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Hepatosplenic and Subcutaneous Panniculitis-Like γ/δ T Cell Lymphomas Are Derived from Different Vδ Subsets of γ/δ T Lymphocytes

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Gamma/delta T cell lymphomas (γ/δ TCL) represent rare, often aggressive types of T cell malignancy that are clinically and pathologically diverse. Most γ/δ TCL occur as a hepatosplenic or subcutaneous type. To date, analysis of the T cell receptor δ (TCRδ) gene repertoire of hepatosplenic γ/δ TCL (γ/δ HSTCL) and subcutaneous panniculitis-like γ/δ TCL (γ/δ SPTCL) has been reported only in a limited number of cases. In this study we analyzed 11 γ/δ HSTCL and 4 γ/δ SPTCL by polymerase chain reaction and immunostaining to determine their usage of the Vδ subtypes (Vδ1–6). It is noteworthy that 10 of 11 γ/δ HSTCL expressed the Vδ1 gene. The remaining case also expressed T cell receptor δ (TCRδ) as determined by flow cytometry and TCRδ rearrangement in Southern blot. However, the Vδ gene expressed by this lymphoma could not be determined, which suggests usage of an as yet unidentified Vδ gene. In striking contrast to the γ/δ HSTCL, all 4 γ/δ SPTCL expressed the Vδ2 gene. Our data demonstrate that γ/δ HSTCL are preferentially derived from the Vδ1 subset of γ/δ T lymphocytes, whereas γ/δ SPTCL are preferentially derived from the Vδ2 subset. The pattern of Vδ gene expression in HSTCL and SPTCL corresponds to the respective, predominant γ/δ T cell subsets normally found in the spleen and skin. This finding suggests that γ/δ TCL are derived from normal γ/δ T lymphocytes which reside in the affected tissues. Furthermore, the selective, lymphoma type-specific Vδ gene segment usage may provide a molecular tool to distinguish better among various types of γ/δ TCL lymphoma particularly in the clinically advanced, widely disseminated cases. *(J Mol Diag 2000, 2:11–19)*

Similar to normal T lymphocytes, T cell lymphomas (TCL) may express two different types of T cell receptor (TCR), α/β or γ/δ. Four TCR genes (α, β, γ, and δ) are composed in their germline configuration of noncontiguous segments of variable (V), diversity (D), joining (J), and constant (C) regions. During T cell differentiation, somatic VDJ rearrangements occur and thereby generate variability of the TCR.[1] Only complete in-frame TCR gene rearrangements, consisting of V, D, and J regions, may form a functional TCR. Incomplete rearrangements between two D regions (D-D), between V and D region (V-D), and between D and J region (D-J) are nonfunctional. The majority of normal T lymphocytes express the α/β heterodimer; however, approximately 5% of the T cells express the γ/δ heterodimer.[2] In contrast to α/β T lymphocytes, development of γ/δ T cells is not dependent on expression of major histocompatibility complex (MHC) I or MHC II molecules.[3,4] Unlike α/β T cells, which develop almost exclusively in thymus, γ/δ T cells can be generated in extrathymic sites such as intestinal epithelium, skin, spleen, and fetal liver.[5–8] The exact function of γ/δ T lymphocytes has not been fully elucidated, but some studies suggest a role for these cells in early immune responses to infections, autoimmune disorders, and cancer immune surveillance.[9] γ/δ T cells share some features with CD8+ α/β T lymphocytes and with natural killer (NK) cells. They show MHC-dependent and MHC-independent cytotoxity, produce lymphokines, and exhibit NK-like lytic activity.

γ/δ TCL represent a rare type of T cell malignancy. They comprise less than 10% of peripheral T cell lymphomas[10] and occur mostly at extranodal sites in hepatosplenic, subcutaneous, or intestinal form. Hepatosplenic

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Table 1. List of Primers

	Primers	Source
5′ primers		
Vδ1	5′-ACT CAA GCC CAG TCA TCA GT	Yokota et al[35]
Vδ1b	5′-GCA AAG TAC TTT TGT GCT CTT G	Yokota et al[35]
Vδ2	5′-GAG TCA TGT CAG CCA TTG AG	Yokota et al[35]
Vδ2b	5′-GCA CCA TCA GAG AGA GAT GA	Yokota et al[35]
Vδ3	5′-ACA GCA GAT CAG AAG GTG CA	Przybylski et al[36]
Vδ4	5′-CCA GTG ATC CAA GTT ATG GTC	Przybylski et al[36]
Vδ5	5′-CTG AAG GTC CTA CAT TCC TG	Przybylski et al[36]
Vδ6	5′-TAT CAT GGA TTC CCA GCC TG	Przybylski et al[36]
Dδ2	5′-AGA GGG TTT TTA TAC TGA TGT	Schmidt et al[37]
3′ primers		
Jδ1	5′-GAG TTA CTT ACT TGG TTC CAC	Yokota et al[35]
Dδ3	5′-AGG GAA ATG GCA CTT TTG CC	Yokota et al[35]
Reference primers		
RAG1(5′)	5′-GCC ATG AAG AGC AGT GAA TTA	Salhany et al[26]
RAG1(3′)	5′-AGG AAT TAA CTC ACA AAC TGC	Salhany et al[26]
RAG2(5′)	5′-TTG GCA TAT ACC AGG AGA CAA T	Salhany et al[26]
RAG2(3′)	5′-ACT ATT TGC TTC TGC ACT GA	Salhany et al[26]

