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Characterization of Clonality of Epstein–Barr Virus–Induced Human B Lymphoproliferative Disease in Mice with Severe Combined Immunodeficiency

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To improve the diagnostic accuracy and understanding of the pathogenesis of lymphoproliferative diseases (LPDs) occurring in immunosuppressed transplant recipients (post-transplantation LPD), clonality of Epstein-Barr virus-induced human LPDs in mice with severe combined immunodeficiency was examined by analyzing: 1) human immunoglobulin genes and their products, 2) the clonality of Epstein–Barr virus DNA, and 3) genetic alteration of c-myc or bcl-2 genes. A spectrum of clonality was found in the LPDs comparable with that reported for post-transplantation LPDs, although rearrangements of c-myc or bcl-2 genes were not detected. It is confirmed that this system is useful in terms of clonality for understanding the early phases in the pathogenesis of post-transplantation LPD or LPD in immune deficient patients. (Am J Pathol 1993, 142:139–147)

The prevalence of lymphoproliferative disease (LPD) in immunocompromised patients is significantly higher than that of non-Hodgkin's lymphoma in immunocompetent individuals.[1,2] LPD is increasing in frequency because of the growing number of allogeneic organ transplant recipients. The frequent etiological association of Epstein–Barr virus (EBV) with the LPD in immunosuppressed patients has been documented,[1,2] and the disease differs clinicopathologically from non-Hodgkin's lymphoma in immunocompetent individuals. The LPD in immunosuppressed transplant recipients (post-transplantation LPD; PTLPD) often regresses after reduction of immunosuppression.[3–5] Moreover, monoclonality is not always detectable in PTLPD, although the disease may be fatal[2,4,6] before the lesions evolve from a benign polyclonal process to a malignant monoclonal process.[7–10] These features may cause confusion in the management of patients with PTLPD. Thus, an *in vivo* model is needed to achieve a better understanding of the pathogenesis of these clonal changes evolving in the lesions as well as for defining criteria for providing a correct diagnosis of PTLPD in transplant patients.

Subsequent to the first report of human LPD in mice with severe combined immunodeficiency (SCID),[11] which fail to develop and sustain mature T or B cells,[12] we and others have reported clinical, histopathological, phenotypic, and virological features of LPD in these chimeric mice.[14–18] In this system, the LPDs often develop at multiple sites and histologically resemble human large-cell lymphoma, especially of an immunoblastic type. The proliferating cells express both B cell-related and EBV-related molecules. These features are similar to those of PTLPD. However, most of these studies investigated

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the pathogenetic role of EBV in the development of LPD. Features of clonality, which are important to characterize this system as an *in vivo* model of PTLPD, have not been systematically examined, even though multiple methods for the assessment of clonality are currently available.

We therefore describe the clonality of human LPDs that were induced in *scid* mice as assessed by 1) immunofixation electrophoresis of human immunoglobulin (Ig) in mouse sera, 2) immunohistochemical examination for the expression of Ig light chains, 3) molecular genetic analysis of Ig heavy- and light-chain genes, and 4) determination of the restriction fragments of EBV DNA[19] present in the proliferating human cells. Furthermore, we examined possible rearrangements of c-*myc* and *bcl*-2 genes. The purpose of this study was to examine whether these chimeric mice can be used as an *in vivo* model of human PTLPD in terms of their clonality.

Materials and Methods

Induction of LPD in scid Mice

C.B-17 mice homozygous for the SCID mutation[20] (*scid* mice) were obtained from McLaughlin Research Institute (Great Falls, MT), bred, and maintained in the Animal Resource Facility at the University of Nebraska Medical Center in a specific pathogen-free environment.[16–18]

Heparinized peripheral blood was collected from six healthy individuals and three patients with X-linked lymphoproliferative disease[1,21,22] after informed consent. The status of the donors to EBV infection was determined by examining IgG antibody against EBV capsid antigen and EBV-determined nuclear antigen in the sera. Peripheral blood mononuclear cells (PBMC) were isolated from blood samples by density-gradient centrifugation using Lymphoprep (Nycomed As, Oslo, Norway) and resuspended in RPMI 1640 medium.

