Correlative Morphologic and Molecular Genetic Analysis Demonstrates Three Distinct Categories of Posttransplantation Lymphoproliferative Disorders

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The posttransplantation lymphoproliferative disorders (PT-LPDs) are a morphologically heterogeneous group of Epstein-Barr virus (EBV)-driven lymphoid proliferations of varying clonal composition. Some PT-LPDs regress after a reduction in immunosuppression, while others progress in spite of aggressive therapy. Previously defined morphologic categories do not correlate with clonality, and neither morphology nor clonality has reliably predicted the clinical behavior of PT-LPDs. We investigated 28 PT-LPD lesions occurring in 22 patients for activating alterations involving the bet-1, bet-2, c-myc, and H-, K-, and N-ras proto-oncogenes and for mutations involving the p53 tumor suppressor gene. We correlated the results of these studies with the morphology of the lesions, their clonality based on Ig heavy and light chain gene rearrangement analysis, and the presence and clonality of EBV infection. We found that the PT-LPDs are divisible into three distinct categories as follows: (1) plasmacytic hyperplasia: most commonly arise in the oropharynx or lymph nodes, are nearly always polyclonal, usually contain multiple EBV infection events or only a minor cell population infected by a single form of EBV, and lack oncogene and tumor suppressor gene alterations; (2) polymorphic B-cell hyperplasia and polymorphic B-cell lymphomas: may arise in lymph nodes or various extranodal sites, are nearly always monoclonal, usually contain a single form of EBV, and lack oncogene and tumor suppressor gene alterations; and (3) immunoblastic lymphoma or multiple myeloma: present with widely disseminated disease, are monoclonal, contain a single form of EBV, and contain alterations of one or more oncogene or tumor suppressor genes (N-ras gene codon 61 point mutation, p53 gene mutation, or c-myc gene rearrangement). The PT-LPDs are divisible into three categories exhibiting distinct morphologic and molecular genetic characteristics. Alterations involving the N-ras and c-myc proto-oncogenes and the p53 tumor suppressor gene may play an important role in the development and/or progression of the PT-LPDs.

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phic. These morphologic categories often have not been useful in predicting the clonality of these lymphoid proliferations, however, and neither morphology nor clonality has proven capable of reliably predicting disease behavior.

Locker and Nalesnik observed, however, that those non-monomorphic PT-LPDs displaying a strong clonal Ig gene rearrangement band on Southern blotting and c-myc gene rearrangement exhibit disease progression. This observation is important, as proto-oncogene and tumor suppressor gene alterations are highly associated with the development and progression of lymphoid malignancy. We hypothesized that the inability of the current criteria to reliably subcategorize PT-LPDs as benign and malignant and predict clinical behavior may be partially related to the failure to identify the structural alterations of these genes in the PT-LPDs and to correlate them with the morphologic features and an accurate molecular assessment of the clonality of the PT-LPDs. Therefore, we investigated 28 PT-LPD lesions occurring in 22 patients for activating alterations involving the bcl-1, bcl-2, c-myc, and H-, K-, and N-ras proto-oncogenes and for mutations involving the p53 tumor suppressor gene. We correlated the results of these studies with the morphology of the lesions, their clonality based on Ig heavy and light chain gene rearrangement analysis, and the presence and clonality of EBV infection. We discovered that the PT-LPDs are divisible into three categories exhibiting distinctive morphologic and molecular genetic characteristics and identified some of the genetic alterations that may play a role in the development and/or progression of PT-LPDs.

MATERIALS AND METHODS

Patients. One lung, two kidney, and 19 heart transplant recipients who developed one or more lymphoproliferative disorders after organ transplantation at the Columbia-Presbyterian Medical Center (New York, NY) during the period from April 1988 to February 1993 were included in this study. Their inclusion was based on the availability of a minimum of one fresh unfixed tissue specimen sufficient in quantity to permit the molecular genetic and other studies described below. All relevant clinical information was collected by reviewing the medical records of each patient. Portions of the clinical data concerning 15 of these 22 patients have been reported previously. The complete clinical data of all of these patients will be presented in detail elsewhere.

The one lung, one of the two kidneys, and all 19 cardiac transplant recipients were maintained on an immunosuppressive regimen comprised of cyclosporine, azathioprine, and prednisone, as previously described. One renal transplant recipient was maintained on an immunosuppressive regimen comprising of cyclosporine and prednisone. In addition, 10 of the patients received anti-CD3 Ig (Orthoclone OKT3; Ortho Pharmaceutical Corp, Raritan, NJ). The mean doses of each individual immunosuppressive drug at the time of transplantation and at the time of PT-LPD diagnosis in this cohort of PT-LPD patients did not differ significantly from that of the entire cardiac transplant patient population at the Columbia-Presbyterian Medical Center during this time period. Rejection episodes were treated with oral steroids, intravenous steroids, OKT3, and/or antithymocyte globulin as previously described.

Pathologic specimens. Twenty-eight PT-LPD specimens were collected from the 22 patients during the course of clinical evaluation using standard diagnostic procedures: nine from lymph nodes, six from lungs, five from tonsils and/or adenoids, one from jejunum, one from colon, one from liver, one from brain, one from breast, one from skin, one from bone marrow, and one from soft tissues of the thigh. Of the 28 specimens, 27 were collected under sterile conditions, placed in tissue culture media RPMI 1640 or physiologic saline, and immediately delivered to the Hematopathology Laboratory (Columbia-Presbyterian Medical Center). Representative portions of these 27 specimens were snap-frozen in a cryopreservative solution (OCT compound; Miles, Eckhart, IN) and stored at -70°C. The remaining specimen, a sample of aspirated bone marrow, was collected in a heparinized syringe. A viable mononuclear cell suspension was prepared from this sample by ficoll-hypaque (Pharmacia, Piscataway, NJ) density gradient centrifugation. The cells were cryopreserved in dimethylsulfoxide and fetal calf serum in vapor-phase liquid nitrogen at -170°C. Portions of each of the 28 specimens were routinely fixed in formalin, B5, and/or Bouin's and embedded in paraffin, from which hematoxylin- and eosin-stained sections were prepared.

