

# The Frequency of B- and T-Cell Gene Rearrangements and Epstein-Barr Virus in T-Cell Lymphomas

## *A Comparison Between Angioimmunoblastic T-Cell Lymphoma and Peripheral T-Cell Lymphoma, Unspecified With and Without Associated B-Cell Proliferations*

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**We report on a series of 58 cases of angioimmunoblastic T-cell lymphoma (AILT) and 59 cases of peripheral T-cell lymphoma, unspecified (PTCL-NOS). Subsets of cases from both diagnostic groups were complicated by associated B-cell proliferations, and we performed B- and T-cell clonality studies and *in situ* hybridization for Epstein-Barr virus (EBV) to investigate the relationship between B-cell proliferation, B-cell clonality, and EBV. Using multiplex polymerase chain reaction assays based on the BIOMED-2 collaborative study, we detected *TCRγ* T-cell clones in 78 and 81% of AILT and PTCL-NOS cases, respectively, and *IGH* B-cell clones in 34 and 35% of AILT and PTCL-NOS cases, respectively. The majority of cases contained EBV-positive cells, including 50% of AILT and 57% of PTCL-NOS cases, and cases with B-cell proliferations were more often EBV-positive. Although a relatively high rate of B-cell clonality has been shown for AILT, our findings for PTCL-NOS differ from previous reports in that B-cell clonality was relatively frequent. Overall, a positive B-cell clone correlated, in part, with the presence of a B-cell proliferation but not with EBV. Our findings demonstrate that B-cell clonality is a common finding in AILT and PTCL-NOS, and its presence should not negate the diagnosis established by morphologic, immunophenotypic, and clinical findings. (*J Mol Diagn* 2006, 8:466–475; DOI: 10.2353/jmoldx.2006.060016)**

histologic, immunophenotypic, and genetic findings.<sup>1</sup> However, the diagnosis is often difficult for pathologists because PTCLs are uncommon, and the histologic findings can be nonspecific and can overlap with reactive conditions. Perhaps the most challenging aspect is the lack of good immunophenotypic markers to establish clonality for T-cell lineage neoplasms, and pathologists often rely on DNA-based studies for evidence of clonality.

Given the difficulty in diagnosing PTCL, pathologists often perform both B- and T-cell clonality studies to clarify lineage and provide support for malignancy. Currently, the most commonly used method is the polymerase chain reaction (PCR), which has largely replaced Southern blot-based clonality assays. However, there are several caveats to this approach. In particular, studies evaluating large numbers of PTCLs by PCR show that simultaneous B- and T-cell clones occur relatively often (9 to 16%).<sup>2–6</sup> These cases, with both B- and T-cell clones, present a diagnostic dilemma for the pathologist and treating oncologist.

There are numerous possible explanations for the detection of two clones in some cases, including those that are technical in nature, so-called lineage infidelity of a single clone, and the presence of two different clones in a sample.<sup>7</sup> The phenomenon referred to as lineage infidelity, in this context, is the result of recombination of both T-cell receptor (*TCR*) and immunoglobulin (*IG*) genes in the same clone.<sup>7</sup> This finding is more frequently seen in immature hematolymphoid neoplasms and is uncommon in mature B- and T-cell non-Hodgkin lymphomas.<sup>7–10</sup> In recent years, cases of PTCL with associated B-cell proliferations have been described. Many of these cases are positive for both B- and T-cell clones, and in our series of peripheral T-cell lymphoma, unspecified (PTCL-NOS), a subset of B-cell proliferations were asso-

Peripheral T-cell lymphoma (PTCL) is an uncommon malignancy, accounting for less than 10% of non-Hodgkin lymphomas worldwide. By current World Health Organization criteria, PTCLs are subclassified based on clinical,

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ciated with Epstein-Barr virus (EBV).<sup>11</sup> Others have reported similar B-cell proliferations in PCTL-NOS as well as angioimmunoblastic T-cell lymphoma (AILT), and many of these cases are EBV-positive and exhibit B-cell clones.<sup>12-14</sup>

We hypothesized that the relative frequency of B-cell clones in PTCL may be because of associated B-cell proliferations and/or EBV. To address this issue, we performed PCR-based B- and T-cell clonality studies on a total of 117 cases of PTCL. The series is composed of 58 cases of AILT, 12 of which show an associated B-cell proliferation, and 59 cases of PTCL-NOS, 11 of which show an associated B-cell proliferation. In addition, we performed *in situ* hybridization for EBV to characterize the relationship between the B-cell proliferation, EBV, and underlying T-cell lymphoma.

In this article, we describe the results of these studies, which include a high overall frequency of B-cell clones in both AILT (34%) and PTCL-NOS (35%) that correlates, in part, with an associated B-cell proliferation but not with EBV. Interestingly, cases without demonstrable B-cell proliferations also exhibited B-cell clonality, although with less frequency than cases with B-cell proliferations. This finding suggests that additional factors may contribute to B-cell clonality.

## Materials and Methods

### Cases

Fifty-eight cases of AILT and fifty-nine cases of PTCL-NOS were selected from the Laboratory of Hematopathology, Stanford University Medical Center, Stanford, CA. The cases were received between January 1, 2000, and April 4, 2005, and a diagnosis was rendered based on criteria established by the World Health Organization.<sup>1</sup> AILT and PTCL-NOS cases were further subclassified as to whether they showed an associated-B-cell proliferation. Briefly, a B-cell proliferation was defined as a readily identifiable infiltrate of B cells complicating a PTCL as described by Higgins and colleagues.<sup>11</sup> Cases were selected based on availability of paraffin-embedded tissue and/or residual DNA remaining from clonality studies performed for the original diagnosis. Use of tissue for this study was approved by the Stanford University Panel on Medical Human Subjects [protocol ID 79034; institutional review board number 348 (panel 1)].

