 to 36). The demographic data of those 36 patients are summarized in Table 2.

Histopathology

EBV-associated PT-LPD was diagnosed within 1.5 to 196 months (mean, 34.5 months) after solid organ transplantation. The morphology of the PT-LPDs is summarized in Table 2. PT-LPDs were classified into three morphological categories as suggested by Knowles et al[11]; 14 specimens collected from 11 patients (28% of patients) were classified as PH. These included 10 from tonsils and/or adenoids, 3 from lymph nodes, and 1 from the lung. PBCH or PBCL was diagnosed in 21 patients (54%). Seven specimens obtained from seven patients were classified as PBCH, and twenty-nine specimens collected from fourteen patients were classified as PBCL. The 35 specimens in this group represented biopsy samples from diverse sites: colon, n = 10; lymph node, n = 7; lung, n = 6; skin, n = 5; gingiva, n = 2; tonsil and/or adenoid, n = 1; stomach, n = 1; liver, n = 1; kidney, n = 1; breast, n = 1; and brain, n = 1. ML or MM was diagnosed in eight specimens from seven patients (18%). Two patients had MM, diagnosed in one bone marrow and one soft tissue biopsy, two patients had BL diagnosed in two lymph node biopsies and one bone marrow biopsy, and three patients had diffuse large-cell lymphoma (DLL) diagnosed in three lymph node biopsies. Three patients exhibited disease progression within 4 to 57 months. In one patient, PBCH was diagnosed in a lung biopsy 36 months after heart transplantation and PBCL was diagnosed in two skin biopsies 4 months later. A second patient developed PH 13 months after heart transplantation (three biopsies from the tonsils and adenoids were studied), and PBCL was diagnosed in a lymph node biopsy 57 months later. In a third patient, PH was diagnosed in a lymph node biopsy 2 months after heart transplantation and MM presented as a soft tissue mass in the thigh 13 months later.

Table 2. Demographic Data and LMP1 Status of PT-LPD Patients

Patient	Age (years)	Sex	Organ transplanted	Biopsy site	Morphology	Time (months)*	LMP1
1	61	F	Heart	Lymph node	PH	2	W
2	5	M	Heart	Tonsil, adenoid	PH	13	D
3	46	F	Heart	Lymph node	PH	1.5	W
4	21	M	Heart	Lung	PH	4	D
5	18	M	Heart	Adenoid	PH	58	W
6	24	F	Kidney	Lymph node	PH	1.5	D
7	4	M	Heart	Tonsil	PH	57	D
8	42	F	Heart	Colon	PBCH	5	D
9	55	F	Kidney	Lymph node	PBCH	96	W
10	14	F	Heart	Lung	PBCH	20	W
11	61	M	Heart	Lung	PBCH	11	W
12	7	M	Heart	Lymph node	PBCH	48	W
13	44	M	Heart	Lymph node	PBCL	46	D
14	20	M	Lung	Lung	PBCL	5	W
15	48	M	Heart	Lung	PBCL	11	D
16	52	F	Heart	Lung	PBCL	5	D
17	2	M	Heart	Liver	PBCL	6.5	W
18	55	F	Heart	Brain, breast	PBCL	12	W
19	17	M	Heart	Lymph node	PBCL	2	D
20	58	M	Kidney	Skin	PBCL	49	W
21	62	M	Heart	Lymph node	ML	27	D
22	68	M	Heart	Bone marrow	MM	18	D
23	15	M	Heart	Colon, lymph node	PBCL	3.5	W
1†	63	F	Heart	Soft tissue thigh	MM	15	W
24	53	M	Kidney	Lymph node	ML	39	W
25	3	M	Heart	Lymph node	ML	33	D
26	52	M	Kidney	Lymph node, bone marrow	ML	60	D
27	48	M	Kidney	Lymph node	ML	115	D
28	34	F	Kidney	Tonsil, gingiva	PBCL	196	D
2†	9	M	Heart	Lymph node	PBCL	70	D
29	54	M	Kidney	Stomach	PBCL	4.5	W
30	72	M	Heart	Lung	PBCH	36	W
30†	72	M	Heart	Skin	PBCL	40	W
31	9	M	Heart	Lymph node	PBCL	51	W
32	3	F	Heart	Tonsil, adenoid	PH	19	W
33	2	M	Heart	Adenoid	PH	20	W
34	64	F	Kidney	Renal allograft	PBCH	2	D
35	16	M	Heart	Tonsil, adenoid	PH	11	W
36	5	F	Heart	Tonsil, adenoid	PH	30	W

F, female; M, male; W, w-LMP1; D, del-LMP1.
*Time between solid organ transplantation and diagnosis of PT-LPD.
†Patients with disease progression.