γ/δ TCL (γ/δ HSTCL) is recognized as a provisional subset of peripheral T cell lymphoma in the Revised European-American Classification of Lymphoid Neoplasms (REAL),[11] although a few identified cases of α/β HSTCL appear to have similar clinicopathological characteristics. Histologically, γ/δ HSTCL is characterized by a mixture of small to medium-sized atypical lymphocytes. To date only about 40 cases of γ/δ HSTCL have been reported.[10,12–23] These lymphomas frequently show two nonrandom chromosomal abnormalities, isochromosome 7q [i(7)(q10)] and trisomy 8 (8+).[19,20] Affected individuals are usually young males. Patients commonly present with B symptoms and hepatosplenomegaly, but not lymphadenopathy. The disease usually follows an aggressive course with poor response to chemotherapy and short time of survival.

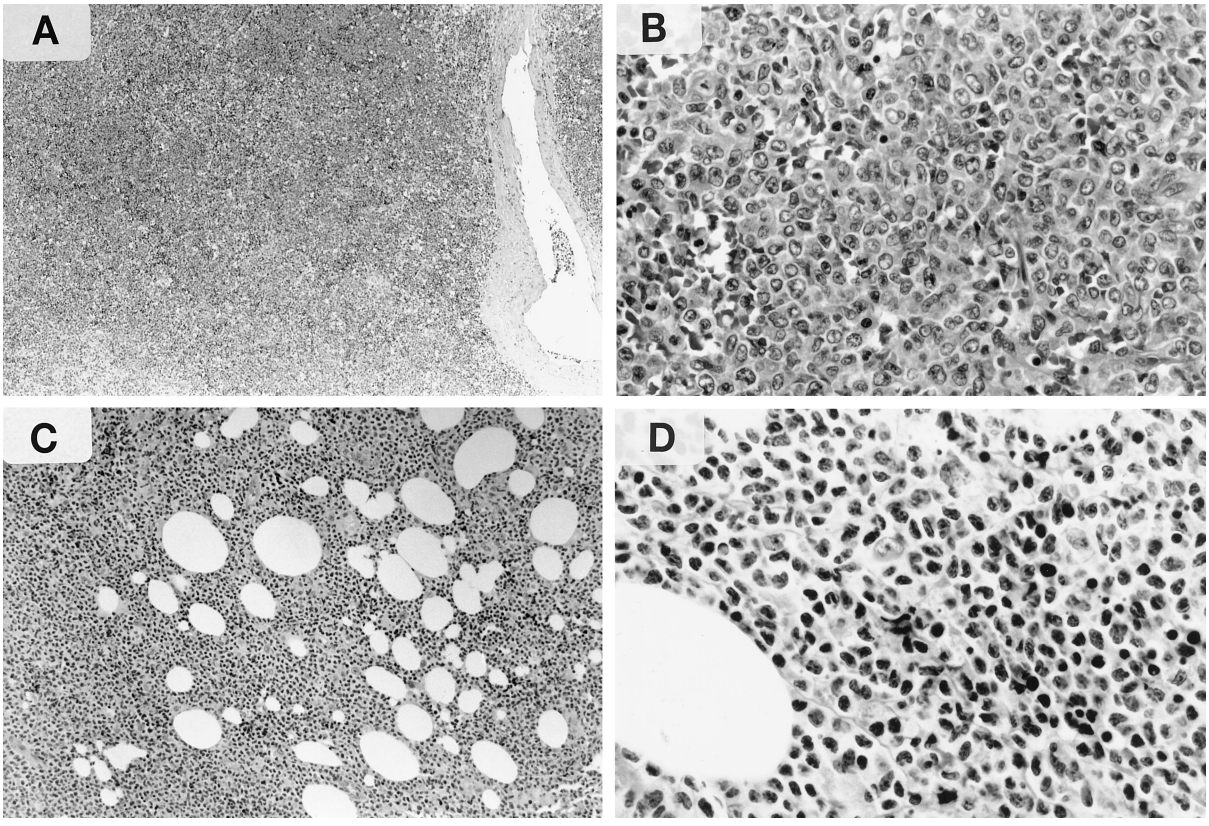

Figure 1. Histology of representative cases of γ/δ HSTCL (**A** and **B**, Patient 5 in Table 2) and γ/δ SPTCL (**C** and **D**, Patient 12). **A:** Diffuse involvement of splenic parenchyma. **B:** Relatively monomorphic population of medium-size lymphocytes with round to oval nuclei, small nucleoli, and a moderate amount of pale cytoplasm. **C:** Lobular panniculitis-like pattern. **D:** Predominance of pleomorphic, small lymphocytes with irregular and hyperchromatic nuclei and scant cytoplasm.