### Histology and Immunohistochemistry

Histologic sections were prepared from formalin-fixed, paraffin-embedded tissue by cutting 3- to 4- $\mu$ m-thick sections and staining with hematoxylin and eosin. Sections were stained for immunohistochemistry on a Ventana BenchMark instrument (Ventana Medical Systems, Tucson, AZ) using the biotin-avidin technique in which diaminobenzidine was used as a chromogen.<sup>15</sup> All cases were stained with at least one B- and one T-cell marker. The following monoclonal antibodies were used: 4C7 (anti-CD5; Vision Bio-System, Norwell, MA), 56C6 (anti-

CD10; Vision Bio-System), L26 (anti-CD20; DAKO, Carpinteria, CA), IF8 (anti-CD21; DAKO), IB12 (anti-CD23; Vision Bio-System), JCV117 (anti-CD79a; DAKO), and B-B4 (anti-CD138; Serotec, Raleigh, NC). Polyclonal antibodies directed against the following antigens were used: CD3 (Cell Marque, Hot Springs, AR) and  $\kappa$  and  $\lambda$  immunoglobulin light chains (DAKO). Antigen retrieval was achieved through automated heat pretreatment for 4C7, 56C6, L26, IB12, JCV117, B-B4, and antibody against CD3 (Ventana Medical Systems). Antigen retrieval was achieved through automated protease pretreatment for IF8 and antibodies against immunoglobulin light chains (Ventana Medical Systems).

### Isolation of DNA

DNA was obtained from formalin-fixed, paraffin-embedded tissue blocks by cutting four to eight 20- $\mu$ m-thick sections followed by deparaffinization by extracting three times in 1.0 ml of either xylene or HistoClear (National Diagnostics, Atlanta, GA). The extracted tissue was washed two times in 1.0 ml of 100% ethanol and then dried at 65°C. The tissue was resuspended in 2 vol (30 to 250  $\mu$ l) of a mixture of 200 mmol/L potassium chloride, 40 mmol/L Tris [Tris(hydroxymethyl)aminomethane]-hydrochloride (pH 8.5), 0.1% sodium dodecyl sulfate, and 0.6 mg/ml proteinase K and incubated at 55 to 65°C overnight. The sample was then boiled for 8 minutes and centrifuged at 15,000  $\times g$  for 10 minutes, and the DNA-containing supernatant was collected. To evaluate the yield of DNA, a 5- $\mu$ l aliquot of supernatant was separated by electrophoresis on a 0.8% agarose gel (Invitrogen, Carlsbad, CA), stained with ethidium bromide, and visualized by ultraviolet illumination. Based on an estimate of DNA concentration, the crude extract was diluted with an appropriate volume of reagent-grade water (Teknova, Hollister, CA) and used directly in a PCR. Typically, a 1:10 to 1:20 dilution was prepared, and 5  $\mu$ l was used for the PCR.

### B-Cell and T-Cell Clonality Studies

Cases were evaluated for B- and T-cell clonality using two commercially available PCR-based kits that detect clonal rearrangements in *IGH* and *TCR $\gamma$* , respectively (InVivoScribe Technologies, San Diego, CA). These multiplex PCRs are based on a European collaborative study (BIOMED-2 Concerted Action).<sup>16</sup> The PCRs were performed according to the manufacturers' protocols. Briefly, a 5- $\mu$ l aliquot of DNA sample was added to 45  $\mu$ l of each reaction mixture and 1.25 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA). Amplification was performed in a Perkin-Elmer 9700 thermocycler (Perkin-Elmer Applied Biosystems, Foster City, CA) by initially heating at 95°C for 7 minutes, followed by 35 cycles of 95°C for 45 seconds, 60°C for 45 seconds, and 72°C for 90 seconds. The final step was incubation at 72°C for 10 minutes.

After amplification, 1  $\mu$ l of PCR product was added to 10  $\mu$ l of HI-Deionized Formamide (Applied Biosystems)

and 1  $\mu$ l of ROX-250 internal size standard (Applied Biosystems). The mixture was then denatured at 95°C for 5 minutes, chilled on ice for 5 minutes, and resolved by capillary electrophoresis on an ABI 3100 instrument using performance-optimized polymer-4 (POP-4; Applied Biosystems). The data were stored electronically and analyzed using GeneScan analysis software (Applied Biosystems). Printed electropherograms were reviewed independently by two separate pathologists (B.T.T. and D.A.A.) who were blinded to the identity of specimens. Criteria for assigning a clone were similar to those used for clinical specimens at the Stanford molecular pathology laboratory. Briefly, a clone was required to yield one or two distinct peaks within the expected size range for a given primer set. In some cases, a polyclonal background was present in addition to the distinct peak(s). To meet criteria for a clone, the height of at least one distinct peak had to exceed that of the polyclonal background by at least twofold. When a primer set yielded three or more distinct peaks, the results were classified as oligoclonal, regardless of whether a polyclonal background was present or absent. Cases with a clonal peak demonstrated by one or multiple primer sets were scored as positive. Cases with only polyclonal and/or oligoclonal results were scored as negative.

Control specimens included clonal DNA isolated from cell lines (InVivoScribe), polyclonal DNA isolated from human tonsil specimens (InVivoScribe), a sensitivity control composed of clonal DNA diluted in polyclonal DNA, and a blank composed of reagent-grade water (Teknova). Multiple polyclonal DNA specimens, each isolated from different patients (InVivoScribe), consistently yielded polyclonal signals and no clonal signals. For a run of clonality studies to be accepted into the data set, a positive clone had to be detectable from the sensitivity control, which was prepared at 2 or 3% (expressed as  $\mu$ g of clonal DNA/ $\mu$ g of polyclonal DNA) for *TCR $\gamma$*  or *IGH* assays, respectively. Individual DNA specimens had to yield detectable products of at least 300 nucleotides from the control amplification tube, which contained primers to amplify *HLA-DQ*. Samples yielding inadequate amplification were purified using a QIAamp DNA blood mini kit (Qiagen, Valencia, CA), and the purified DNA typically yielded adequate amplification. In our validation of the InVivoScribe *TCR $\gamma$*  and *IGH* kits, we were able to detect clones in 80 to 90% of known B- and T-cell neoplasms, and amplification of the provided polyclonal control DNA (InVivoScribe) or DNA isolated from paraffin-embedded reactive tonsil specimens in our laboratory yielded only polyclonal results.