EBV Clonality and Type

The presence and clonality of EBV infection was determined by analysis of fused termini of the EBV genome, which, because of its variable number of tandem repeated sequences, is a distinct marker for different EBV infection events. Of the 55 PT-LPDs that were studied for the terminal repeat, 53 showed evidence of EBV infection. Of the 13 lesions classified as PH, 4 showed a hybridization smear, 1 showed a hybridization smear and a band, and 6 showed a single band. Of the 34 PBCH/PBCLs, 4 exhibited a hybridization smear and a band, 22 exhibited a single band, 7 exhibited two bands, and 1 case was negative. Two MMs, three BLs, and two DLLs showed a single band, and one DLL was negative. EBV was detected by PCR analysis in the two negative cases as well as in the three cases not studied for EBV terminal repeat. Multiple (two to 10) separate lesions were biopsied at the same time from eight patients. In two of these patients, one with 2 and the other with 10 biopsied lesions, analysis of the EBV terminal repeat revealed that these lesions contained a different number of repeats in each separate lesion, representing different clonal EBV-driven lymphoid proliferations. The remaining patients exhibited the same EBV clone in the samples collected simultaneously from different biopsy sites.

The type of EBV was determined by size difference of the PCR amplification products for EBNA-2 and EBNA-3c in 46 lesions. Forty-five lesions were infected by type A EBV, and one DLL was infected by type B EBV.

IgH Gene Rearrangements

Clonal rearrangement of the IgH gene was studied by Southern blot hybridization of the *Eco*RI- and *Hin*dIII-digested DNAs using an IgH gene joining region (J_H) probe in 54 lesions. Applying this method, 1 of the 12 PHs, 29 of the 34 PBCH/PBCLs, and all 8 ML/MMs exhibited clonal IgH gene rearrangements indicated by one or two non-germline hybridization bands on Southern blotting. In the multiple lesions biopsied simultaneously

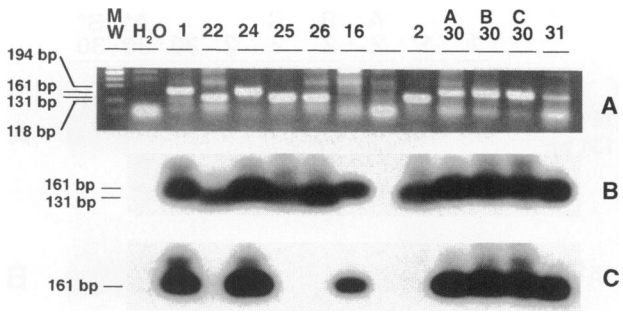

Figure 1. A: PCR analysis of the LMP1 gene region harboring the characteristic 30-bp deletion of EBV using primers flanking this region. A 161-bp and a 131-bp PCR product are representative of the wild-type (w-LMP1) and the deleted variant (del-LMP1) of the LMP1 gene, respectively. DNA from patients 1, 24, 16, 30, and 31 exhibit the 161-bp PCR product indicating w-LMP1. From patient 30 three different biopsies (A, B, and C) were studied and all showed the same LMP1 status. DNA from patients 22, 25, 26, and 2 showed the 131-bp PCR product indicating del-LMP1. **B** and **C:** Hybridization of PCR products after amplification with primers to the LMP1 gene region harboring the characteristic 30-bp deletion. **B:** Results of hybridization using an oligonucleotide probe specific for a region flanking the deleted segment and, thus, hybridizing in all amplifiable cases. **C:** Results of hybridization with an oligonucleotide probe specific for the segment of the LMP1 gene harboring the specific 30-bp deletion and, thus, hybridizing in those cases that do not carry the deletion (cases 1, 24, 16, 30A, 30B, 30C, and 31).

from eight different patients, the results of EBV clonality of these lesions found by EBV terminal repeat analysis were confirmed by IgH gene rearrangement analysis.