Table 2. Clinical Features of Hepatosplenic and Subcutaneous γ/δ T Cell Lymphomas

Case	Age/sex	Primary site	Clinical symptoms	Cytopenias	Therapy and response	Survival
Hepatosplenic γ/δ T-Cell Lymphoma						
1	68 M	Spleen, liver, BM	Bacterial infections, fever, weight loss, hepatosplenomegaly	Neutropenia: WBC 800; ANC 128, anemia: Hgb: 7 g/l, thrombocytopenia: plt 35,000, polyclonal gammophaty	Splenectomy, CHOP: CR 22 mo	22 mo; died of sepsis due to aplastic anemia NED at autopsy
2	32 M	Spleen, liver, BM, PB	Fever, night sweats, weight loss, hepatosplenomegaly	Leukopenia: WBC 700 anemia: Hgb 8.3 thrombocytopenia: plt 43,000 circulating blasts in blood	Prednisone + acyclovir: increase in platelet number, progressive hepatosplenomegaly and leukemia CHOP:NR	5 mo; DOD
3	59 M	Spleen, liver	Weight loss, splenomegaly, abdominal pain	Neutropenia: ANC 1440 anemia: Hgb 6 thrombocytopenia: plt 44,000	CHOP: NR, progressive disease Fludarabine: minimal response	10 mo; DOD
4	42 F	Spleen, liver, BM, PB	Fever	Anemia: Hgb 7.6	Reduction in immunosuppression: NR; modified CHOP: PR 4 mo.	6 mo; DOD
5	46 M	Spleen, liver, BM	Fever, chills, sweats	Anemia: Hgb 9.0, thrombocytopenia: plt 45,000	Reduction in immunosuppression: NR CHOP: PR, rapid relapse allo-BMT with response	5 mo; died 21 days after BMT from complications of BMT
6	18 M	Spleen, liver	Autoimmune hepatitis		BMT: rapid relapse	
7	33 M	Spleen, liver	Fever, jaundice hepatosplenomegaly	WBC: 6600, thrombocytopenia: plt 70,000	Splenectomy	1 mo; died during surgery
8	30 M	Spleen, liver, lung, axillary LN	Fever, night sweats, weight loss, hepatosplenomegaly	WBC: 7200, anemia: Hgb 9.1, thrombocytopenia: plt 80,000	CHOP: NR BMT: PR XRT	30 mo; died of pulmonary hemorrhage during radiotherapy
9	41 M	Spleen, liver, BM, PB	Weight loss, abdominal pain	Leukopenia: WBC 1,200, anemia: Hct: 30%, thrombocytopenia: plt 72,000	Splenectomy, multiagent chemotherapy: CR 19 mo. Relapse, untreated	22 mo; DOD
10	32 M	Spleen, liver, BM, PB	Fever, night sweats, hepatosplenomegaly	Leukopenia: WBC 2,000, anemia: Hct: 32%, thrombocytopenia: plt 8,000	Splenectomy, Mega IV chemotherapy	2 mo; died, NED on autopsy
11	65 M	Spleen	Prolonged gingival bleeding post dental procedure, splenomegaly	Leukopenia: WBC 1,600, anemia: Hct: 34%, thrombocytopenia: plt 66,000	Splenectomy, CHOP	10 mo; NED
Subcutaneous γ/δ T-Cell Lymphoma						
12	47 M	skin/subcutis arms, back, abdomen, face	Fever, night sweats HPS	Neutropenia; ANC 1054, anemia; Hct 37%, thrombocytopenia: plt 52,000	CHOP: PR	3 mo; DOD
13	53 F	skin/subcutis thighs	HPS	Neutropenia; ANC 1660, anemia: Hct 34%	CHOP: CR–2 mo, local recurrence XRT: CR 3 mo 2nd recurrence Polychemotherapy: CR	18 mo; DOD
14	36 M	skin/subcutis extremities, trunk	HPS	Leukopenia: WBC 1800, Hct, Hgb, plt count: normal	CHOP: PR, 2-CDA: PR, XRT: PR, Multiagent chemotherapy: NA	36 mo; DOD
15	45 M	skin/subcutis extremities, torso	Fever, night sweats, weight loss, arthralgias	Normal (ANC) to low normal (Hb, plt) counts	CHOP, ESHAP, FLAG-CR, 9 mo relapse ABMT, 13 mo relapse	15 mo; DWD

BM, bone marrow; PB, peripheral blood; HPS, hemophagocytic syndrome; WBC, white blood cells; ANC, absolute neutrophil count; plt, platelet count; Hct, hematocrit; Hgb, hemoglobin; NR, no response; CR, complete remission; PR, partial remission; XRT, radiation therapy, BMT, bone marrow transplantation; NED, no evidence of disease; DOD, dead of disease; DWD, dead with disease; NA, information not available.

Table 3. Immunophenotype of Hepatosplenic and Subcutaneous γ/δ T Cell Lymphomas

	T-cell-associated								TCR-specific		NK-associated			
Case	CD2	CD3	CD4	CD5	CD7	CD8	CD43	CD45R0	TCRα/β	TCR γ/δ	CD11c	CD16	CD56	CD57
Hepatosplenic γ/δ T cell lymphoma														
1	++++	++++	+	+	+++	−			−	++++	++++	+	−	−
2	++++	++++	−	−	+	−			−	++++	+	++++	+++	−
3	++++	++++	−	−	++++	−			−	++++	−	++++	++++	−
4	++++	++++	+	+	++++	++			+	+++		+++	+++	−
5	++++	++++	−	−	++++	−			−	++++	+	+++	+++	−
6	++++	++++	−	+	+++	+			−	+++				
7		++++	−			−	++	+	−				−	−
8		++++	−			−	++++	+++	−				−	−
9		+++				−	+++	+++	−				++	−
10	++++	++++	−	+++	++++	++++	+++	−	−		++++		+++	−
11	++++	++++	−	−	++++	−			−	++++		++++	++++	
Subcutaneous γ/δ T cell lymphoma														
12	+++	++++	−	−	++	−	+++	+++	−	++++	−	−	++++	−
13	++++	++++	−/+	+++	++++	+	++++	+++	−	+++	−	−	+++	−
14	++++	++++	+			−		+++	−	+++			+++	
15	++++	++++	−	−	−	−			−	++++		−	++++	−

TCR, T-cell receptor; NK, natural killer; −, <10% positive; +, 10–25% positive; ++, 26–50% positive; +++, 50–75% positive; ++++, >75% positive.