DNA extraction and Southern blot hybridization analysis. Genomic DNA was extracted from cryopreserved tissue blocks and mononuclear cell suspensions using a salting-out procedure. Aliquots (5 μg) of genomic DNA were digested with the appropriate restriction 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 the method of Southern. The filters were hybridized in 50% formamide/× standard sodium citrate (SSC) at 37°C to probes that had been 32P-labeled by the random primer extension method. The filters were washed in 0.2× SSC/0.5% sodium dodecyl sulfate (SDS) at 60°C for 2 hours and then autoradiographed at -70°C for 16 to 48 hours, as previously described.

DNA probes. The Ig heavy chain, kappa light chain, and lambda light chain genes were investigated by hybridization of EcoRI- and HindIII-digested DNAs to an Ig heavy chain gene joining region (JH) probe, BamHI-digested DNAs to a kappa light chain joining region (Jκ) probe, and EcoRI-digested DNAs to a lambda light chain constant region (Cλ) probe. The T-cell receptor beta chain (Tα) gene was investigated by hybridization of EcoRI- and BamHI-digested DNAs to a DNA probe that hybridizes to the constant region of the Tα gene. The organization of the c-myc gene was analyzed by hybridization of EcoRI- and HindIII-digested DNAs to the human c-myc probe MC413RC, representative of the third exon of the c-myc gene. The presence of bcl-1 gene rearrangements was analyzed by hybridization of Bcl-1-digested DNAs to a 2.3-kb XbaI fragment from the bcl-1 locus, as previously described. The presence of bcl-2 gene rearrangements was analyzed by hybridization of HindIII-digested DNAs to the pFL-1 probe, representing a portion of chromosome 18 at the major bcl-2 breakpoint region, and to the pFL-2 probe, representing a portion of chromosome 18 at a minor bcl-2 breakpoint region. The presence of EBV genome and the clonality of EBV infection was investigated by hybridization of BamHI-digested DNAs to a probe specific for the EBV genomic termini (5.2-kb BamHI-EcoRI fragment isolated from the fused BamHI-terminal fragment NJ-het).4,5

Oligonucleotide primers. All of the oligonucleotides used for polymerase chain reaction (PCR) amplification were synthesized by the solid-phase triester method. Pairs of primers derived from published sequences were used to analyze the c-myc gene first exon-intron boundary region, p53 gene exons 5 through 9,7,8 and the H-, K-, and N-RAS gene codons 12, 13, and 61.21 Single-strand conformation polymorphism (SSCP) analysis. SSCP analysis was accomplished according to an adapted version8 of a previously reported method. Briefly, PCRs were performed with 100 ng of genomic DNA, 10 pmol of each primer, 2.5 μmol/
Fig 1. The histopathology of the PT-LPDs. (A, B) Plasmacytic hyperplasia (original magnification [OM]: \( \times 40, \times 630 \), respectively). (C) Polymorphic B-cell hyperplasia (OM: \( \times 630 \)). (D, E, and F) Polymorphic B-cell lymphoma (OM: \( \times 630, \times 630, \times 40 \), respectively). (G, H) Immunoblastic lymphoma (OM: \( \times 630, \times 630 \), respectively). (I) Multiple myeloma (OM: \( \times 630 \). Plasmacytic hyperplasia of a lymph node is characterized by diffuse expansion, with sparing of the sinuses (A), by a predominant population of plasmacytoid lymphocytes associated with plasma cells, and by sparse immunoblasts (B). Polymorphic B-cell hyperplasia is composed of a mixture of plasmacytoid lymphocytes and abundant plasma cells and immunoblasts without cytologic atypia in which necrosis is limited to single cells or small foci (C). Polymorphic B-cell lymphoma is composed of a mixture of lymphoid cells lacking prominent plasmacytic differentiation and displaying significant cytologic atypia (D) and large, atypical immunoblasts (E) and containing large confluent areas of necrosis (F).

L dNTPs (for p53 and c-myc primers) or 50 \( \mu \)mol/L dNTPs (for RAS primers), 1 \( \mu \)Ci of [\( \alpha ^{32} \)P]dCTP [New England Nuclear (NEN), Boston, MA; specific activity, 3,000 Ci/\( \mu \)mol], 10 \( \mu \)mol/L Tris (pH 8.8), 50 \( \mu \)mol/L KCl, 1 \( \mu \)mol/L MgCl\(_2\) (for p53, N-RAS 12-13, N-RAS 61, K-RAS 12-13, and H-RAS 12-13), or 1.5 \( \mu \)mol/L MgCl\(_2\) (K-RAS 61 and c-myc), 0.01% gelatin, 0.5 U Taq polymerase, in a final volume of 10 \( \mu \)L. Thirty cycles of denaturation (94°C), annealing (63°C for p53 exons 5, 6, and 9; 62°C for p53 exon 7; 58°C for p53 exon 8; 58°C for all RAS amplifications; and 63°C for c-myc), and extension (72°C) were performed on an automated heat-block (DNA Thermal-Cycler; Perkin-Elmer Cetus, Norwalk, CT). The reaction mixture (2 \( \mu \)L) was diluted 1:25 in 0.1% SDS, 10 \( \mu \)mol/L
EDTA, and further mixed 1:1 with a sequencing stop solution (95% formamide, 20 mmol/L EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol). Samples were heated at 85°C for 5 minutes, chilled on ice, and immediately loaded onto a 5% acrylamide-Tris-borate EDTA (TBE) gel containing 10% glycerol. The gels were run at 4 to 8 W for 14 to 16 hours at room temperature. They were then fixed in 10% acetic acid and air-dried, and autoradiography was performed at −70°C with an intensifying screen for 6 to 24 hours.