The method for inducing human LPD in the mice has been described elsewhere.[11] Briefly, scid mice aged 6 to 12 weeks were inoculated with 25 to 90 $\times$ 10^6 human PBMC by an intraperitoneal injection. One to three mice received PBMC from each donor. For EBV-seronegative donors (who were negative for IgG antibody against EBV capsid antigen and EBV-determined nuclear antigen in the sera), supernatants of cultured B95-8 cells[23] were inoculated into the recipient mice by both intravenous (2×10^5 immortalizing units of EBV) and intraperitoneal (8×10^5 immortalizing units of EBV) routes 6 weeks after transfer of PBMC. Thirty-eight mice and five control mice, which had not been inoculated with human PBMC, were examined after death by 14 weeks after cell transfer, whereas three mice were examined during the 19th week after transfer.

We used histopathological criteria of Nalesnik et al[4] and Frizzera et al,[6] which have been applied for the diagnosis of PTLPD. The LPD which developed in 22 of 41 mice was found to be of human B cell origin by immunohistochemical examination. Histopathological and phenotypic features of LPD in 10 of these 22 mice, which have been described in detail,[18] and LPD in 12 additional mice, were similar. Sufficient DNA was available from a single representative tumor (Table 1) of 10 LPD of 22 mice.

Serum Analysis for Human Ig

Serum samples of the mice were examined by immunofixation electrophoresis using Titan gel immunofixation kits (Helena Laboratories, Beaumont, TX). A serum was characterized to be monoclonal when a single clonal protein or two or more clonal proteins

Table 1. *Transfer of Human Peripheral Blood Mononuclear Cells into* scid *Mice*

Mouse*	Previous no.†	Donor	EBV status	Number of PBMC transferred ($\times 10^6$)	Sacrifice (days)‡
A1		Healthy	+	25	130
A2				25	130
B1		Healthy	–	25	83
B2				25	84
B3	2–1			60	76
C	3–1	Healthy	–	60	94
D	6–1	Carrier of XLP	+	90	62
E1	9–1	XLP	+	60	53
E2	9–2			60	69
E3	9–3			60	69

XLP, X-linked lymphoproliferative disease.
* Mice with the same letter received PBMC from the same donor.
† Histopathological and phenotypic features of the lymphoproliferative diseases in the mice with these numbers have been described in reference 18.
‡ Period between the date of PBMC injection and that of sacrifice.

with a single Ig light chain were detected. A serum was characterized to be oligoclonal when two or more clonal proteins with different Ig light chains were detected.[17]

Immunohistochemistry

Expression of human Ig by the proliferating cells was examined on snap-frozen or formalin-fixed tissues by the avidin-biotin-peroxidase complex method[24] using rabbit polyclonal antibodies against human Ig light chains (DAKOPATTS, Glostrup, Denmark), a biotinylated secondary antibody (goat Ig), and the complex (Vector Laboratories, Burlingame, CA). The antigen localization was visualized by incubation of the sections with a 3,3′-diaminobenzidine-H_2O_2 solution. The sections were then counterstained with either methyl green or hematoxylin and examined with a light microscope. Sections wherein the primary antibodies were omitted served as negative controls.

Evaluation of clonality was based on the reported criteria for LPDs in organ transplant recipients.[4,6] The process was considered to be monoclonal when a κ/λ ratio of 5:1 or greater and a λ/κ ratio of 3:1 or greater was observed.[3]