### Sequencing of PCR Products

Eight cases yielding clonal V <sub>$\gamma$</sub> 10 rearrangements by GeneScan analysis were reamplified from the original DNA sample using a *TCR $\gamma$*  clonality kit that uses primers of identical sequence to those used for GeneScan analysis, but the primers lack a 5'-fluorescent label, which would otherwise interfere with subcloning (InVivoScribe). The amplification products were separated by electro-

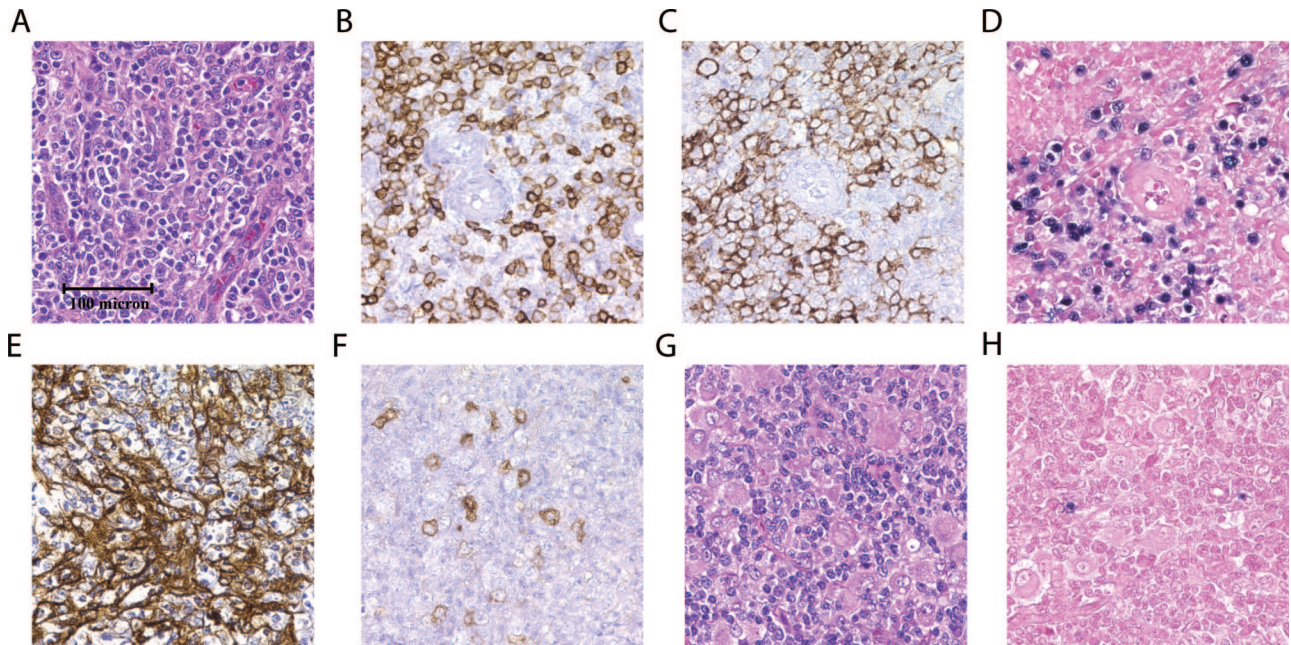
phoresis on a 2% low melting point agarose gel (Invitrogen), visualized by ethidium bromide staining, excised from the gel, and purified using a QIAquick gel extraction kit (Qiagen). The purified products were ligated to a plasmid vector using a TOPO TA cloning kit for sequencing (Invitrogen), and the ligation products were transformed into *Escherichia coli*. From each transformation, three bacterial colonies were picked, bacterial cultures were grown, and plasmid DNA was prepared using a PureLink quick plasmid miniprep kit (Invitrogen). The DNA was sequenced using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) with a bacteriophage T7 primer (Invitrogen). The resulting sequences were compared to those of V <sub>$\gamma$</sub> 10 listed on the international ImMunoGeneTics website (<http://imgt.cines.fr/as> accessed on January 23, 2006).

### In Situ Hybridization for EBV

EBV-encoded small RNA (EBER) was detected from 3- to 4- $\mu$ m-thick, formalin-fixed, paraffin-embedded tissue sections by *in situ* hybridization using a Ventana Benchmark instrument running a standardized program incorporating deparaffinization, hybridization to the Inform EBER probe cocktail, and staining with ISH iVIEW nitro blue tetrazolium (Ventana Medical Systems). Cases were evaluated for EBV-labeled cells under visible light microscopy, and the number of cells with blue-colored nuclei was visually estimated at 0, <1, 1 to 10, >10 to 100, or >100 per medium-power field using a  $\times$ 15 ocular and  $\times$ 20 objective lens on an Olympus BX50 microscope (Olympus, Tokyo, Japan). The minimum number of labeled nuclei required for a nonzero score was three per section, which is more than that typically seen in a lymph node or tonsil removed from a healthy individual without evidence of immunodeficiency (B.T.T. and R.A.W., unpublished observations). In approximately half of the cases studied for EBV (depending on availability of sample), a control hybridization was performed with an oligodeoxythymidine probe to detect polyadenylated mRNA. In addition, an external positive control consisting of an EBV-positive malignancy (NK/T-cell lymphoma or nasopharyngeal carcinoma) was run with each set of hybridizations.

### Statistical Analysis

The data were analyzed using the software program JMP-IN version 5.1 (SAS Institute, Cary, NC). Age was entered as a continuous numerical parameter, and all other data were entered as categorical parameters. Differences in age between categorically defined groups were evaluated by *t*-test and *F*-test, and the lower *P* value was recorded. Correlations between categorical parameters were evaluated by Pearson's  $\chi^2$  test and likelihood ratio test, and the lower *P* value was recorded. Only *P* values <0.15 were reported, except for some correlations with EBV, which were based on a lower number of cases.