Subcutaneous panniculitis-like TCL (SPTCL) is an uncommon form of cutaneous lymphoma, involving mainly subcutis and often mimicking lobular panniculitis.[24] SPTCL also has been proposed as a provisional subset of peripheral T cell lymphoma in the REAL classification.[11] It is sometimes associated with aggressive clinical behavior and poor prognosis, particularly when accompanied by a hemophagocytic syndrome.[24–26] Based on TCR expression, SPTCL can be divided into α/β and γ/δ SPTCL subsets, which are not recognized as distinct entities in the REAL classification. To date only a few γ/δ SPTCL cases have been reported.[26–31]

The TCRδ gene consists of at least six Vδ gene segments.[32,33] A detailed analysis revealed that over 95% of the γ/δ T cells express either Vδ1 or Vδ2 gene.[2,9] Interestingly, normal γ/δ T lymphocytes present in spleen, thymus, and intestinal epithelium predominantly express the Vδ1 gene, whereas the majority of γ/δ T cells in peripheral blood, tonsils, and skin express the Vδ2 gene.[2] The reason for this dichotomy in the Vδ gene usage repertoire remains unclear. Vδ gene usage by γ/δ TCL has not been studied so far in the great detail, and the small number of the reported cases does not allow any definitive conclusions in regard to Vδ usage by the specific subtype of γ/δ TCL. In this study we analyzed the Vδ usage in 11 hepatosplenic and 4 subcutaneous γ/δ TCL using polymerase chain reaction (PCR) and flow cytometry. Preferential usage of Vδ1 gene was found in γ/δ HSTCL (10/11 cases) and of Vδ2 gene in γ/δ SPTCL (4/4 cases). Biological and diagnostic implications of this finding are discussed.

Materials and Methods

Patient Samples

We investigated 15 patients with γ/δ TCL of hepatosplenic (11 cases) and subcutaneous (4 cases) type in this study. The cases were derived from files of the participating institutions: the University of Pennsylvania, Vanderbilt University, Johns Hopkins University, the University of Pittsburgh, and Harvard University. Data on cases 1–3, 12, and 13 including Vδ subtype expression were previously reported in part.[20,26]

Cytogenetic Analysis and Fluorescence in Situ *Hybridization (FISH)*

Metaphase cytogenetic analysis was performed by standard trypsin Giemsa banding using unstimulated cell cultures of spleen or bone marrow cells. Slides for FISH were prepared from spleen or peripheral blood mononuclear cells according to a standard method. In brief, liquid nitrogen-stored, dimethyl sulfoxide-preserved frozen cells from three patients were cultured overnight, cytospun onto slides at a concentration of 10^4 cells/slide, air-dried, fixed in Carnoy's for 20 minutes, and air-dried overnight. The cytospins were analyzed with VYSIS (Downers Grove, IL) CEP 8 probe to enumerate chromosome 8 centromeres or combination of CEP 7 and LSI D7S486 probes to detect simultaneously chromosome 7 centromere and band 7q31 on the chromosome's long arm. The staining was performed as recommended by the probe manufacturer.

Immunophenotype Analysis

Flow cytometry and frozen section or paraffin immunohistochemistry were used for immunophenotyping of the lymphomas. All cases were studied by flow cytometry and/or frozen section immunohistochemistry with a set of standard anti-T cell and anti-NK cell antibodies as previously described.[20,26] To confirm the γδ phenotype, we used antibodies to the α/β and γ/δ TCR (Endogen, Woburn, MA).[20,26] Vδ subtype expression was also determined by