Molecular Genetic Analysis

High-molecular-weight DNA was prepared either from fresh frozen tissue (mice A1, A2, B1, and B2) or from stored tissue which had been used for phenotypic analysis (mice B3, C, D, E1, E2, and E3).[18] After proteinase K digestion, phenol/chloroform (1:1) extraction, and ethanol precipitation, 10-μg aliquots of DNA were digested with restriction enzymes including *Bam*HI, *Eco*RI, *Pst*I, *Sst*I (Bethesda Research Laboratories, Gaithersburg, MD), and *Hin*dIII (Behringer Mannheim, Indianapolis, IN). DNA fragments were then electrophoresed on 0.7% agarose gel and transferred to nylon filters by the method of Southern.[25] Cloned DNA restriction fragments were radiolabeled with [α-^{32}P]dCTP by random hexamer priming for use as probes.[26] After hybridization to ^{32}P-labeled DNA probes, filters were exposed to XAR-2 films (Eastman Kodak Company, Rochester, NY) for 2 to 5 days at −70 C using intensifying screen. DNA probes for the Ig heavy-chain joining region gene (5.4-kb fragment of human genomic DNA, J_H), T cell receptor β-chain constant region gene (0.4-kb fragment of human complementary DNA, Cβ2), c-*myc* (0.4-kb fragment of human complementary DNA spanning exon 2), and *bcl-2* major breakpoint region (2.7-kb fragment of human genomic DNA) were obtained from Oncogene Science Inc. (Manhasset, NY). DNA probes for the Ig κ light-chain joining region gene (1.8-kb *Sst*I fragment of human DNA, Jκ)[27] and Ig λ light-chain constant region gene (3.5-kb *Eco*RI-*Hin*dIII fragment of human DNA, Cλ2)[28] were kindly provided by Dr. P. Leder. A probe to EBV DNA (*Xho*I 1.9-kb fragment) was supplied from Dr. Raab-Traub.[19] DNA probes to *bcl-2* minor cluster region (0.4-kb *Bam*HI-*Hin*dIII fragment of human DNA, probe d)[29] and *bcl-2* 5′ breakpoint region (1.6-kb *Eco*RI fragment of human DNA, pB16)[30] were generous gifts from Dr. Y. Tsujimoto. Human placental DNA was used as a control to confirm the germline configuration of DNA fragments. Mouse liver DNA was also used because of the possible cross-hybridization of human DNA probes to mouse DNA. For *Xho*I 1.9 probe, a DNA sample from a cell line (Namalwa) carrying normal clonal EBV DNA served as positive control.

For interpreting the results obtained by J_H, Jκ, Cλ and Cβ2 probes, clonality was determined when one non-germline band was detected in at least two enzyme digests, or two or more non-germline bands were detected in one enzyme digest. Furthermore, LPDs were considered to be monoclonal or non-monoclonal based on the structure of the genomic termini of EBV. Non-monoclonal LPDs were considered oligoclonal when two or three well-spaced episomal bands with one predominant form were observed. When at least six closely spaced bands of about equal intensity were found, the lesions were regarded as polyclonal.[31]

Results

Immunofixation Electrophoretic Findings

Restricted or multiple clonal Ig proteins were detected in all of the sera, and four sera were considered monoclonal. One of them (E1) contained a single clonal protein, whereas the other three (A1, A2, and B1) contained two or more clonal proteins with the same light chain types with/without different isotypes (Table 2). The remaining six sera showed either two or three (B2, E2, and E3), or four or more (B3, C, and D) clonal proteins with different light chain types.

Phenotypic Findings

Surface and cytoplasmic Igs were detected in all of the LPDs, but approximately 30 to 50%, but not all, of the large lymphoid cells were positive for either κ or

Table 2. *Comparison of Clonality of Lymphoproliferative Diseases in* scid *Mice*

Mouse	Clonality by serum immunofixation electrophoresis	Site of lymphoproliferative disease	Surface and cytoplasmic light chains	Immunoglobulin genes*			EBV DNA† (*Xho*I 1.9) clonality
				J_H	Jκ	Cλ	
A1	Monoclonal [IgM(λ), 2IgG(λ)]	Abdominal lymph node	Monoclonal (λ)	R (2–4)	R (2)	R (1)	Oligoclonal (3)
A2	Monoclonal [2IgM(λ)]	Abdominal lymph node	Monoclonal (λ)	R (1–3)	R (1–3)	NR	Oligoclonal (2)
B1	Monoclonal [2IgM(κ), IgG(κ)]	Mediastinal mass	Monoclonal (κ > λ)	R (1–3)	R (1)	NR	Monoclonal
B2	Oligoclonal	Abdominal lymph node	Monoclonal (κ > λ)	R (1–3)	R (3–4)	NR	Oligoclonal (3)
B3	Oligoclonal	Liver	Polyclonal	R (1–4)	R (2–5)	NR	Oligoclonal (3)
C	Oligoclonal	Spleen	Polyclonal	NR	NR	NR	Oligoclonal‡ (3)
D	Oligoclonal	Kidney	Polyclonal	R (2)	R (1–2)	R (1)	Oligoclonal (2)
E1	Monoclonal [IgM(κ)]	Spleen	Monoclonal (κ > λ)	R (1)	R (1–2)	NR	Monoclonal
E2	Oligoclonal	Pancreas	Monoclonal (κ < λ)	R (1–2)	NR	R (1)	Monoclonal‡
E3	Oligoclonal	Kidney	Polyclonal	NR	NR	NR	Oligoclonal‡ (2)

* R, rearranged bands; NR, no rearranged bands. Numbers in parentheses indicate the number of rearranged bands seen in two to four enzyme digests.
† Numbers in parentheses indicate the number of distinct bands.
‡ Several weak, but discrete, bands were also present.
§ Several bands of lower molecular sizes were also seen.