**Figure 1.** Histology of representative AILT and PTCL-NOS cases. All panels shown at identical magnification. **A–F:** AILT with EBV-associated large B-cell proliferation stained with H&E (**A**), immunohistochemistry with anti-CD5 (**B**), anti-CD20 (**C**), *in situ* hybridization for EBV (**D**), anti-CD21 (**E**), or anti-CD10 (**F**). **G and H:** PTCL-NOS stained with H&E (**G**) or examined by *in situ* hybridization for EBV (**H**). Images were captured with a Nikon Eclipse E1000 microscope (Nikon, Tokyo, Japan) using a  $\times 20/0.75$  objective lens (Nikon) and a Spot CCD camera (Diagnostic Instruments, McHenry, IL) with Spot software version 4.0.5 for image acquisition.

## Results

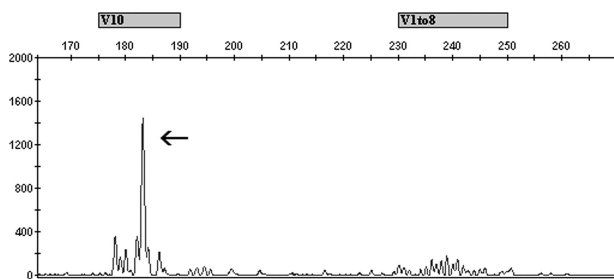
### Cases

We collected 58 cases of AILT and 59 cases of PTCL-NOS from the Stanford hematopathology laboratory. Each case corresponded to a different patient, and all patients were adults. The mean ages of patients with AILT and PTCL-NOS were 64 and 62 years, respectively. Men slightly outnumbered women, accounting for 60 and 61% of patients with AILT and PTCL-NOS, respectively. Comparison of the AILT versus PTCL-NOS groups yielded no statistically significant difference in patient age or sex. The majority of specimens were from lymph nodes, and cases with involvement limited to skin, central nervous system, bone marrow, or body fluid/cavity were excluded. There were a total of 11 extranodal specimens, including five lung, two liver, one mediastinum, one spleen, one stomach, and one subcutaneous tissue specimen. The subcutaneous specimen was in the AILT group, and other extranodal specimens were in the PTCL-NOS group.

All cases of AILT showed the histologic features of an atypical mixed infiltrate within a background of prominent arborizing vessels. In Figure 1, A–F, representative histologic, immunohistochemical, and EBV *in situ* studies are shown for a case of AILT with an EBV-associated large B-cell proliferation. For 56 of the 58 total AILT cases, a network of extrafollicular dendritic cells was demonstrated by immunohistochemical staining for CD21 and/or CD23. In the remaining two cases, the diagnosis was rendered based on characteristic morphology and staining demonstrating T-cell lineage. A stain for CD10 was performed in 33 cases, demonstrating scattered

CD10-positive cells unassociated with follicles in 20 cases, negative results in seven cases, and equivocal results in six cases. Eleven cases of AILT were complicated by an associated proliferation of B cells with seven composed of large B cells and four composed of small B cells. Two large B-cell proliferations and one small B-cell proliferation were extensive and met criteria for B-cell lymphoma based on the sheet-like architecture of the B-cell proliferation. One additional case showed an associated proliferation of plasma cells and was considered in the subgroup of cases with B-cell proliferations. The B-lineage proliferations were demonstrated by stains for various antigens including CD20, CD79a, CD138, and  $\kappa$  and  $\lambda$  immunoglobulin light chains.

The PTCL-NOS group was composed of cases that met criteria for peripheral T-cell lymphoma, unspecified, by the current World Health Organization classification.<sup>1</sup> These criteria are exclusionary in nature, and cases with morphologic, immunophenotypic, or clinical features diagnostic or suggestive of specific subtypes of PTCL were excluded from the PTCL-NOS group. In Figure 1, G and H, representative histologic and EBV *in situ* studies are shown for a case of PTCL-NOS. For 21 of the 59 total PTCL-NOS cases, an immunohistochemical stain for CD21 was performed and was negative in all 21 cases. A stain for CD10 was performed in 12 cases, demonstrating scattered CD10-positive cells in three cases, negative results in five cases, and equivocal results in four cases. Eleven cases of PTCL-NOS were complicated by an associated proliferation of B cells with seven composed of large B cells, three composed of small B cells, and one composed of mixed small and large B cells. One large B-cell proliferation and one small B-cell proliferation were classified as B-cell lymphoma based on



**Figure 2.** Electropherogram of PCR for *TCRγ* rearrangements. The *x* axis indicates DNA length in nucleotides. The *y* axis indicates relative fluorescence units. Shown is electrophoresis of products from one multiplex tube, which contains primers to amplify *Vγ*1-8 and/or *Vγ*10 rearrangements, with expected product sizes above the *x* axis. The case was interpreted as positive with a clonal peak detected by *Vγ*10 primers (arrow) and polyclonal signals detected by *Vγ*1-8 primers.

the sheet-like architecture of the B-cell proliferation. The B-cell proliferations were demonstrated by stains for various antigens including CD20, CD79a, and  $\kappa$  and  $\lambda$  immunoglobulin light chains.

### T-Cell Clonality Studies

We performed T-cell clonality studies using a multiplex PCR developed in a European BIOMED-2 collaborative study that detects clonal rearrangements in the T-cell receptor  $\gamma$  chain gene (*TCRγ*).<sup>16</sup> Data from a representative *TCRγ* clonality study are shown in Figure 2, and the results for all cases are summarized in Table 1. The proportion of cases with a positive T-cell clone was 78 and 81% for total AILT and total PTCL-NOS cases, respectively. These figures included cases with associated B-cell proliferations, and when considered separately, 75% of AILT cases with B-cell proliferations and 73% of PTCL-NOS cases with B-cell proliferations were positive for T-cell clonality. There was no statistically significant correlation between T-cell clonality and diagnostic group, presence or absence of a B-cell proliferation, or the results of B-cell clonality or EBV studies. Interestingly, a positive T-cell clone correlated with older age ( $P = 0.045$ ) and male sex ( $P = 0.009$ ).