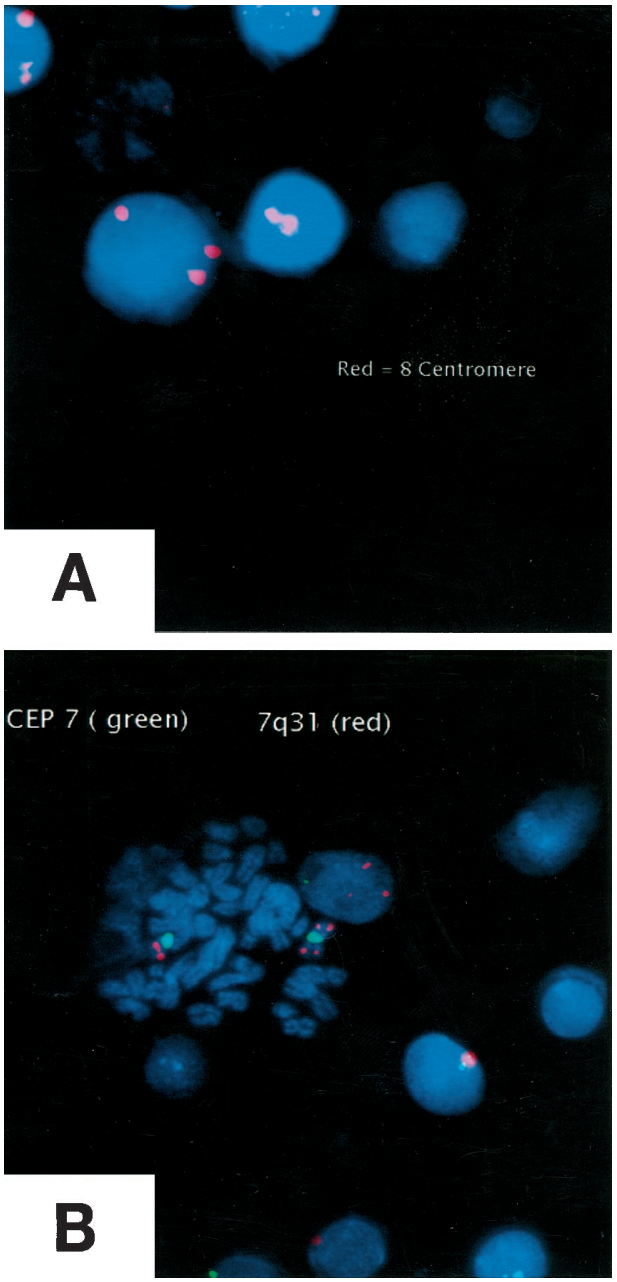

Figure 2. FISH analysis of γ/δ HSTCL (Patient 2). **A:** CEP 8 probe to enumerate chromosome 8 centromeres showed the presence of trisomy 8 in 20% of the analyzed cells. **B:** Combination of CEP 7 probe (green staining) and LSI D7S486 probe (red staining) to detect simultaneously chromosome 7 centromere and band 7q31 on the chromosome's long arm showed the presence of isochrome 7 in 28% of the analyzed cells. A positive cell in metaphase is seen in the center of this photograph. Similar results showing the presence of both trisomy 8 and isochromosome 7 were obtained in patients 4 and 5 (not shown).

flow cytometry or frozen section immunohistochemistry using commercially available monoclonal antibodies specific for different V regions of the TCRδ chain (Vδ1 and Vδ2, Endogen; Vδ3, Immunotech, Westbrook, ME).

Southern Blot Analysis

Southern blot analysis for TCRδ gene rearrangements was performed on genomic DNA extracted from frozen tumor in five cases of γ/δ HSTCL. The DNA was treated with restriction enzymes *Eco*RI, *Hin*dIII and *Bam*HI, transferred to a nylon membrane and hybridized to a TCRδ gene probe TCRDJ1 (Dako Corp., Carpinteria, CA), which corresponds to the Jδ1 exon and its 3′ flanking region. A nonoverlapping 3.0-kb probe, which corresponds to the Jδ2 exon and its 5′ flanking region (pjk 3.0s, kindly provided by Dr. Carlo Croce, Philadelphia, PA)[34] was also used in one case that did not show a TCRδ rearrangement using the TCRDJ1 probe.

PCR

PCR of 50 μl total volume was performed in a Trio-Thermoblock (Biometra, Goettingen, Germany) with 0.1 μg of genomic DNA, 10 pmol of each primer, 5 nmol each dATP, dCTP, dGTP, dTTP (Perkin Elmer-Cetus, Norwalk, CT), 1.5 U *Taq* polymerase, and PCR Buffer (Perkin Elmer-Cetus) including 10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, and 0,001% (w/v) gelatin. After 3′ denaturation at 94°C, 35 PCR cycles were performed, each cycle consisting of denaturation at 94°C, annealing at 60°C, and extension at 72° (for 1 minute each), followed by a final 7-minute extension at 72°C. Oligonucleotide primers used for PCR were previously described[26,35–37] and are listed in Table 1. Their specificity was confirmed by sequencing of their products[36,37] (and data not shown) and nested PCR which yielded products of expected molecular weight (data not shown). To exclude DNA contamination, negative controls were included, and to avoid false negative results an internal positive control was run in each reaction. Amplification of recombinase activating gene (RAG1) served as such a positive control. PCR products were visualized by 2% agarose gel electrophoresis containing ethidium bromide. Under these experimental conditions, lymphoma-derived, clonal TCRδ gene rearrangements, which are present in the large proportion of cells in the samples, are seen as distinct, well-defined bands in the gel. Because the incidence of a specific Vδ rearrangement is low in normal reactive polyclonal T cells, TCRδ genes from reactive T cells present in the samples are amplified, but not visible as distinct bands.

Results

Multiparameter Analysis of the γ/δ TCL

All cases were evaluated independently by at least two hematopathologists. According to the REAL classification, 11 cases were classified as γ/δ HSTCL and 4 cases as γ/δ SPTCL. Typical histological findings from two representative cases are shown in Figure 1. Detailed clinical features of all cases are summarized in Table 2. Thirteen of the patients were male and only two were female; patient age ranged from 18 to 68 years (median, 42 years). Most patients presented with B symptoms, anemia, thrombocytopenia, and/or leukopenia. Three γ/δ SPTCL patients developed severe hemophagocytic syndrome. All patients followed an aggressive clinical course

Table 4. Immunophenotypic and Molecular Analysis of the TCR Vδ Gene Subset Expression in Hepatosplenic and Subcutaneous γ/δ T Cell Lymphoma