λ, or both light chains. There was no discordance between surface and cytoplasmic Ig expression in terms of the light chain types. According to the criteria for the evaluation of clonality, six LPDs were found to be monoclonal (Table 2). However, the degree of the predominance of one light chain type varied among the LPDs. The proliferating cells were stained almost exclusively for one light chain in mice A1 and A2, whereas the other four LPDs contained a small but a significant number of cells positive for a less predominant light chain type. The morphology of these cells was examined in paraffin sections and found to show immunoblastic features similar to those having a predominant light chain. When we compare the Ig light chain of the LPD in different mice having received PBMC from the same donor, we found that two mice that had received cells from either donor A or B developed LPDs with the same light chain types (A1 and A2; B1 and B2), but two mice that had received cells from donor E developed LPDs which had different light chain types (E1 and E2).

Molecular Genetic Findings

For the analysis of human Ig genes, J_H and Jκ probes were hybridized with *Bam*HI, *Eco*RI, *Hin*dIII, and *Pst*I digests, whereas a Cλ probe with *Eco*RI and *Hin*dIII digests. The intensity of the bands seen in LPD DNA was weak when compared with that of the placental DNA, but none of these probes showed cross-hybridization with the *scid* mouse DNA. Clonal

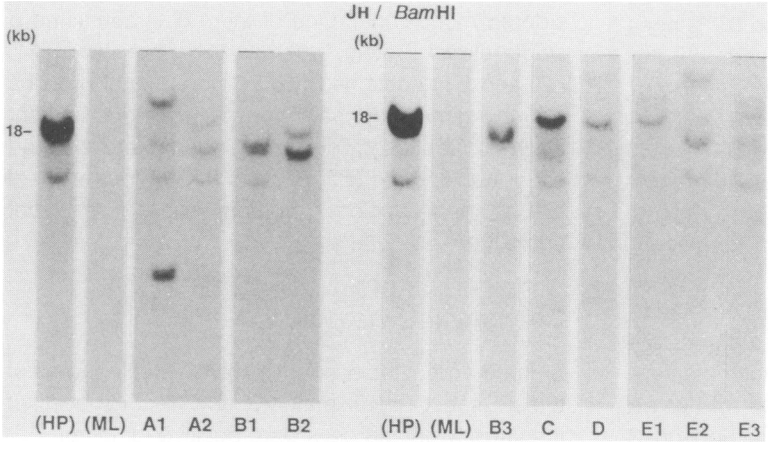

Figure 1. *Southern blot hybridization analysis of human lymphoproliferative disease induced in the* scid *mice. DNAs were extracted from tumors or tumor-containing tissues in the mice (lanes A1 to E3) listed in Tables 1 and 2. Lesions in mice A1 to B2 and those in mice B3 to E3 were examined separately. Human placental DNA (lane HP) and mouse liver DNA (ML) were used for controls. These DNAs were digested with* Bam*HI. After Southern blot transfer, the filters were hybridized to a human J_H probe. The size of the expected germline band is shown in kilobases.*