Cloning and sequencing of PCR products. PCR products were cloned in the pCR 1000 vector using the TA cloning system (Invitrogen Corp. San Diego, CA) following the manufacturer’s instructions. The DNA sequencing was performed directly from a small-scale plasmid preparation after determining the presence of an insert. The Sequenase version 2.0 system (US Biochemical, Cleveland, OH) was used, and a modification of the procedure in the manufacturer’s instructions was performed as previously described.11

RESULTS

Histopathology. Drs Knowles, Chadburn, and Frizzera reviewed all of the histologic sections prepared from each specimen and classified each PT-LPD without knowledge of the clinical characteristics of the patients or the results of any of the additional studies performed here. Five morphologic categories became apparent from this review: (1) plasmacytic hyperplasia, (2) polymorphic B-cell hyperplasia, (3) polymorphic B-cell lymphoma, (4) immunoblastic lymphoma, and (5) multiple myeloma (Fig 1). We designated the term “plasmacytic hyperplasia” to indicate those lesions exhibiting retention of the underlying architecture of the lymph node or other tissue and expansion of the interfollicular area or diffuse infiltration of other tissue by a predominant population of plasmacytoid lymphocytes, associated with plasma cells and sparse immunoblasts. Germinal centers were hyperplastic, involuted, or were absent in these cases. The histopathologic criteria for inclusion into categories 2 and 3 were those previously described by Frizzera and Frizzera et al. Briefly, lesions producing extensive disturbance of the organ architecture and composed of a mixture of lymphoid cells with prominent plasmacytoid differentiation and abundant immunoblasts without cytoplogic atypia, in which necrosis was limited to single cells or small foci, were classified as polymorphic B-cell hyperplasia. Lesions composed of a mixture of lymphoid cells lacking prominent plasmacytoid differentiation and displaying significant cytoplogic atypia, atypical immunoblasts, and containing large confluent areas of coagulative necrosis were classified as polymorphic B-cell lymphoma. Lesions composed of a monomorphic collection of cytologically malignant cells were classified as immunoblastic lymphoma. Lesions composed of a monomorphic population of cytologically atypical plasma cells were classified as multiple myeloma.

Ten specimens collected from eight patients were classified as plasmacytic hyperplasia. These included five from tonsils and/or adenoids, four from lymph nodes, and one from lung (Table 1). Five specimens obtained from five pa-
patients were classified as polymorphic B-cell hyperplasia, and eight specimens collected from seven patients were classified as polymorphic B-cell lymphoma. The anatomic sites involved by these two categories of PT-LPDs were diverse but overlapped: five from lungs, three from lymph nodes, one from jejunum, one from colon, one from liver, one from brain, and one from breast. Three PT-LPDs occurring in lymph nodes and skin in two patients (cases 3 and 14) were classified as immunoblastic lymphoma. The immunoblastic lymphomas in patient 3 and one of the two in patient 14 were characterized as pleomorphic because of large, bizarre nuclei. The other immunoblastic lymphoma in patient 14 had distinctly plasmacytoid features. The lesions occurring in the soft tissues of the thigh of patient 4 and the bone marrow of patient 19 were classified as multiple myeloma. Patient 4 had plasmacytic hyperplasia of a lymph node 14 months before presenting with disseminated multiple myeloma. Patient 11 had plasmacytic hyperplasia of a pericolonic lymph node synchronous with polymorphic B-cell hyperplasia of the colon.

**Antigen receptor gene rearrangements.** Of the 10 specimens classified morphologically as plasmacytic hyperplasia, 9 lacked clonal rearrangements of the Ig heavy chain and kappa and lambda light chain genes on Southern blot hybridization (Table 1; Fig 2). The tenth specimen (patient 1, adenoids) displayed a solitary faint Ig heavy chain gene rearrangement band and two faint kappa light chain gene rearrangement bands, consistent with the presence of a small clonal B-cell population. The 17 remaining specimens studied here, which included all the PT-LPDs classified as polymorphic B-cell hyperplasia, polymorphic B-cell lymphoma, immunoblastic lymphoma, and multiple myeloma, exhibited clonal Ig heavy chain gene rearrangements detectable as one or two new, non-germline hybridization bands on Southern blotting. Of these 17 PT-LPDs, 14 also displayed clonal kappa and/or lambda light chain gene rearrangements, and 2 of these 14 PT-LPDs exhibited kappa light chain deletion in conjunction with lambda light chain rearrangement. The hybridization signals were clear and of variable but generally strong intensity, indicating the presence of a significant clonal B-cell population in each of these pathologic specimens. The one exception was specimen 17, which displayed