Patient	Vδ gene rearrangement (Southern blot)	Vδ subtype protein (flow cytometry)	Vδ subtype DNA (PCR)	Vδ subset classification
Hepatosplenic γ/δ T Cell Lymphoma				
1	R/R	neg. Vδ(1–3)	neg. Vδ(1–6)Jδ (1,2)	undetermined
2	R/D	Vδ1+	Vδ1Jδ1	Vδ1
3	R/R	Vδ1+	Vδ1Jδ1/Dδ2Jδ1	Vδ1
4	R/G	Vδ1+	Vδ1Jδ1	Vδ1
5	R/R	Vδ1+	Vδ1Jδ1	Vδ1
6			Vδ1Jδ1	Vδ1
7			Vδ1Jδδ1	Vδ1
8			Vδ1Jδ1/Dδ2Jδ1	Vδ1
9			Vδ1Jδ1/Dδ2Jδ1	Vδ1
10			Vδ1Jδ1	Vδ1
11			Vδ1Jδ1/Dδ2Jδ1	Vδ1
Subcutaneous −/− T Cell Lymphoma				
12		Vδ2+	Vδ2Jδ1	Vδ2
13		Vδ2+	Vδ2Jδ1/Vδ3Jδ1	Vδ2
14			Vδ2Jδ1/Dδ2Jδ1	Vδ2
15			Vδ2Jδ1	Vδ2

PCR, polymerase chain reaction; Sb, Southern blot; Vδ, variable region; Jδ, joining region; Dδ, diversity region; R, rearranged; D, deletion; G, germline; ND, not done.

with short survival of 1 to 36 months (median, 10 months). Immunophenotyping data of the cases are presented in Table 3. All lymphomas expressed T-cell-associated markers, mainly CD3, CD2, and/or CD7. Most were negative for CD4 and CD8. The γ/δ T cell phenotype was confirmed in all cases by positive staining with anti-TCRγ/δ antibody and/or negative staining with anti-TCRα/β antibody. Although case 4 showed staining with anti-TCRβ in a small subset of cells, these probably represented reactive T cells, because most cells were TCRγ/δ+. Lymphoma cells usually expressed at least one of NK-associated marker, chiefly CD56. Interestingly, whereas the majority of γ/δ HSTCL expressed also CD16 and, to a lesser degree, CD11c, γ/δ SPTCL expressed only CD56.

We were also able to perform cytogenetic analysis in three cases of γ/δ HSTCL (cases 2, 4, and 5). By FISH all three cases have shown cytogenetic abnormalities characteristic for HSTCL: isochromosome 7q and trisomy 8[19,20,38,39] (Figure 2). Standard metaphase cytogenetic analysis demonstrated an abnormal karyotype in case 2 including the presence of isochromosome 7q and trisomy 8 (46, X, −Y, i(7)(q10), +8, del(10)(q?22) and failed to show any abnormalities in the remaining two cases.

TCRδ Gene Rearrangements in γ/δ T Cell Lymphomas

Southern blot analysis using the TCRDJ1 probe and the pjk 3.0s probe revealed clonal TCRδ gene rearrangements in all 5 γ/δ HSTCL cases investigated (cases 1–5; see Table 4). In three cases two rearranged bands were visible, indicating rearrangement of both TCRδ alleles. In case 4 the presence of a rearranged and a germline band in Southern blot probably represented a monoallelic rearrangement or, less likely, contribution of the germline sequence by the non-lymphoma cells combined with deletion of one rearranged allele in the lymphoma cells. Finally, in case 2, a single rearranged band was observed but the germline band was absent, suggesting deletion of the second allele. Such deletion occurs on rearrangement of the surrounding TCRα gene; however, immunophenotyping indicated that the TCRα gene was not expressing functional protein in this case.

Usage of Vδ Segments in Hepatosplenic and Subcutaneous γ/δ T Cell Lymphomas

To determine the usage of TCRδ gene segments by γ/δ T cell lymphomas, PCR was performed with Vδ1-Vδ6 specific 5′ primers and a Jδ1 3′ primer. We also looked for incomplete TCRδ gene rearrangements: Dδ2Jδ1, frequently occurring in T-ALL,[37,40] and Vδ2Dδ3 and Dδ2Dδ3 rearrangements, which were frequently found in B-precursor ALL.[41]. To each reaction control primers which amplify a 600-bp fragment of recombinase activating gene-1 (RAG1) were added to exclude false negative results. In this experimental setup, both TCRδ and RAG1 amplification products should be detected in the samples which contain a dominant T cell clone. In the reactive, polyclonal conditions detection of RAG1 but not TCRδ amplification product is expected. Because in cases 7–11 no fresh tissue was available, DNA was extracted from archival formalin-fixed, paraffin-embedded samples. Because tissue fixation frequently leads to partial DNA degradation, PCR was performed with internal Vδ1b and Vδ2b primers which amplify a shorter fragment (~140 bp) and RAG2 control primers, which amplify a 220-bp DNA fragment. We were also able to perform analysis of the Vδ usage on the protein level by flow cytometry using anti-Vδ1, -Vδ2, and -Vδ3 antibodies in 7 cases. Representative results for γ/δ HSTCL PCR using fresh-frozen tissue are shown in Figure 3 and results using formalin-fixed tissue in Figure 4. Representative results for γ/δ SPTCL PCR are shown in Figure 5. All PCR