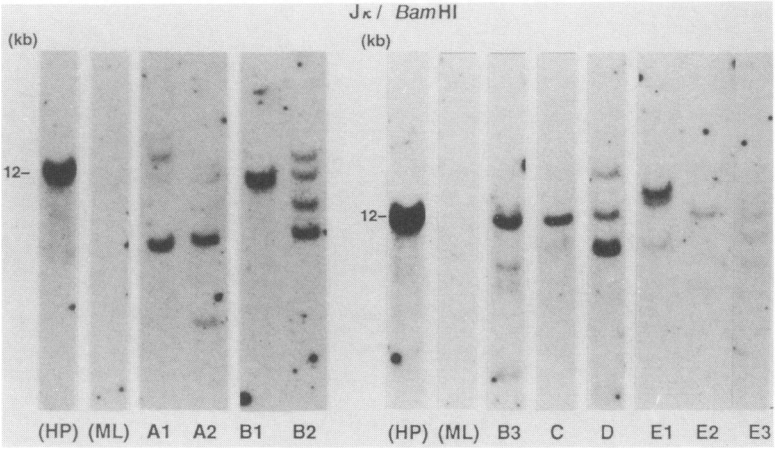

Figure 2. *Southern blot hybridization analysis of human lymphoproliferative disease induced in the* scid *mice. The DNAs were digested with* Bam*HI and examined with a human Jκ probe. See the legend to Figure 1 for detail.*

rearrangements of J_H were detected in 8 of 10 LPDs, and five of them showed three or more non-germline bands in each DNA sample. A *Bam*HI digest hybridized with J_H probe was shown in Figure 1. The pattern of Jκ rearrangements was similar to that of J_H rearrangements, except for mouse E2, in which non-germline bands were not detected (Figure 2). Cλ was clonally rearranged in three LPDs (A1, D, and E2; Figure 3).

EBV genome was examined in a *Bam*HI digest hybridized with a *Xho*I 1.9 fragment. Because of the variability in the numbers of copies of EBV DNA in each sample, filters were exposed to films at various duration (4 to 48 hours), and the genome was detected in all of the 10 LPDs. In a 24-hour exposure (Figure 4), a single band with a higher molecular size corresponding to a circular form was found in two LPDs (B1 and E1), which were characterized to be monoclonal. LPD in one mouse (B3), with three distinct bands of higher molecular sizes was characterized to be oligoclonal. In two other LPDs (C and E3), two to three distinct bands together with multiple indistinct bands with higher molecular sizes were identified and these were also considered oligoclonal. Evaluation of clonality of the remaining five LPDs was difficult because of strong hybridization, but a 6-hour exposure (Figure 5) disclosed that LPDs in four mice (A1, A2, B2, and D) were oligoclonal. LPD in mouse E2 was characterized to be monoclonal even though several bands of lower molecular weights were also present (Figures 4 and 5).

Analysis of T cell receptor β-chain gene was performed in *Bam*HI, *Eco*RI, *Hin*dIII, and *Pst*I digests. Clonal rearrangements of Cβ2 was not identified in any of the LPDs, although the probe showed cross-hybridization with mouse DNA, giving germline bands of mouse origin which were different from those of human origin. Analysis of the c-*myc* proto-oncogene was performed following *Bam*HI and *Eco*RI digestion. A c-*myc* probe showed hybridization with mouse DNA as well as human DNA, but no rearrangements were detected. No rearrangements of the *bcl-2* was identified in *Bam*HI, *Eco*RI, *Hin*dIII,

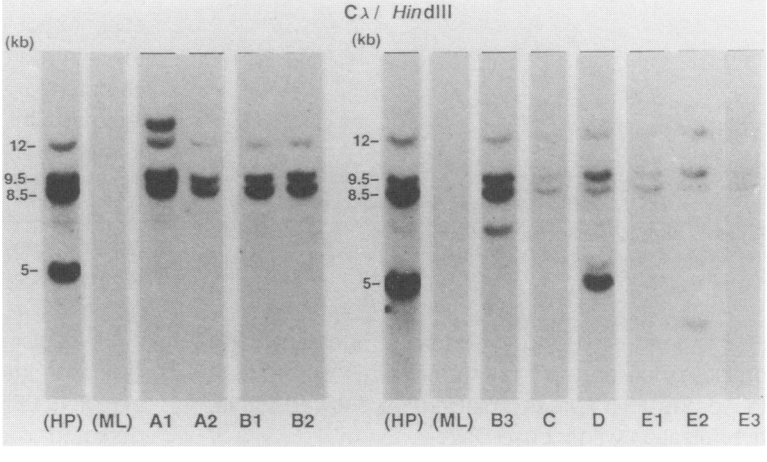

Figure 3. *Southern blot hybridization analysis of human lymphoproliferative disease induced in the* scid *mice. The DNAs were digested with HindIII and examined with a human Cλ probe. See the legend to Figure 1 for detail.*