### Table 1. Results of Molecular Genetic Analysis of 28 PT-LPDs Occurring in 22 Patients

<table>
<thead>
<tr>
<th>Patient/Specimen No.</th>
<th>Histopathology</th>
<th>Specimen</th>
<th>Jα EcoRI HindIII Jβ Cα Tχ EBV</th>
<th>Oncogenes and Tumor Suppressor Genes</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>bet-1</td>
</tr>
<tr>
<td>22</td>
<td>PH</td>
<td>Adenoids</td>
<td>G G G G G G NEG</td>
<td>G G G WT WT WT WT WT</td>
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<tr>
<td>4A</td>
<td>PH</td>
<td>LN</td>
<td>G G G G G G Smear</td>
<td>G G G WT WT WT WT WT</td>
</tr>
<tr>
<td>8A</td>
<td>PH</td>
<td>Tonsil</td>
<td>G G G G G G 1 band*</td>
<td>G G G WT WT WT WT WT</td>
</tr>
<tr>
<td>8B</td>
<td>PH</td>
<td>Tonsil</td>
<td>G G G G G G 1 band* + smear</td>
<td>G G G WT WT WT WT WT</td>
</tr>
<tr>
<td>8C</td>
<td>PH</td>
<td>Adenoids</td>
<td>G G G G G G 1 band*</td>
<td>G G G WT WT WT WT WT</td>
</tr>
<tr>
<td>18</td>
<td>PH</td>
<td>LN</td>
<td>G G G G G G Smear</td>
<td>G G G WT WT WT WT WT</td>
</tr>
<tr>
<td>16</td>
<td>PH</td>
<td>Lung</td>
<td>G G G G G G 1 band*</td>
<td>G G G WT WT WT WT WT</td>
</tr>
<tr>
<td>1</td>
<td>PH</td>
<td>Adenoids</td>
<td>R1* R1* R2* G G G</td>
<td>G G G WT WT WT WT WT</td>
</tr>
<tr>
<td>10</td>
<td>PH</td>
<td>LN</td>
<td>G G G G G G NEG</td>
<td>G G G WT WT WT WT WT</td>
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<tr>
<td>11A</td>
<td>PBCH</td>
<td>Colon</td>
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<td>G G G WT WT WT WT WT</td>
</tr>
<tr>
<td>17</td>
<td>PBCH</td>
<td>Lung</td>
<td>R1 R1 R1* G G G</td>
<td>G G G WT WT WT WT WT</td>
</tr>
<tr>
<td>2</td>
<td>PBCH</td>
<td>Lung</td>
<td>R1 R2 R1* G G G</td>
<td>G G G WT WT WT WT WT</td>
</tr>
<tr>
<td>15</td>
<td>PBCH</td>
<td>Lung</td>
<td>R1 R1 G R1 G NEG</td>
<td>G G G WT WT WT WT WT</td>
</tr>
<tr>
<td>21</td>
<td>PBCH</td>
<td>LN</td>
<td>R1 R1 G G G G</td>
<td>G G G WT WT WT WT WT</td>
</tr>
<tr>
<td>5</td>
<td>PBCL</td>
<td>LN</td>
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<td>G G G WT WT WT WT WT</td>
</tr>
<tr>
<td>6</td>
<td>PBCL</td>
<td>Lung</td>
<td>R2 R1 R2 R1 G G</td>
<td>G G G WT WT WT WT WT</td>
</tr>
<tr>
<td>7</td>
<td>PBCL</td>
<td>Lung</td>
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<td>G G G WT WT WT WT WT</td>
</tr>
<tr>
<td>12</td>
<td>PBCL</td>
<td>Jejunum</td>
<td>R2 R2 R2 G G G</td>
<td>G G G WT WT WT WT WT</td>
</tr>
<tr>
<td>13</td>
<td>PBCL</td>
<td>Lung</td>
<td>R2 R2 R1 G G G</td>
<td>G G G WT WT WT WT WT</td>
</tr>
<tr>
<td>20</td>
<td>PBCL</td>
<td>Liver</td>
<td>R1 R1 R1 G G G</td>
<td>G G G WT WT WT WT WT</td>
</tr>
<tr>
<td>9A</td>
<td>PBCL</td>
<td>Brain</td>
<td>R2 R2 R1 G G G</td>
<td>G G G WT WT WT WT WT</td>
</tr>
<tr>
<td>9B</td>
<td>PBCL</td>
<td>Breast</td>
<td>R11 R11 R11 G G</td>
<td>G G G WT WT WT WT WT</td>
</tr>
<tr>
<td>3</td>
<td>PIb</td>
<td>LN</td>
<td>R1 R1 R1 G G G</td>
<td>G G G WT WT WT M-Ex8</td>
</tr>
<tr>
<td>14A</td>
<td>IB-P</td>
<td>LN</td>
<td>R1 R1 D R1 G G</td>
<td>G G G WT WT WT M-C61 WT</td>
</tr>
<tr>
<td>14B</td>
<td>PIb</td>
<td>Skin</td>
<td>ND ND ND ND ND ND</td>
<td>ND ND ND ND WT WT WT M-Ex7</td>
</tr>
<tr>
<td>4B</td>
<td>MM</td>
<td>Thigh</td>
<td>R2 R1 G G G G</td>
<td>G G G WT WT M-C61 WT</td>
</tr>
<tr>
<td>19</td>
<td>MM</td>
<td>BM</td>
<td>R2 R2 D R2 G G G</td>
<td>G G G WT WT M-C61 WT</td>
</tr>
</tbody>
</table>

Abbreviations: PH, plasmacytic hyperplasia; PBCH, polymorphic B-cell hyperplasia; PBCL, polymorphic B-cell lymphoma; PIb, pleomorphic immunoblastic lymphoma; IB-P, plasmacytoid immunoblastic lymphoma; MM, multiple myeloma; LN, lymph node; BM, bone marrow; G, germline; R, rearrangement; ND, not done; D, deletion; NEG, negative; WT, wild type; M-C, mutation, codon; M-Ex, mutation, exon.

* Faint band.

† Different pattern in same patient.
only a solitary faint Ig heavy chain rearrangement band in the absence of light chain gene rearrangements. Interestingly, two separate PT-LPDs (brain and breast) classified as polymorphic B-cell lymphoma obtained from patient 9 only 6 days apart displayed different Ig heavy and light chain gene rearrangement patterns, suggesting distinct clonal derivations. None of the 27 PT-LPDs studied here contained clonal T<sub>g</sub> gene rearrangements (data not shown). These results indicate that the large majority of plasmacytic hyperplasias are monoclonal lymphoid proliferations or contain only minor clonal B-cell populations, while all other PT-LPDs are monoclonal B-cell proliferations.