Also listed in Table 1 are the frequencies of clonal detection for individual primer sets, which targeted rearrangements between specific variable genes (*Vγ*) and the joining segment (*Jγ*) of *TCRγ*. For a given case, clonality was often demonstrable by more than one

**Table 2.** T-Cell Clonality by PCR for *TCRγ* by Specific Variable Gene Rearrangement

Rearrangement(s)	Number of cases		
	AILT	PTCL-NOS	Total (%)
<i>Vγ</i> 1-8	8	12	20 (17.1)
<i>Vγ</i> 1-8, <i>Vγ</i> 10	9	7	16 (13.7)
<i>Vγ</i> 1-8, <i>Vγ</i> 9, <i>Vγ</i> 10	7	7	14 (12.0)
<i>Vγ</i> 1-8, <i>Vγ</i> 9	2	8	10 (8.5)
<i>Vγ</i> 1-8, <i>Vγ</i> 9, <i>Vγ</i> 10, <i>Vγ</i> 11	2	1	3 (2.6)
<i>Vγ</i> 1-8, <i>Vγ</i> 10, <i>Vγ</i> 11	0	2	2 (1.7)
<i>Vγ</i> 1-8, <i>Vγ</i> 11	1	0	1 (0.9)
<i>Vγ</i> 10	3	3	6 (5.1)
<i>Vγ</i> 9	2	0	2 (1.7)
<i>Vγ</i> 11	1	0	1 (0.9)
<i>Vγ</i> 9, <i>Vγ</i> 10	7	7	14 (12.0)
<i>Vγ</i> 9, <i>Vγ</i> 11	0	1	1 (0.9)
Negative	16	11	27 (23.1)

primer set, as summarized in Table 2. There was no statistically significant difference in variable gene distribution between AILT and PTCL-NOS cases. Overall, rearrangements in *Vγ*1-8 were most common, detected in 50% and 63% of total AILT and PTCL-NOS cases, respectively. In addition, rearrangements in *Vγ*10 were observed frequently, occurring in 48% and 46% of total AILT and PTCL-NOS cases, respectively. Of the 93 total T-cell clones, 66 (71%) were detected by *Vγ*1-8 primers and 55 (59%) were detected by *Vγ*10 primers. The majority of *Vγ*10 rearrangements were seen in combination with *Vγ*1-8 and/or *Vγ*9 rearrangements, and cases with only *Vγ*10 rearrangements were uncommon, occurring in three AILT and three PTCL-NOS cases. Clonal rearrangements involving *Vγ*10 are reportedly uncommon in PTCL.<sup>17-19</sup> Therefore, we sequenced eight PCR products corresponding to clonal *Vγ*10 rearrangements to confirm that the findings were not the result of nonspecific amplification or amplification of non-*Vγ*10 rearrangements by primers in the multiplex PCR. In all eight cases, the resulting sequence matched that of the *Vγ*10 segment.

### B-Cell Clonality Studies

We performed B-cell clonality studies using a BIOMED-2-derived multiplex PCR that detects clonal rearrange-

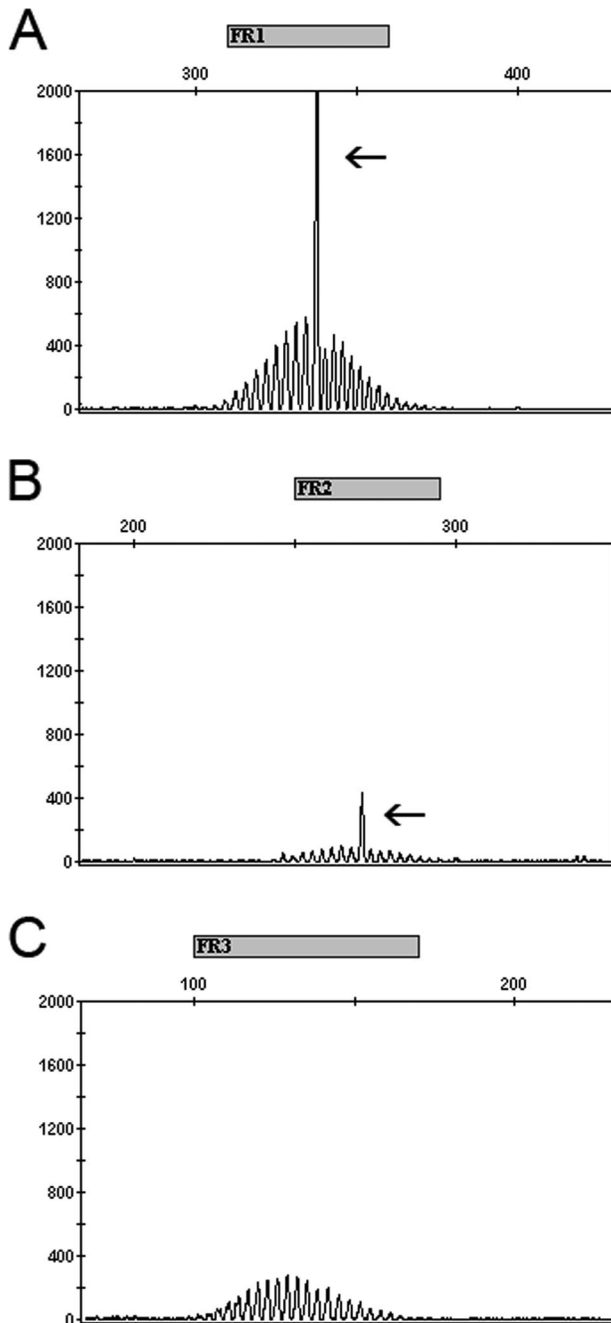
**Table 1.** T-Cell Clonality by PCR for *TCRγ* by Diagnosis

Diagnosis	Number of cases studied	Number of positive cases (%)	Number of positive cases detected by specific primer sets (%)				
			<i>Vγ</i> 1-8	<i>Vγ</i> 9	<i>Vγ</i> 10	<i>Vγ</i> 11	<i>Vγ</i> 9/11*
AILT							
Without B-cell proliferation	46	36 (78)	23 (50)	18 (39)	22 (48)	4 (9)	6 (13)
With B-cell proliferation <sup>†</sup>	12	9 (75)	6 (50)	2 (17)	6 (50)	0 (0)	2 (17)
Total	58	45 (78)	29 (50)	20 (34)	28 (48)	4 (7)	8 (14)
PTCL-NOS							
Without B-cell proliferation <sup>‡</sup>	48	40 (83)	29 (60)	20 (42)	21 (44)	4 (8)	2 (4)
With B-cell proliferation	11	8 (73)	8 (73)	4 (36)	6 (55)	0 (0)	1 (9)
Total	59	48 (81)	37 (63)	24 (41)	27 (46)	4 (7)	3 (5)

\**Vγ*9/11 indicates a clonal PCR product within a range of sizes that is indeterminate between *Vγ*9 and *Vγ*11.