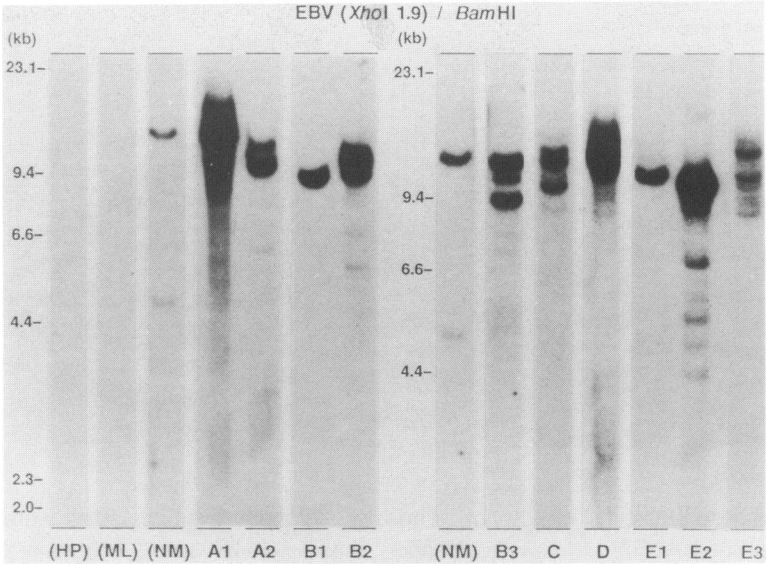

Figure 4. *Southern blot hybridization analysis of EBV termini in human lymphoproliferative disease in the* scid *mice. DNA extracted from the Namalwa cell line was used as a positive control. The DNAs were digested with* BamHI *and the filters hybridized to a probe specific for the terminal sequence of linear EBV genome (*XhoI *1.9) were exposed to films for 24 hours. Approximate molecular sizes based on the* HindIII*-digested bacteriophage λ DNA are shown in kilobases. See the legend to Figure 1 for detail.*

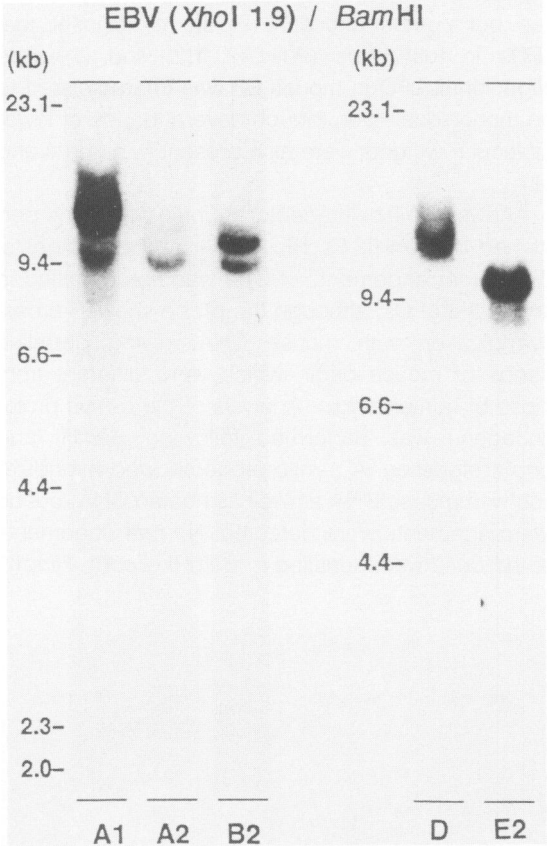

Figure 5. *The filters hybridized similarly to those in Figure 4 were exposed for 6 hours. See the legend to Figure 4 for detail.*

and *SstI* digests using three DNA probes spanning major, minor, and 5′ break point regions, although the probe to 5′ break point region showed cross-hybridization with mouse DNA.

Discussion

In the present study, clonality of EBV-induced human LPD in *scid* mice were analyzed by several indepen dent methods to examine whether this system can be an *in vivo* model of PTLPD. Molecular genetic analyses disclosed that the development of oligoclonal tumors was not unusual in this system as in the PTLPDs.