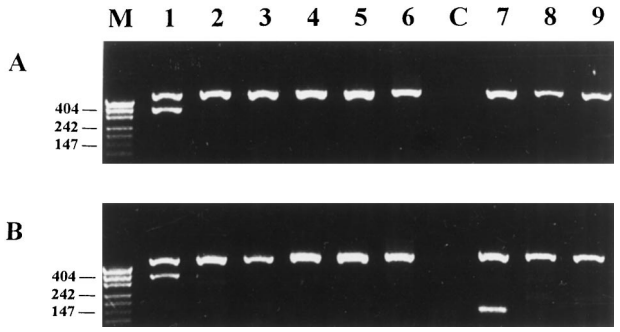

Figure 3. PCR analysis of Vδ gene usage in hepatosplenic γ/δ T cell lymphoma. **A:** Patient 2. **B:** Patient 3. **Lane M**, molecular weight marker; **lanes 1–6**, PCR products obtained with Vδ1-Vδ6 specific primers, respectively, and Jδ1 primer; **lane C**, negative control; **lane 7**, Dδ2Jδ1, **lane 8**, Vδ2Dδ3; **lane 9**, Dδ2Dδ3. The upper band of ~600 bp represents RAG1 reference gene, bands of ~400 bp in **lanes A1** and **B1** represent a Vδ1Jδ1 rearrangement, band of ~140 bp in **lane B7** represents an incomplete Dδ2Jδ1 rearrangement.

and flow cytometry data are summarized in Table 4. Strikingly, 10 of 11 γ/δ HSTCL showed a Vδ1Jδ1 rearrangement and in 4 of them an additional, incomplete Dδ2Jδ1 rearrangement of the second allele was detected. In one γ/δ HSTCL (patient 1; see Table 4) no amplification product was obtained. This case expressed the TCRγ/δ (Table 3); however, it was negative for Vδ1–3

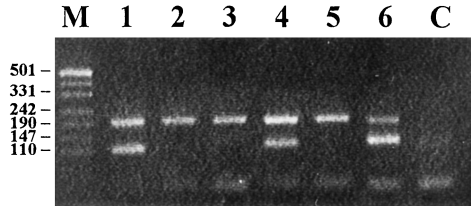

Figure 4. PCR analysis of Vδ gene usage in archival paraffin-embedded samples of hepatosplenic γ/δ T cell lymphoma. **Lane M**, molecular weight marker; **lanes 1–3**, Patient 7; **lanes 4–6**, Patient 8; **lane C**, negative control. PCR was performed with the Jδ1 primer and the Vδ1b primer (**lanes 1** and **4**), the Vδ2b primer (**lanes 2** and **5**), and the Dδ2 primer- (**lanes 3** and **6**). The upper band of ~220 bp represents RAG2 reference gene, bands of ~120 bp in **lanes 1** and **4** represent a Vδ1Jδ1 rearrangement, and the band of ~140 bp in **lane 6** represents an incomplete Dδ2Jδ1 rearrangement.

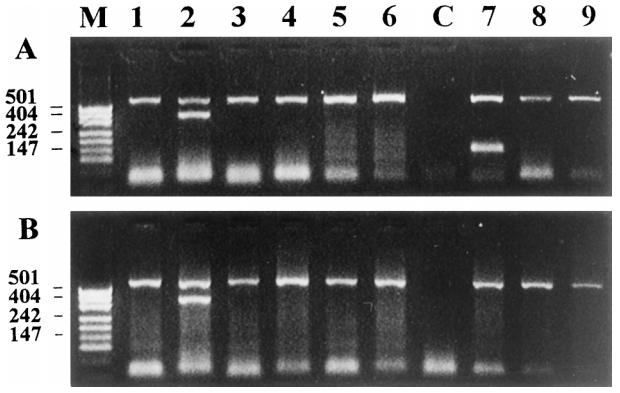

Figure 5. PCR analysis of Vδ gene usage in subcutaneous panniculitis-like γ/δ T cell lymphoma. **A:** Patient 14. **B:** Patient 15. **Lane M**, molecular weight marker; **lanes 1–6**, PCR products obtained with Vδ1-Vδ6 specific primers, respectively, and the Jδ1 primer; **lane C**, negative control; **lane 7**, Dδ2 and Jδ1 primers, **lane 8**, Vδ2 and Dδ3 primers; **lane 9**, Dδ2 and Dδ3 primers. The upper band of ~600 bp represents RAG1 reference gene, bands of ~400 bp in **lanes A2** and **B2** represent a Vδ2Jδ1 rearrangement, and the band of ~140 bp in **lane A7** represents an incomplete Dδ2Jδ1 rearrangement.

expression (Table 4), as determined by immunophenotyping. Furthermore, on Southern blot, it showed a rearrangement pattern different from other γ/δ HSTCLs analyzed in this study. These findings suggest that a variable TCRδ gene segment other than Vδ1-Vδ6 might have been used in this case. In contrast to γ/δ HSTCL, all 4 γ/δ SPTCL showed rearrangement of the Vδ2Jδ1 gene segment (Figure 5 and Table 4). Case 14 showed also the Dδ2Jδ1 rearrangement and case 13 the Vδ3Jδ1 rearrangement of the second allele. Because flow cytometry analysis in case 13 showed a Vδ2 expression, the Vδ3Jδ1 rearrangement was recognized to be nonfunctional.