<sup>†</sup>One case yielded oligoclonal *Vγ*10 and clonal *Vγ*9/11 products and was scored as positive.

<sup>‡</sup>One case yielded oligoclonal *Vγ*1-8 products only and was scored as negative.



**Figure 3.** Electropherograms of PCRs for *IGH* rearrangements. The *x* axis indicates DNA length in nucleotides. The *y* axis indicates relative fluorescence units. Shown are electrophoresis of products from three multiplex tubes, which contain primers targeting the FR1 (A), FR2 (B), and FR3 regions (C), with expected product sizes above the *x* axis. The case was interpreted as positive with clonal peaks detected by FR1 and FR2 primers (arrows) and polyclonal signals detected by FR3 primers.

ments in the immunoglobulin heavy chain gene (*IGH*).<sup>16</sup> Data from a representative *IGH* clonality study are shown in Figure 3, and the results for all cases are summarized in Table 3. The proportion of cases with a positive B-cell clone was 34 and 35% for total AILT and total PTCL-NOS cases, respectively. Of the 39 total cases with B-cell clones, 34 were positive for T-cell clonality and 5 were negative for T-cell clonality. Cases with associated B-cell

proliferations showed a higher frequency of B-cell clonality than those without B-cell proliferations. For AILT cases, the frequencies of B-cell clonality were 50% for those with B-cell proliferations and 30% for those without B-cell proliferations, and for PTCL-NOS cases, the frequencies were 45% for those with B-cell proliferations and 33% for those without B-cell proliferations. When cases from both diagnostic groups were considered, B-cell clonality correlated, in part, with the presence of a B-cell proliferation ( $P = 0.13$ ). There was no correlation between B-cell clonality and T-cell clonality or EBV.

Also listed in Table 3 are the frequencies of clonal detection for different primer sets, amplifying either a complete  $V_H-D_H-J_H$  rearrangement in the case of FR primers or an incomplete  $D_H-J_H$  rearrangement in the case of  $D_H$  primers. Cases with both a complete ( $V_H-D_H-J_H$ ) rearrangement and incomplete ( $D_H-J_H$ ) rearrangement can be explained by a productive rearrangement on one *IGH* allele and a nonproductive rearrangement on the other allele. Clonality was typically demonstrable by multiple primer sets, as summarized in Table 4. Among FR primers, the FR1 and FR3 primers were most sensitive, detecting 18 and 21 of the 39 total B-cell clones, respectively. The FR2 primers detected only nine clones, and the low sensitivity is likely technical in nature because these primers typically yielded weaker PCR products than the FR1 and FR3 primers in our laboratory (Figure 3). Among  $D_H$  primers, the  $D_H$ 1-6 and  $D_H$ 7 primers detected 17 and 15 clones, respectively. Most clones detected by  $D_H$  primers were also detected by FR primers. However, 14 of the 39 total B-cell clones were detected by  $D_H$  primers only, consistent with the presence of an unamplifiable  $V_H-D_H-J_H$  rearrangement on one allele and an amplifiable  $D_H-J_H$  rearrangement on the other allele. Of these 14 cases, 9 of 11 were EBV-positive, and for the remaining three cases, tissue was not readily available for *in situ* studies. Clonality of *IGH* demonstrable only by  $D_H$  primers correlated with EBV ( $P = 0.015$ ) but not with diagnosis, B-cell proliferation, or T-cell clonality.

### EBV Studies

We performed *in situ* hybridization for EBV EBER on approximately half of the cases. Representative *in situ* hybridizations for an AILT with an EBV-associated B-cell proliferation and a PTCL-NOS case are shown (Figure 1, D and H, respectively), and the results for all cases studied are summarized in Table 5. A positive result, which was defined as more than three EBV-labeled cells per section, was observed in 50% and 57% of total AILT and total PTCL-NOS cases, respectively. Cases with B-cell proliferations were more often EBV-positive and tended to show greater numbers of EBV-labeled cells. Among AILT cases, 45% without B-cell proliferations were EBV-positive, whereas 63% with B-cell proliferations were EBV-positive. For PTCL-NOS cases, 52% without B-cell proliferations were EBV-positive, and 75% with B-cell proliferations were EBV-positive. Overall, a positive EBV study correlated, in part, with the presence of a B-cell proliferation ( $P = 0.20$ ) and male sex ( $P = 0.12$ ).

**Table 3.** B-Cell Clonality by PCR for *IGH* by Diagnosis

Diagnosis	Number of cases studied	Number of positive cases (%)	Number of positive cases detected by specific primer sets (%)					
			FR1	FR2	FR3	D <sub>H</sub> 1-6	D <sub>H</sub> 7	
AILT								
Without B-cell proliferation <sup>†</sup>	44	13 (30)	3 (7)	2 (5)	6 (14)	4 (9)	6 (14)	
With B-cell proliferation	12	6 (50)	5 (42)	3 (25)	4 (33)	1 (8)	2 (17)	
Total	56*	19 (34)	8 (14)	5 (9)	10 (18)	5 (9)	8 (14)	
PTCL-NOS								
Without B-cell proliferation <sup>†</sup>	46	15 (33)	8 (17)	3 (7)	8 (17)	10 (22)	4 (9)	
With B-cell proliferation	11	5 (45)	2 (18)	1 (9)	3 (27)	2 (18)	3 (27)	
Total	57*	20 (35)	10 (18)	4 (7)	11 (19)	12 (21)	7 (12)	

Framework region (FR) primer sets amplified rearrangements in *IGH* between variable segment (*V<sub>H</sub>*), diversity segment (*D<sub>H</sub>*), and joining segment (*J<sub>H</sub>*). *D<sub>H</sub>* primer sets amplified rearrangements between *D<sub>H</sub>* and *J<sub>H</sub>*.