**EBV.** The presence and the clonality of EBV infection was determined by analysis of the structure of the fused termini of the EBV genome. Because EBV genomic termini contain a variable number of tandem repeated sequences, the molecular configuration of the fused termini serves as a distinct marker for each EBV infection event. Multiple identical viral episomes with the same fused termini are maintained in the progeny of the infected cell, providing a means of analyzing clonality in the EBV-infected specimens. Differences in the configuration of EBV-fused termini are detectable as differently sized fragments on Southern blots. The detection of a hybridization smear, two bands, or a single band indicates infection by multiple forms, two distinct forms, or a single form of EBV, respectively.15,34

Eight of 10 PT-LPDs classified as plasmacytic hyperplasia, three of five classified as polymorphic B-cell hyperplasia, and all 12 classified as polymorphic B-cell lymphoma, immunoblastic lymphoma, and multiple myeloma studied here contained evidence of EBV infection (Table 1; Fig 2). The four EBV-negative lesions were rehybridized twice more, but EBV was still not demonstrable in these cases. The clonal patterns of EBV infection were highly variable among the eight EBV-containing plasmacytic hyperplasias. The patterns were as follows: hybridization smear alone, three; hybridization smear accompanied by a solitary faint band, one; solitary faint band, three; and a solitary intense band, one. The faint bands detected in the three PT-LPD specimens obtained simultaneously from patient 8 (right tonsil, left tonsil, and adenoids) were identical, suggesting the presence of the same single form of EBV in all three lesions. In contrast with the plasmacytic hyperplasias, none of the PT-LPDs belonging to the other categories that were studied here displayed hybridization smears. The clonal pattern of EBV infection in these 15 PT-LPDs was as follows: solitary intense band, 12; one faint and one intense band, one; two faint and one intense bands, one; and one faint and two
intense bands, one. The two PT-LPDs occurring in patient 9 that displayed different clonal Ig gene rearrangement patterns exhibited different EBV band patterns, suggesting that these two clonally distinct PT-LPDs were infected by different forms of EBV. These results indicate that the plasmacytic hyperplasias, which are polyclonal, represent a spectrum of largely EBV-containing lymphoid proliferations, ranging from those containing multiple distinct EBV infections to those containing only a single form of EBV. In contrast, virtually all lesions belonging to the other PT-LPD categories, which are monoclonal B-cell proliferations, are infected by a single form of EBV or occasionally contain evidence of a second or third infection event.

Proto-oncogenes and tumor suppressor genes. All the PT-LPDs except one were investigated for bcl-1, bcl-2, and c-myc gene rearrangements by Southern blot hybridization. A c-myc gene rearrangement was detected in the plasmacytoid immunoblastic lymphoma occurring in patient 14 (specimen 14A; data not shown). All the remaining PT-LPDs lacking c-myc gene rearrangements were analyzed by SSCP for small deletions/insertions or point mutations in the region surrounding the first exon-first intron of the c-myc gene. In addition, all the PT-LPDs were analyzed by SSCP for mutations occurring within codons 12 and 13 of the H-, K-, and N-ras genes, codon 61 of the K- and N-ras genes, and exons 5 through 9 of the p53 gene—the regions known to contain the majority of the mutations involving these genes.38,39 This technique allows the detection of single base pair mutations as the wild type and mutant radiolabeled PCR products display different migration patterns because of the formation of alternative secondary structures after denaturation and subsequent electrophoresis in a polyacrylamide gel under non-denaturing conditions. This method was previously shown to be highly specific and sensitive to the 1% level.38 In those cases displaying an abnormal migration pattern suggesting mutations, the DNA fragments of altered electrophoretic mobility were reamplified in a separate reaction, cloned, and sequenced to verify the presence of a mutation and delineate its nature. The three PT-LPDs classified as plasmacytoid immunoblastic lymphoma or multiple myeloma exhibited N-ras gene mutations, and the two PT-LPDs classified as pleomorphic immunoblastic lymphoma exhibited p53 tumor suppressor gene mutations (Table 1). The plasmacytoid immunoblastic lymphoma occurring in patient 14 (specimen 14A) and the two multiple myelomas (specimens 4B and 19) contained identical point mutations: a C to A transversion in the first nucleotide of codon 61 leading to a change in the encoded amino acid from glutamine to lysine (Table 1, Fig 3). The pleomorphic immunoblastic lymphoma occurring in patient 3 contained an insertion of a C nucleotide in codon 301 of p53 gene exon 8 leading to a frameshift (data not shown). The pleomorphic immunoblastic lymphoma occurring in patient 14 (specimen 14B) contained a missense mutation in p53 gene exon 7: a C to A transversion in the second nucleotide of codon 248 leading to a change in the encoded amino acid from arginine to leucine (Fig 4).

The presence of different genetic lesions in the two separate PT-LPDs occurring in patient 14 suggest that these lesions are of distinct clonal derivation. However, we could not prove this unequivocally because of insufficient DNA from specimen 14B to perform Ig gene rearrangement analysis. None of the 24 PT-LPDs classified as plasmacytic hyperplasia, polymorphic B-cell hyperplasia, or polymorphic B-cell lymphoma contained evidence of mutations involving the c-myc, the N-ras, or the p53 genes. None of the PT-LPDs studied here exhibited bcl-1 or bcl-2 gene rearrangements or H- or K-ras gene mutations. The results of these molecular genetic studies are summarized in Table 2.

DISCUSSION

The results of the studies described here confirm that the PT-LPDs represent a heterogeneous group of EBV-driven lymphoid proliferations of varying clonal composition. However, these results extend all prior observations by identifying some of the molecular genetic events that may play an important role in the development and/or progression of the PT-LPDs. By demonstrating that the PT-LPDs are broadly divisible into three categories, each exhibiting distinctive morphologic and molecular genetic characteristics, these findings also provide an explanation for some of the previously reported incongruities between the morphology, clonality, and clinical behavior of PT-LPDs.

In summary, we found that those PT-LPDs that we classified as plasmacytic hyperplasia most commonly arise in the oropharynx or lymph nodes, are nearly always polyclonal, usually contain evidence of multiple EBV infection events and/or only a minor cell population infected by a single form of EBV, and lack oncogene and tumor suppressor gene alterations. Those PT-LPDs that we classified as polymorphic B-cell hyperplasia or polymorphic B-cell lymphoma according to earlier morphologic criteria12,13 exhibit overlapping molecular genetic characteristics that unite them into one category. These PT-LPDs may arise in lymph nodes or in a variety of extranodal sites, especially the lungs and the gastrointestinal tract, are nearly always monoclonal, usually contain evidence of infection by a single form of EBV, and lack oncogene and tumor suppressor gene alterations. Those PT-LPDs that we classified as immunoblastic lymphoma or multiple myeloma usually present with widely disseminated disease, are monoclonal, contain evidence of infection by a single form of EBV, and contain alterations of one or more oncogene or tumor suppressor genes.