LMP1 Gene in PT-LPDs

Either the wild-type LMP1 gene (w-LMP1) or the deleted variant of the LMP1 gene (del-LMP1), indicated by two bands of different sizes in the PCR amplification (Figure 1), was found in all 58 lesions studied. Simultaneous presence of both EBV strains was not detected. The LMP1 status of the PT-LPDs from our 36 patients is summarized in Table 2. The presence of a 161-bp amplification product, indicating w-LMP1, was found in 56% of the cases, and the presence of a 131-bp fragment, indicating del-LMP1, was demonstrated in the remaining 44% of the cases. The PCR amplification results were confirmed by hybridization (Figure 1) using an oligonucleotide probe for a region flanking the deleted segment of the LMP1 gene, hybridizing to the w-LMP1 and to the del-LMP1. The other oligonucleotide probe used was specific for the deleted segment and hybridized only to the w-LMP1. del-LMP1 was found within the three different categories of PT-LPDs with the following frequency: 4 of 11 patients with PH (36%), 8 of 21 patients with PBCH/PBCL (38%), and 5 of 7 patients with ML/MM (71%). w-LMP1 was detected in one MM and del-LMP1 in one MM. del-LMP1 was detected in the two BLs, and among the three DLLs, one showed w-LMP1 and two showed del-LMP1. Although there were differences of the del-LMP1 occurrence within the three morphological groups of PT-LPDs, those were shown not to be statistically significant using the Fisher's exact test ($P = 0.344$).

The morphology, LMP1 status and clonality of PT-LPDs in patients with multiple simultaneous biopsies are summarized in Table 3. In the two cases with multiple (2 to 10) biopsied lesions representing different clonal proliferations by IgH gene rearrangement and EBV terminal repeat analysis, the same LMP1 status was found within all

Table 3. Morphology, LMP1 Status, and Clonality in Patients with Multiple Simultaneous Biopsies

Patient	Morphology	Biopsy site	LMP1	EBV-TR*	IgH gene*
2A	PH	Tonsil	D	Smear	G
2B	PH	Tonsil	D	Smear	G
2C	PH	Adenoid	D	Smear	G
18A	PBCL	Brain	W	3 bands	R
18B	PBCL	Breast	W	3 bands	R
20A	PBCL	Skin	W	1 band	R
20B	PBCL	Skin	W	1 band	R
20C	PBCL	Skin	W	1 band	R
23A	PBCL	Colon	W	1 band	R
23B	PBCL	Colon	W	1 band	R
23C	PBCL	Colon	W	2 bands	R
23D	PBCL	Colon	W	2 bands	R
23E	PBCL	Colon	W	1 band	R
23F	PBCL	Colon	W	2 bands	R
23G	PBCL	Colon	W	2 bands	R
23H	PBCL	Colon	W	1 band	R
23 I	PBCL	Lymph node	W	1 band	R
23 J	PBCL	Colon	W	2 bands	R
26A	ML	Lymph node	D	1 band	R
26B	ML	Bone marrow	D	1 band	R
28A	PBCL	Tonsil	D	1 band (faint)	G
28B	PBCL	Gingiva	D	1 band (faint)	G
28C	PBCL	Gingiva	D	1 band (faint)	G
30A	PBCH	Lung	W	1 band + smear	ND
30B	PBCL	Skin	W	1 band + smear	R
30C	PBCL	Skin	W	1 band + smear	R
32A	PH	Tonsil	W	1 band	G
32B	PH	Adenoid	W	1 band	G

W, w-LMP1; D, del-LMP1; EBV-TR, EBV terminal repeat; IgH, immunoglobulin heavy chain; G, germ line; R, rearranged; ND, not done.
*Patients 18 and 23 are different clones; patients 20, 23, 26, 28, 30, and 32 are the same clones.