\*Two cases of AILT and two cases of PTCL-NOS were not studied because of insufficient DNA.

<sup>†</sup>One case yielded oligoclonal products with FR1 only and was scored as negative.

<sup>‡</sup>One case yielded clonal products with FR1 and oligoclonal products with DH<sub>1-6</sub> and was scored as positive.

There was no correlation between EBV and diagnostic group, age, or the results of B- or T-cell clonality studies ( $P = 0.50$  for correlation between EBV and B-cell clonality).

For each case with an associated B-cell proliferation, the results of the EBV and clonality studies along with the type of B-cell proliferation are listed in Table 6. Overall, 11 of 16 cases with B-cell proliferations were positive for EBV, and 11 of 23 had B-cell clones. In cases with high numbers of EBV-labeled cells, the EBV staining pattern correlated with that of CD79a or CD20, consistent with an EBV-associated B-cell proliferation (Figure 1, C and D). In cases with few numbers of labeled cells, the staining pattern for EBV was typically scattered and did not clearly correlate with that of B- or T-cell lineage markers. Essentially all combinations of type of B-cell proliferation and results of EBV and clonality studies were seen. For cases with small B-cell proliferations, three of five were EBV-positive, and five of seven had a B-cell clone. For cases with large B-cell proliferations, seven of nine were EBV-positive, and 5 of 14 had a B-cell clone. Among the EBV-positive cases, 4 of 11 had a B-cell clone, whereas for EBV-negative cases, three of five had a B-cell clone.

Within the subset of cases with B-cell proliferations, there was no statistically significant correlation between EBV, type of B-cell proliferation, B-cell clonality, or T-cell clonality.

### Discussion

We detected T-cell clones in 78 and 81% of AILT and PTCL-NOS cases, respectively. These figures are consistent with those of previous reports,<sup>2,20-24</sup> including a recent study on the BIOMED-2 PCR,<sup>25</sup> which used the same *TCRγ* primers to demonstrate clonality in 86% of T-cell lymphoproliferative disorders. An important aspect of our study is the variable gene usage data, which highlight the advantage of multiplex PCR assays that target a wide range of variable genes over those that target only *V<sub>γ</sub>*1-8. Although 71% of T-cell clones were detected by *V<sub>γ</sub>*1-8 primers, the remaining 29% were only detectable by a combination of *V<sub>γ</sub>*9, *V<sub>γ</sub>*10, and *V<sub>γ</sub>*11 primers. Lawnicki and colleagues<sup>19</sup> reported similar findings with 25% of T-cell clones undetectable using *V<sub>γ</sub>*1-8 primers alone. Interestingly, we also observed rearrangements in *V<sub>γ</sub>*10 relatively frequently, occurring in 59% of T-cell clones. However, previous studies on variable gene usage in PTCL report rearrangements in *V<sub>γ</sub>*10 as uncommon, occurring in 2 to 17% of cases.<sup>17-19</sup> Because of this apparent discrepancy, we considered the possibility of nonspecific amplification or amplification of non-*V<sub>γ</sub>*10 rearrangements by *V<sub>γ</sub>*10 primers or other primers in the multiplex PCR. Therefore, we sequenced eight PCR products corresponding to *V<sub>γ</sub>*10 rearrangements. In all eight cases, the sequence matched that of *V<sub>γ</sub>*10, confirming the specificity of the BIOMED-2 primers for this region. Our findings suggest a higher sensitivity of the BIOMED-2 PCR for *V<sub>γ</sub>*10 than methods used in previous reports, which was also observed in the initial BIOMED-2 study.<sup>16</sup>

Increased numbers of EBV-positive cells were observed in 50 and 57% of AILT and PTCL-NOS cases, respectively. These frequencies are comparable to those of previous reports on PTCL<sup>26-29</sup> and more recent studies on AILT.<sup>20,30,31</sup> In both AILT and PTCL-NOS groups,

**Table 4.** B-Cell Clonality Detection by PCR for *IGH* by Specific Primer Sets

Primer sets yielding <i>IGH</i> clones	Number of cases		
	AILT	PTCL-NOS	Total (%)
FR1	2	0	2 (1.8)
FR1, FR2	0	1	1 (0.9)
FR1, FR3	1	0	1 (0.9)
FR1, FR2, FR3	3	0	3 (2.7)
FR3	2	2	4 (3.5)
FR1, D <sub>H</sub> 1-6	0	1	1 (0.9)
FR3, D <sub>H</sub> 7	2	1	3 (2.7)
FR1, FR3, D <sub>H</sub> 1-6	0	4	4 (3.5)
FR1, FR3, D <sub>H</sub> 1-6, D <sub>H</sub> 7	0	1	1 (0.9)
FR1, FR2, FR3, D <sub>H</sub> 1-6	1	2	3 (2.7)
FR1, FR2, FR3, D <sub>H</sub> 7	0	1	1 (0.9)
FR1, FR2, FR3, D <sub>H</sub> 1-6, D <sub>H</sub> 7	1	0	1 (0.9)
D <sub>H</sub> 1-6	2	3	5 (4.4)
D <sub>H</sub> 1-6, D <sub>H</sub> 7	1	1	2 (1.8)
D <sub>H</sub> 7	4	3	7 (6.2)
Negative	37	37	74 (65.5)

**Table 5.** EBV Studies by *in Situ* Hybridization for EBER

Diagnosis	Number of cases studied	Number of positive cases (%)	Number of EBV-labeled cells per medium-power field				
			0	Less than 1	1 to 10	Greater than 10 to 100	Greater than 100
AILT							
Without B-cell proliferation	22	10 (45)	12	2	3	3	2
With B-cell proliferation	8	5 (63)	3	0	3	0	2
Total	30	15 (50)	15	2	6	3	4
PTCL-NOS							
Without B-cell proliferation	29	15 (52)	14	2	4	7	2
With B-cell proliferation	8	6 (75)	2	0	2	3	1
Total	37	21 (57)	16	2	6	10	3

cases with associated B-cell proliferations were more often EBV-positive with higher numbers of labeled cells. In many cases, the majority of the B-cell proliferation was EBV-positive (Figure 1, C and D), suggesting that the virus stimulates B-cell proliferation. However, some B-cell proliferations were EBV-negative, indicating additional mechanisms for B-cell proliferation in some T-cell lymphomas.