The PT-LPDs that we designated plasmacytic hyperplasia appear to be morphologically identical to the diffuse reactive plasma cell hyperplasia of lymph nodes described by Nalesnik et al1 in some patients who either had a concurrent or subsequently developed a polymorphic or monomorphic PT-LPD. In both series, these lesions were characterized by retention of the underlying lymph node architecture, a diffuse, benign-appearing plasmacytoid cellular infiltrate exhibiting minimal polymorphism, and an invariably polyclonal composition, except in one of our cases where a small clonal B-cell population was identified. Nalesnik et al1 were uncertain of the relationship between these lesions and their polymorphic and monomorphic PT-LPDs and proposed that their presence "should suggest the possibility of early PT-LPD
Fig 3. Analysis of N-ras codon 61 mutations by PCR-SSCP (A) and sequencing (B). (A) Representative samples corresponding to radiolabeled, PCR-amplified fragments from exon 2 of the N-ras gene, after denaturation and electrophoresis on a 6% acrylamide gel containing 10% glycerol, are shown. Lanes 1 through 19 indicate the case number analyzed, and the letters under these numbers denote different specimens from the same patient. ND, amplified DNA that was not denatured before electrophoresis; —, the same PCR reaction in the absence of DNA; C, wild-type control, for which DNA extracted from a reactive lymph node was used. The histologic classification of each case is indicated above each lane as follows: #, plasmacytic hyperplasia; *, polymorphic B-cell hyperplasia; **, polymorphic B-cell lymphoma; &, pleomorphic immunoblastic lymphoma; @, plasmacytoid immunoblastic lymphoma or multiple myeloma. Several identical bands were seen in all of the samples containing the wild type sequence. Specimens 4B, 14A, and 19 showed a different pattern (arrowheads). These cases were interpreted as positive for mutations and were further studied by generic sequence analysis (B). (B) Each mutation is matched to a control wild type DNA. Arrowheads point to the mutated bases. The nucleotide change in cases 46, 14A, and 19 is identical and consists of a C to A transversion in codon 61 of the N-ras gene leading to an amino acid substitution from glutamine to lysine.

or the presence of PT-LPD elsewhere and prompt a search for EBV infection.” However, we believe that these lesions are part of the PT-LPD spectrum as they occur as clinically detectable lymphoid proliferations posttransplantation, usually contain EBV, are associated with other morphologic forms of PT-LPD in the same patient, and regress after a reduction in immunosuppression. Indeed, it is highly likely that these lesions represent the earliest recognizable lymphoid proliferations that occur posttransplantation, as they often appear to contain occult B-cell clones, based on the analysis of EBV-fused termini, that are undetectable by Ig gene rearrangement analysis. Cleary et al13 already suggested that analysis of the EBV-fused termini may be a more sensitive approach than Ig gene rearrangement analysis to detect occult B-cell clones. The term plasmacytic hyperplasia seems appropriate for these lesions based on their benign morphology, molecular characteristics, and clinical behavior.

Unlike Nalesnik et al,7 we also recognized these lesions
in tonsils and adenoids. In fact, in our experience, all PT-LPDs occurring in the tonsils and adenoids are plasmacytic hyperplasias. It is noteworthy in this regard that of the 10 PT-LPDs occurring in this region in the series reported by Nalesnik et al., all were described as polymorphic, only one was monoclonal by virtue of a faint Ig gene rearrangement band, and nine regressed or resolved. Therefore, some, many, or even all of their oropharyngeal PT-LPDs may correspond to our category of plasmacytic hyperplasia. This would explain why a large proportion of the PT-LPDs that they classified as polymorphic were polyclonal and partially explain why a polymorphic appearance did not correlate more consistently with monoclonality in their series, as it did in ours. The latter discrepancy can also be partially explained by the fact that Nalesnik et al. concluded that six additional polymorphic PT-LPDs were polyclonal based on immunophenotypic analysis alone, which is notoriously less sensitive than antigen receptor gene rearrangement analysis.

The results of the studies described here support the contention of Frizzera and Frizzera et al. that the majority of PT-LPDs possess a distinctive, albeit highly variable, polymorphic lymphoid cellular composition that distinguishes them from the various forms of malignant lymphoma, including immunoblastic lymphoma, that are observed in immunocompetent hosts. However, our results do not support the separation of polymorphic PT-LPDs into benign and malignant categories according to the degree of plasmacytic differentiation, the presence or absence of cytologic atypia, and the type and degree of necrosis as proposed by Frizzera and Frizzera et al. Instead, our results demonstrate that both polymorphic B-cell hyperplasias and polymorphic B-cell lymphomas are monoclonal B-cell proliferations that are homogeneous with respect to the presence and clonality of EBV infection and the absence of oncogene and tumor suppressor gene alterations, and thus appear to represent a single category of PT-LPDs.

Cleary et al. proposed that the PT-LPDs are malignant lymphomas ab initio because of their monoclonality and, as such, should be treated with antineoplastic therapy. Nevertheless, these monoclonal PT-LPDs often regress after a reduction in immunosuppression. Furthermore, while it is true that nearly all malignant lymphomas are monoclonal, the converse, namely that all monoclonal proliferations are malignant lymphomas, may not be true. For example, clonal B-cell populations have been documented in human immunodeficiency virus (HIV)-associated hyperplastic lymphadenopathy, systemic Castleman’s disease, and extranodal pseudolymphomas, and clonal T-cell populations have been documented in a variety of cutaneous lymphoproliferative disorders that may regress spontaneously. Recently, Kamel et al. described EBV-associated lymphoid proliferations morphologically resembling malignant lymphomas that arise during methotrexate therapy for rheumatoid arthritis or dermatomyositis and apparently regress after cessation of methotrexate. Finally, our studies demonstrate that the monoclonal polymorphic PT-LPDs invariably lack structural alterations of the proto-oncogenes and tumor suppressor genes that are commonly associated with lymphoid malignancy. For all of these reasons, the benign or malignant nature of the polymorphic PT-LPDs has been controversial in the past and will probably remain so in the near future. Therefore, we prefer to use the noncommittal designation “polymorphic” PT-LPD for this category of lymphoid proliferations until the controversy is resolved.