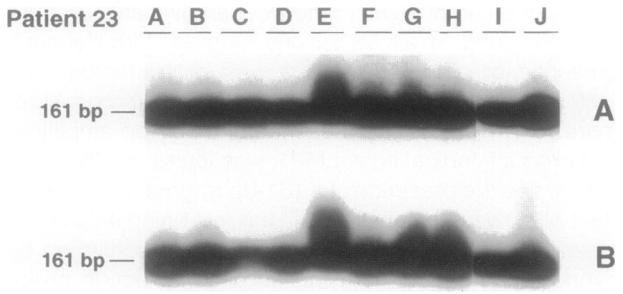

Figure 2. Hybridization of PCR products from 10 different biopsy sites (**lanes A**) to **J sampled simultaneously from patient 23 after amplification with primers for the LMP1 gene region harboring the characteristic 30-bp deletion. A:** Results of hybridization using an oligonucleotide probe specific for a region flanking the deleted segment and, thus, hybridizing to w-LMP1 as well as to del-LMP1. All 10 biopsies exhibit bands of identical size that were shown by PCR analysis to be of the size of w-LMP1 (161 bp). **B:** Results of hybridization with an oligonucleotide probe specific for the segment of the LMP1 gene harboring the specific 30-bp deletion and, thus, hybridizing to w-LMP1 only. All 10 samples show hybridization bands.

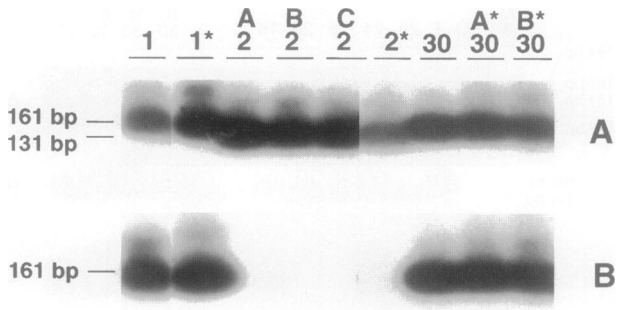

Figure 3. Hybridization of PCR products after amplification with primers to the LMP1 gene region harboring the characteristic 30-bp deletion. Patients exhibiting disease progression are studied (indicated by **asterisk**). *In patient 1, PH was diagnosed 2 months after heart transplantation and MM 13 months later. In patient 2, PH developed 13 months after heart transplantation (three biopsies were analyzed,* **lanes A** to **C**) and PBCL 57 months later. In patient 30, PBCH was diagnosed 36 months after heart transplantation and PBCL 4 months later (results of two biopsies are shown here, **lanes A** and **B**). **A:** Results of hybridization using an oligonucleotide probe specific for a region flanking the deleted segment and, thus, hybridizing to w-LMP1 as well as to del-LMP1. All cases shown here hybridized. **B:** Results of hybridization with an oligonucleotide probe specific for the segment of the LMP1 gene harboring the specific 30-bp deletion as well as all three biopsies from patient 2 did not hybridize, indicating the presence of del-LMP1. Within all three patients, the LMP1 status did not change during disease progression.

lesions (Figure 2). Furthermore, in two patients with PH, two and three lesions biopsied at the same time, respectively, were polyclonal by EBV terminal repeat analysis and therefore harbored multiple infectious events. However, one type, either w-LMP1 or del-LMP1 gene, was found in these lesions (Figure 2). The lesions of four other patients, from which two or three biopsies were obtained at the same time, exhibited infection with a single clone of EBV and showed w-LMP1 in two cases and del-LMP1 in two cases.

Two of the three patients exhibiting disease progression displayed w-LMP1 and one displayed del-LMP1 both in the low- and the high-grade lesions (Figure 3).

EBV and LMP1 Gene Analysis in Uninvolved Tissues from Patients with PT-LPDs

Tissue from sites not involved by PT-LPD was available from 10 patients. EBV and LMP1 status in the uninvolved tissue and the PT-LPDs from those patients are summarized in Table 4. EBV type A was demonstrated in six cases corresponding to the EBV type found in the PT-LPD lesions. We failed to detect EBV in three cases, and one case was amplifiable for EBER only. Wild-type LMP1 was found in five cases and del-LMP1 was detected in two cases. The LMP1 status of the uninvolved tissues was always the same as the LMP1 type present in the PT-LPD lesions of the same patient.