The B-cell clonality studies provided the most interesting results, with B-cell clones detected in 34 and 35% of AILT and PTCL-NOS cases, respectively. Although previously reported frequencies of B-cell clonality in AILT are similar,<sup>20,23,32,33</sup> those for PTCL range from 9 to 16%.<sup>2,4,5,22,34,35</sup> We considered that some cases of PTCL-NOS were misclassified and actually represent AILT. However, PTCL-NOS cases did not have classic

morphologic features of AILT. Furthermore, of the 20 cases of PTCL-NOS with a B-cell clone, an immunohistochemical stain for CD21 was performed in 12 cases, yielding negative results in all 12. In contrast, 56 of 56 AILT cases were positive for CD21 expansions, consistent with previous reports that CD21 staining is a virtually constant finding in AILT.<sup>36</sup> Nevertheless, we cannot exclude that a morphologic spectrum exists between AILT and PTCL-NOS.

Overall, B-cell clonality correlated, in part, with the presence of a B-cell proliferation but not with EBV. However, cases without demonstrable B-cell proliferations also exhibited B-cell clones relatively frequently, suggesting other contributing factors. One factor contributing to the relatively high frequency of B-cell clones detected in the current study may be the inherent sensitivity

**Table 6.** Summary of Results for Cases with Associated B-Cell Proliferations

Diagnosis and type of B-cell proliferation	<i>TCRγ</i> clonality	<i>IGH</i> clonality	No. EBV-labeled cells/mpf	Lineage of EBV-labeled cells
AILT				
Large*	+	+	ND	NA
Large	+	-	ND	NA
Large	+	-	ND	NA
Small	+	+	1 to 10	Unclear
Small	-	+	>100	Subset of B cells
Plasma cells	+	+	Negative	NA
Large	+	+	Negative	NA
Large*	+	-	Negative	NA
Large	-	-	1 to 10	Unclear
Large	+	-	>100	Subset of B cells
Small	+	-	1 to 10	Unclear
Small*	-	+	ND	NA
PTCL-NOS				
Small	+	+	Negative	NA
Large	+	+	1 to 10	Unclear
Large	+	+	>10 to 100	Large B cells
Large	+	+	ND	NA
Small*	+	+	ND	NA
Large	+	-	1 to 10	Large B cells
Small	+	-	Negative	NA
Small and Large	-	-	>10 to 100	Large B cells
Large*	-	-	ND	NA
Large	-	-	>100	Large B cells
Large	+	-	>10 to 100	Subset of B cells

ND, not done; NA, not applicable; mpf, medium-power field

\*These B-cell proliferations met criteria for B-cell lymphoma.

The lineage of EBV-labeled cells was determined by comparison of the *in situ* stain to that of B- and T-cell lineage markers. In some cases, the lineage of EBV-labeled cells could not be reliably determined, and the lineage is indicated as unclear.



of the BIOMED-2 *IGH* PCR, which uses primers targeting all three FRs. Most methods used in previous studies targeted only a single FR,<sup>2,5,22</sup> a strategy shown to be less sensitive.<sup>34</sup> If our data are compared by basing clonality on a single FR primer set (Table 3), 15 to 20% of cases would be considered positive for B-cell clonality. Another possibility is so-called lineage infidelity, referring to simultaneous *TCRγ* and *IGH* rearrangements occurring in the same cell.<sup>7</sup> Although uncommon in mature B- and T-cell non-Hodgkin lymphomas,<sup>7</sup> this phenomenon may have contributed more than in previous studies because of the sensitivity of our assays.

In contrast to previous studies,<sup>11,20</sup> we did not observe the frequent presence of B-cell oligoclonalities in cases with EBV. This difference is likely related to methodology, with the BIOMED-2 *IGH* PCR yielding either positive or negative results rather than oligoclonalities. However, EBV was associated with B-cell clones detectable only by *D<sub>H</sub>* primers, a finding likely caused by the presence of an incomplete *D<sub>H</sub>-J<sub>H</sub>* rearrangement on one *IGH* allele and a complete *V<sub>H</sub>-D<sub>H</sub>-J<sub>H</sub>* rearrangement on the other allele that is unamplifiable. It is possible that the previous methods, which used FR primers only and showed frequent oligoclonalities, would have yielded oligoclonal results in these cases. Alternatively, our findings may represent B-cell clones with mutations that interfere with FR primer detection. In either case, understanding the clonal or oligoclonal nature underlying these findings will require correlation with nongenotypic methods such as immunophenotyping.

Collectively, the B-cell clonality and EBV results suggest a model whereby altered immune function in the locale of AILT or PTCL-NOS results in uncontrolled B-cell proliferation. The frequent presence of EBV is evidence of immune dysfunction, and in EBV-positive cases, the virus may further stimulate B-cell proliferation, which is supported by studies localizing EBV to B cells in PTCL.<sup>11,29,37</sup> Uncontrolled B-cell proliferation may result in the emergence of a dominant clone over time. Evidence for this evolution is provided by PTCL patients who initially lack an associated B-cell proliferation but who later have specimens showing a B-cell proliferation, B-cell clone, or even an associated B-cell lymphoma.<sup>11,14</sup> Overall, the model is similar to that of post-transplant lymphoproliferative disorder (PTLD), but it does not explain the occurrence of B-cell clones in PTCL lacking B-cell proliferations. It is possible that B-cell proliferations are not always readily demonstrable by morphology and immunohistochemistry, and because of the sensitivity and lack of quantitation of PCR, a B-cell clone may reflect processes ranging from an incipient or focal B-cell proliferation to overt B-cell lymphoma. Regardless of the mechanisms involved in B-cell clonality, the clinical significance remains unclear. Feller and colleagues<sup>32</sup> reported that clonal *IGH* rearrangements in AILT were associated with increased overall survival. However, this finding was not statistically significant, possibly because of the low number of cases. Thus, until the clinical manifestations are better understood, the most important aspect of B-cell clonality in PTCL is recognizing that it

occurs and that its presence should not negate the diagnosis of PTCL.

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