Nalesnik et al. have proposed that the subset of PT-LPDs that they term monomorphic are morphologically indistin-
guishable from small or large noncleaved cell (Burkitt's or centroblastic) lymphomas occurring in immunocompetent hosts. Hanto et al. and Frizzera have argued that PT-LPDs exhibiting such morphology are uncommon, but that a small number of PT-LPDs resemble immunoblastic lymphoma morphologically. We did not identify any PT-LPDs in this series exhibiting the morphology of small or large noncleaved cell lymphoma, but we did identify three PT-LPDs exhibiting the morphology of immunoblastic lymphoma. We also identified two PT-LPDs composed of a monomorphic collection of atypical plasma cells. Further evaluation showed that both patients had the classical clinical triad of multiple myeloma. These five PT-LPDs were distinguished rather easily from the polymorphic PT-LPDs by their relatively monomorphic and frankly malignant cytopathology. Although the term monomorphic could be applied to these five PT-LPDs, we believe that it is better to avoid ambiguous terminology in referring to these obvious lymphoid malignancies, and it is more precise to use the standard and accepted nomenclature used for identical lymphoid malignancies occurring in immunocompetent hosts. Therefore, we prefer to designate them as PT-LPD, malignant lymphoma, immunoblastic and PT-LPD, multiple myeloma, respectively.

It is now generally believed that structural alterations involving several well-described proto-oncogenes and tumor suppressor genes play a significant role in the development and/or progression of human lymphoid neoplasia. We were able to identify one or more such genetic lesions in each of the five PT-LPDs that we classified as immunoblastic lymphomas or multiple myelomas, presumably confirming their truly malignant status. In contrast, we did not identify comparable genetic lesions in any of the other PT-LPDs studied here, regardless of their clonal composition or the nature of their EBV content. Thus, the presence of one or more proto-oncogene or tumor suppressor gene alterations was the single feature that correlated with clearly malignant morphology in this series.

It is not entirely surprising that we detected p53 gene mutations in the two pleomorphic immunoblastic lymphomas (specimens 3 and 14B) and N-ras gene mutations in the two multiple myelomas (specimens 4B and 19) and in the plasmacytoid immunoblastic lymphoma (specimen 14A) and that these genetic lesions correlate with aggressive clinical behavior and advanced-stage disease in these patients. We previously demonstrated that p53 gene mutations preferentially occur in certain categories of high-grade lymphoid malignancy. Moreover, Nakamura et al. recently documented p53 gene mutations in about 10% of immunoblastic B-cell lymphomas, and Ichikawa et al. reported that p53 gene mutations are present significantly more frequently in B-cell lymphomas in advanced clinical stage (stage IV). The p53 gene mutations that we identified in two PT-LPDs are consistent with the mutational spectrum of the p53 tumor suppressor gene among lymphoid malignancies. In fact, one was a point mutation involving codon 248, a known mutational hot spot that we have identified in other lymphoid malignancies. In addition, we have previously demonstrated that the introduction of ras oncogenes into EBV-immortalized human lymphoblastoid cells results in their malignant transformation and terminal differentiation into clonal plasma cells. Neri et al. have demonstrated that mutations involving N-ras codons 12, 13, and 61 and K-ras codon 12 occur in approximately 32% of plasma cell leukemias and multiple myelomas. We have further shown that multiple myelomas containing ras gene mutations are characterized by advanced-stage disease and adverse prognostic parameters and that ras gene mutations do not occur in monoclonal gammapathies of undetermined significance or in solitary plasmacytomas. Taken together, these findings strongly suggest that ras gene mutations play an important role in the development and progression of multiple myeloma. In the past, we did not identify ras gene mutations in malignant lymphomas, except for occasional AIDS-associated high-grade lymphomas. The presence of an N-ras gene mutation in the immunoblastic lymphoma in this series may reflect the marked plasmacytic differentiation of this particular neoplasm; indeed, we found it difficult to distinguish this neoplasm morphologically from the two myelomas in this series. Point mutations involving N-ras account for approximately 80% of all ras gene mutations among cases of multiple myeloma and plasma cell leukemia, and the majority of these involve codon 61.

### Table 2. Summary of Results of Molecular Genetic Analysis of 28 PT-LPDs Occurring in 22 Patients

<table>
<thead>
<tr>
<th>Histopathology</th>
<th>No. of Specimens</th>
<th>Clonal Ig Gene Rearrangement</th>
<th>EBV</th>
<th>c-myc Gene Rearrangement</th>
<th>N-Ras</th>
<th>p53</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH</td>
<td>10</td>
<td>1 (faint)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBCH</td>
<td>5</td>
<td>5</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PBCL</td>
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<td>8</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PIB</td>
<td>2</td>
<td>1*</td>
<td></td>
<td>0*</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>IB-P, MM</td>
<td>3</td>
<td>3</td>
<td></td>
<td>1</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations: PH, plasmacytic hyperplasia; PBCH, polymorphic B-cell hyperplasia; PBCL, polymorphic B-cell lymphoma; PIB, pleomorphic immunoblastic lymphoma; IB-P, plasmacytoid immunoblastic lymphoma; MM, multiple myeloma. * Only one of the two specimens was evaluated because of insufficient fresh tissue in one case.
several cases of multiple myeloma containing a C to A transversion in the first nucleotide of N-ras codon 61 resulting in the substitution of lysine for glutamine have been documented in the past. Nevertheless, it is rather astonishing that this specific point mutation occurred in three separate PT-LPDs and accounted for all the ras gene mutations identified in the PT-LPDs in this series. This intriguing finding may reflect the highly selective nature of these genetic changes and suggests, among other possibilities, the involvement of a particular, albeit unknown, mutagen or a selective advantage conferred in vivo to malignant plasma cells by a specific N-ras gene mutation.