Analysis of cellular Ig light chains, which is perhaps the most widely accepted and applied method to determine clonality of B cell neoplasia in immunocompetent individuals, was not effective in at least two mice (B3 and D). The problem probably resulted from the inability to detect clonality of tumors consisting of two or more clones with different Ig light chain types. In contrast, immunofixation electrophoresis of the serum could indicate the presence of such tumors and appeared to be suitable for screening for the development of LPDs, since clonal Ig proteins were detected in all of the 10 mice. However, it should be noted that this method measures the total burden of all of the LPDs in a mouse that secretes Ig. Actually, some of the mice had multiple lesions, although molecular analyses could be performed in only a single lesion of each mouse due to the small size of the lesion. Evaluation of the results obtained from patients with PTLPD may be further complicated, since in some bone marrow transplant recipients, clonal serum proteins are thought not to be directly related to LPD.[32,33]

Molecular genetic analysis of the LPD for Ig genes disclosed that clonal B cell populations were present in 8 of 10 LPDs and multiple rearranged bands were detected in each DNA sample. Although there are

several possible interpretations to explain these results in DNA sample containing a single B cell clone,[34] the absence of cytogenetic abnormalities involving chromosomes 2, 14, and 22 as reported in our previous study,[17] along with marked differences in the intensity of rearranged bands in several of our LPDs, favors the presence of multiple clones.[34] Since post-rearrangement alterations such as isotype switching, somatic mutations, and deletion can occur in the Ig heavy chain gene,[35–37] the presence of three or more rearranged J_H bands does not always imply the development of multiple clonal proliferation. However, at least three LPDs (A2, B2, and B3), which showed three or more rearranged bands of Ig κ chain gene, were considered to be oligoclonal, because the occurrence of such post-rearrangement alterations is an extremely rare event in Ig light chain genes.[36,37] We examined a putative oncogene, *bcl-2* because the gene can serve as a more stable clonal marker,[35] but rearrangement of the locus was not detected in any lesion. This negative result supports our postulate based on the phenotypic findings that the LPDs in *scid* mice are restricted to the post-follicular center cell stage of B cell differentiation (18).

Clonality of LPDs was also determined based on the structure of the circularized genomic termini of EBV. Because of considerable inter-case variations in the intensity of hybridization signal, films of hybridized filters were exposed at various durations. According to published criteria,[31] all of the 10 LPDs were characterized to be either monoclonal or oligoclonal. However, the presence of multiple less dominant clones was suggested in at least two LPDs (C and E3), since several weak, but localized, bands of higher molecular weights in addition to two or three intense bands were seen. In addition to circular forms of EBV DNA, a few fragments with intermediate molecular sizes which may be replicative intermediates[31] were seen in three LPDs (A2, B2, and B3) and linear forms were present in one LPD (E2). This finding has also been described in some PTLPDs.[10,31]

When these results were compared with those of Ig gene analysis, discordant features were found in at least two LPDs (C and E3). Although there are several possible explanations for the discordant features,[9,39] the difference in sensitivity inherent in the two independent methods appears to be one of the major factors. It is possible that a lesion composed of multiple B cell clones with a spectrum of different clonal sizes can be characterized as polyclonal, oligoclonal, or monoclonal depending on sensitivity of the method employed.

Our data indicate heterogeneity of clonality in the LPDs. This feature is similar to that reported in PTLPDs.[8–10] However, rearrangement of c-*myc* oncogene could not be detected in our system, but has been described in some PTLPDs[9]; these latter tumors usually had a monomorphic large-cell morphology, were clonal by Ig gene and EBV DNA analyses, and were progressive despite the reduction of immunosuppressive therapy, indicating the occurrence of the c-*myc* rearrangement at the later phase in the possible multi-step process of EBV-induced LPD. Perhaps, this difference can be explained based on the variable duration and the cellular kinetics of the LPD in patients compared with mice. The incubation period for the LPD in mice is generally only about 2 to 3 months, whereas that of the PTLPD is longer, hereby allowing time for genetic errors involving c-*myc* to occur.

In conclusion, the *scid* mice system is a useful model for understanding the early phases in the pathogenesis of PTLPD in terms of clonality as well as histopathological and phenotypic features.[18] Our finding of trisomy 11 in a LPD lesion in a mouse[17] and this finding commonly in PTLPD[7] as well supports our view. This model can also be used to investigate the relationship between clonality and a variety of hematolymphoid malignancies.

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