Discussion

T cell lymphomas expressing γ/δ TCR represent recently recognized, rare subsets of non-Hodgkin's lymphoma.[11] The literature on γ/δ T cell lymphomas remains sparse.[10,14–33] Because only a few cases were analyzed in each of the published studies, any preferences in Vδ gene usage could not have been adequately addressed. Our present report, which describes 11 cases of γ/δ HSTCL and 4 cases of γ/δ SPTCL, is the largest study to date on γ/δ TCL. It is the first study focused on immunophenotypical and molecular subtyping of these rare but clinically distinct subtypes of peripheral TCL. There was an excellent concordance among results obtained by molecular and immunophenotypic approaches (Table 4). Our data demonstrate that the two types of γ/δ TCL express different subset of γ/δ TCR. We found that 10 out of 11 γ/δ HSTCL expressed the Vδ1 gene, whereas all 4 γ/δ SPTCL used the Vδ2 gene ($P = 0.001$, Fisher's exact test). This result indicates that selection of the specific Vδ subtype is lymphoma type-dependent. The results of two previous, rather limited studies on Vδ gene usage which evaluated a total of 6 γ/δ HSTCL[10,15,18] and 2 γ/δ SPTCL,[31] support our conclusion. In these studies, 4/6 γ/δ HSTCL expressed the Vδ1 gene, and in the remaining 2 γ/δ HSTCL cases, an unidentified V gene was used. In contrast, both γ/δ SPTCL expressed the Vδ2 gene. It is well established that normal γ/δ T lymphocytes which reside in spleen express predominantly the Vδ1 gene, whereas most γ/δ T lymphocytes present in subcutis express the Vδ2 gene.[42] The identified unique Vδ gene usage patterns in γ/δ HSTCL and γ/δ SPTCL reflects local predominance of either Vδ1+ or Vδ2+ subset within their normal T cell counterparts. This strongly suggests that both γ/δ HSTCL and γ/δ SPTCL are derived from the local lymphoid tissue. Vδ subtype analysis; rather limited studies on other γ/δ T cell lymphomas also suggested preferences in Vδ usage. Two out of three precursor T cell lymphomas involving lymph nodes expressed the Vδ1 (the third expressed an unidentified Vδ gene),[43] 3/3 nasal γ/δ TCL expressed the Vδ2 gene,[31] 2/3 gastro-intestinal γ/δ TCL expressed the Vδ3, and 1 expressed the Vδ2 gene.[31] In a large study on γ/δ T cell acute lymphoblastic leukemia, the vast majority (26/30) of cases, similarly to γ/δ HSTCL, expressed the Vδ1 gene, 2 cases used the Vδ2 gene, one case used the Vδ3 gene and 2 cases used the Vα gene rearranged to the Jδ1 segment.[44] The Vδ

gene expression pattern in TCR γ/δ+ T-ALL resembled that of TCR γ/δ+ thymocytes and differed markedly from that of peripheral blood γ/δ+ T cells. These data support the conclusion that T-ALL is a malignant counterpart of thymocytes rather than peripheral blood γ/δ+ T cells.

A detailed immunophenotypic analysis, performed in γ/δ HSTCL and γ/δ SPTCL showed a similar pattern of T-cell associated antigens (CD3+, CD2+ CD7+, CD5−, CD4−, CD8−). All lymphomas expressed also NK-associated antigens, but some differences were observed. Five out of six γ/δ HSTCL tested expressed both CD16 and CD56, whereas all 3 γ/δ SPTCL tested expressed only CD56 and, finally, 1 γ/δ HSTCL, which used an unidentified Vδ gene, expressed neither CD16 nor CD56 but did express CD11c antigen. Furthermore, in contrast to γ/δ SPTCL, α/β SPTCL do not express CD56.[26] Taken together, the above data suggest a relationship between the type of TCR and a pattern of expression of NK cell-associated markers among various types of hepatosplenic and subcutaneous TCL. However, the number of the TCL cases analyzed by us and others is still too small to draw any definitive conclusions in this regard.

It is often difficult to diagnose TCL on histological grounds alone, especially the cases involving skin. Molecular analysis has proven to be very useful in this respect. Detection of a clonally rearranged TCRγ gene often allows to distinguish T cell lymphoma from benign, reactive T cell proliferation or B cell lymphoma highly enriched in reactive T lymphocytes.[45,46] Our study indicates that analysis of Vδ gene usage may be helpful in diagnosis and proper classification of γ/δ TCL. The observed dichotomy in the Vδ gene usage between γ/δ HSTCL and γ/δ SPTCL indicates that analysis of expression of the Vδ gene subtype by either molecular or immunological method may permit better discrimination among different types of γ/δ TCL, particularly in the clinically advanced, generalized cases with multi-organ involvement. Furthermore, because Vδ gene rearrangements show an extensive diversity of the joining site, lymphoma-specific probes could be developed to monitor minimal residual disease in γ/δ TCL.[18]

In summary, our results indicate that hepatosplenic and subcutaneous panniculitis-like γ/δ T cell lymphomas are derived from different Vδ subsets of γ/δ T lymphocytes. Whereas γ/δ HSTCL belong usually to the Vδ1 subset, γ/δ SPTCL represent the Vδ2 subset. The exact properties of either normal or malignant Vδ1+ and Vδ2+ γ/δ T cells leading to this different, tissue-specific expression of the Vδ subsets have not been determined. Whether the restricted, highly lymphoma type-specific Vδ gene expression in γ/δ HSTCL and γ/δ SPTCL plays a role in the pathogenesis of these lymphomas also remains to be determined.

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