Similarly, it is not too surprising that we detected rearrangement of the c-myc gene in only one PT-LPD, the plasmacytoid immunoblastic lymphoma (specimen 14A), and that we did not detect bel-1 and bel-2 gene rearrangements in any of the PT-LPDs in this series. Rearrangements of the c-myc gene occur in only a small proportion of diffuse aggressive malignant lymphomas lacking small noncleaved cell (Burkitt’s and Burkitt-like) morphology, except for those occurring in association with AIDS or arising in the gastrointestinal tract, and occur only very rarely in multiple myeloma. This is probably the reason why c-myc gene rearrangements have been detected in only a small number of PT-LPDs in the past. Moreover, all past studies of the c-myc gene in PT-LPDs have been limited to detection of c-myc gene rearrangements by Southern blotting. However, mutational analysis by SSCP in conjunction with direct sequencing is necessary to detect the small mutations in the region surrounding the first exon-first intron of the c-myc gene. Our combined use of both approaches in the studies described here allowed us to determine conclusively that the c-myc gene is infrequently involved in PT-LPDs. This is unlike lymphomagenesis associated with other immunodeficiency states, such as AIDS. The putative oncogene bel-1 is associated with the t(11;14) and occurs in only about 4% of malignant lymphomas and lymphoid leukemias, most commonly in low-grade mantle-cell (centrocytic) lymphomas. The bel-2 oncogene is associated with the t(14;18) and occurs preferentially in malignant lymphomas of follicular center cell derivation. The PT-LPDs do not resemble mantle-cell or follicular center-cell lymphomas morphologically, and our findings suggest that the PT-LPDs are not related to mantle zone or follicular center B cells.

In addition, while it is widely acknowledged that the majority of PT-LPDs are B-cell proliferations, occasional T-cell lymphomas have been reported to occur after transplantation as well. The relationship between their development and posttransplantation immunosuppression is unclear. However, human T cells may be immortalized after transplantation with EBV DNA and several T-cell malignancies containing clonal integration of EBV, including one occurring after transplantation, have been documented. It is conceivable, therefore, that EBV may contribute to the pathogenesis of these posttransplantation T-cell lymphomas in a manner analogous to that of the more common B-cell PT-LPDs. In one of the few prior studies where Southern blot hybridization analysis of PT-LPDs for T-cell receptor gene rearrangements was performed, however, an oligoclonal population of T cells was apparently identified in only one PT-LPD. Moreover, we did not identify clonal T cell gene rearrangements in any of the 28 PT-LPDs studied here. Therefore, clonal T-cell populations appear to be present in very few lymphoid proliferations that arise posttransplantation. Consequently, the pathogenesis of the rare T-cell malignancies that occur after transplantation and whether their development is directly related to posttransplantation immunosuppression or is merely coincidental remains unclear and must await additional studies.

Finally, Locker and Nalesnik previously proposed that the results of molecular genetic analysis of PT-LPDs may have prognostic value. They suggested that monomorphic PT-LPDs containing a strong Ig gene rearrangement band and a c-myc gene rearrangement usually progress in spite of a reduction in immunosuppression. We confirmed that observation in this series in that all four PT-LPDs that contained genetic alterations were classified morphologically as immunoblastic lymphoma or multiple myeloma, and the patients had advanced-stage and progressive disease that did not regress after reduction in immunosuppression. They also suggested that those PT-LPDs possessing high clone strength (a semiquantitative estimate of the proportion of clonal B cells in an individual PT-LPD lesion, based on the intensity of the Ig gene rearrangement hybridization signal on Southern blotting) in the absence of c-myc gene rearrangement also progress despite reduced immunosuppression. However, based on our findings, it is likely that some of Locker and Nalesnik’s clinically progressive monomorphic PT-LPDs exhibiting high clone strength and lacking c-myc gene rearrangements contained alterations of other genes, such as Ras and p53, that were not investigated. Therefore, the biologic characteristics of PT-LPDs that predict their clinical behavior are not entirely clear. We suggest that a comprehensive correlative clinical, morphologic, and molecular genetic analysis of a large cohort of PT-LPDs is necessary to identify those factors that determine disease progression and, thus, are useful in predicting the eventual outcome of patients who develop a PT-LPD.

Several investigators have subscribed to the hypothesis that the development of lymphoid proliferations after transplantation is a multistep process. Immunosuppressive therapy is believed to lead to reactivation of latent EBV or primary infection by EBV, which acts as a continuously present oncogenic agent in these patients. It is theorized that the lack of normal immune surveillance in these patients permits the expansion of multiple EBV-infected and immortalized B-cell clones (polyclonal). Those clones having a proliferative advantage outgrow and obscure the other clones, so that only a few (oligoclonal) or only one (monoclonal) EBV-infected B-cell clone eventually predominates. Such clones are believed to be susceptible to further genetic changes, ie, structural alterations of oncogenes and/or tumor suppressor genes, resulting in the emergence of a fully transformed monoclonal B-cell proliferation containing homogeneous episomal EBV. The results of the studies described here support this hypothesis. Our results strongly suggest...
that the PT-LPDs represent a spectrum of EBV-driven lymphoid proliferations that parallel the stages along this continuum from polyclonal (plasmacytic hyperplasia) to monoclonal (polymorphic PT-LPDs) to frankly malignant continuum from polyclonal (plasmacytic hyperplasia) to monoclonal (polymorphic PT-LPDs) to frankly malignant.

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Correlative morphologic and molecular genetic analysis demonstrates three distinct categories of posttransplantation lymphoproliferative disorders

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