Discussion

Post-transplantation lymphoproliferative disorders are highly associated with EBV infection[1-4] and span a spectrum from polyclonal to monoclonal lesions. Although peripheral blood analysis has shown high prevalence of infection with type B EBV in patients on immunosuppressive therapy,[48] in our experience, type A EBV has been found almost exclusively in the PT-LPD lesions.[37] In this study, type B EBV was detected in only one DLL among 58 PT-LPDs analyzed. The uninvolved peripheral blood and bone marrow samples from six patients with PT-LPDs also contained type A EBV, corresponding to the EBV type of the lymphoid proliferation occurring in the same patient. Furthermore, the LMP status was the same in the lesions and in the available uninvolved tissues, further

Table 4. LMP1 and EBV Status in Uninvolved Tissue and PT-LPD Lesions in the Same Patient

Patient	Biopsy site uninvolved tissue	EBER	EBNA 2a	LMP1	Biopsy site PT-LPD	EBNA2a	LMP1
1	Peripheral blood	+	A	W	Lymph node	A	W
8	Bone marrow	–	–	–	Colon	A	D
11	Peripheral blood	–	–	–	Lymph node	A	W
14	Colon	–	–	–	Lymph node	A	W
15	Bone marrow	+	A	D	Lung	A	D
20	Peripheral blood	+	A	W	Skin	A	W
27	Peripheral blood	+	–	D	Lymph node	A	D
30	Peripheral blood	+	A	W	Lung, skin	A	W
31	Peripheral blood	+	A	W	Lymph node	A	W
35	Peripheral blood	+	A	W	Tonsil, adenoid	A	W

W, w-LMP1; D, del-LMP1.

strengthening the role of EBV in the development of lymphoid malignancies in immunosuppressed patients.

The thought that del-LMP may play an important role in the pathogenesis of post-transplantation lymphoid malignancies was based on several previously mentioned observations. In support of this notion, Kingma et al[35] studied five PT-LPDs and found that the four malignant lymphomas exhibited del-LMP1 whereas the one reactive lesion showed w-LMP1. This led to the suggestion that the deleted form may be preferentially selected during lymphoid transformation in transplant patients. However, in analyzing a larger cohort of PT-LPDs, Smir et al[36] found del-LMP1 in 41% of the cases, with no preferential association with any of the histopathological categories. Although this finding suggests that del-LMP1 does not have a significant impact on the development of PT-LPDs, several important issues helpful in confirming this assertion were not addressed. Here, besides concluding that our results support the findings of Smir et al,[36] we address those issues. In our heart and kidney transplant series, del-LMP1 was detected in 36% of the patients with PH, 38% of the patients with PBCH/PBCL, and 71% of the patients with ML/MM. Thus, our results also fail to support a correlation between the presence or absence of del-LMP1 in PT-LPDs and the histopathological category or the malignant nature of the disorder. Furthermore, we included the results of our analysis of cases undergoing disease progression, of clonally unrelated multiple separate PT-LPD lesions, and of uninvolved tissues. Analysis of peripheral blood samples from patients presenting with various PT-LPDs demonstrated del-LMP1 or w-LMP1 corresponding to the LMP1 status of the PT-LPDs occurring in the same patient. The LMP1 status did not change during disease progression in all three patients analyzed, and the progression occurred with both LMP1 types. We also found in multiple separate lesions occurring at the same time that the LMP1 status remained the same within all PT-LPDs, regardless of their histopathological category and clonality.

Overall, these findings suggest that infection probably occurs with a w- or del-LMP1-type EBV isolate before the development of the lymphoproliferative lesion and does not change once a patient acquires the virus. Isolates harboring either wild-type or deleted LMP1 gene probably cause the initial expansion and transformation of the infected cell.

The occurrence of EBV isolates harboring del-LMP1 in all PT-LPD categories probably reflects the frequency of del-LMP1 in the population.[36] Chen et al[30] showed a high prevalence of del-LMP1 in Brazilian BLs (80%) but also in EBV-positive normal and reactive lymphoid tissue in the same population (59%), supporting the notion that del-LMP1 may not occur preferentially in EBV-driven malignancies. Khanim et al[49] did not find a higher incidence of del-LMP1 in various EBV-associated malignancies than in the geographically appropriate normal population.

In summary, although del-LMP1 has been thought to have a higher oncogenic potential than the wild type, our results suggest that it is probably not an important factor in lymphomagenesis in solid organ transplant recipients. The frequency of del-LMP1 in PT-LPDs does not correlate with the occurrence of MLs, with the aggressiveness of the lesions, with disease progression, or with the responsiveness to the reduction or cessation of immunosuppressive therapy. The infection occurs with either a wild or a deleted type and it remains the same in the infected person. It is far more likely that EBV plays an early role in the induction of lymphoid proliferations in post-transplantation patients regardless of the LMP1 type, as was previously suggested,[11] and additional molecular genetic alterations occur that are important in the development and subsequent progression of post-transplantation malignant lymphoma.

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