T-γ lymphoproliferative disease (T-γ LPD) is a chronic disorder of mature T cells that is associated with neutropenia and autoimmune phenomena. Although the progression of the lymphoproliferation is indolent, it is often associated with a monoclonal proliferation of T-cell–type large granular lymphocytes (LGL) that manifest multiple in vitro suppressor and cytotoxic activities. We considered the possibility that the granulocytopenia or anemia might represent an autoimmune disorder mediated by the monoclonal LGL via T-cell receptor (TCR) recognition of an antigen involved in hemopoiesis. Therefore, in an effort to characterize the usage of the TCR α- and β-chain genes in patients with T-γ LPD, we cloned and sequenced TCR α- and β-chain mRNAs derived from the T-cell type LGL of five patients. The five patients studied did not use a common Vα nor a common Jα segment. However, an unusual finding was observed in one of the patients where the occurrence of a single variable diversity-junctional (VDJ) rearrangement of the β chain confirmed the monoclonal origin of the LGL proliferation. In accord with this evidence for monoclonality, many of the cells studied used a common Vα (Vα19.1). In contrast to this common Vα usage, there was a marked diversity of the Jα segments and N-region addition that were associated with the Vα19.1 segment. This pattern of common Vα usage associated with different N and Jα segments suggests an immune-mediated selection process affecting the TCR α chain occurring after the transformation event that established the clone. We suggest that the T-cell type LGL malignant clone might have developed autoreactivity conferred by the selected TCR α chain and that this autoreactivity might be implicated in this patient’s anemia.

The syndrome of large granular lymphocyte (LGL) proliferation was first described in 1975 by Brouet et al1 in patients with chronic lymphocytic leukemia of T-cell origin and by McKenna et al2 in 1977 in patients with chronic neutropenia associated with an increased number of circulating LGLs. Since the initial reports, many more cases have been described under a variety of names.*

The clinical, cellular, and functional characteristics of these proliferations have been extensively studied1-12 and were recently reviewed.13 In most cases, the disease follows a chronic course characterized by peripheral blood lymphocytosis with splenic and bone marrow infiltration and mild to severe neutropenia with recurrent infections. Although many clinical manifestations such as pure red blood cell aplasia or autoimmune phenomena (Coombs-positive hemolytic anemia and rheumatoid arthritis) may significantly contribute to the morbidity, the overall prognosis is predominantly dictated by the severity of the neutropenia. Two major types of LGL proliferations have been described and the phenotypes correlated to distinct functions.14 The more common type (A or T-cell–type [T-LGL] leukemia) consists of CD2+, CD3+, CD8−, CD40− cells. Most are also CD57+ (HNK1/Leu7), CD16+ (Leu11) (the presence of Fcγ receptor is often only detected by ox erythrocyte rosetting) and CD56− (HNK1). Functionally, these cells do not manifest natural killer (NK) activity but variable degrees of antibody-dependent-cell-mediated cytotoxic activity (ADCC). The less common type (B or NK type or NK-LGL leukemia) usually consists of CD2− and CD56− (HNK1) but CD3+, CD8+, CD16+ and CD57+ cells that usually manifest both NK and ADCC cytotoxic activities. In many cases of LGL proliferations, LGL-mediated suppressor activities can be shown in vitro, including LGL suppression of T-cell mitogen or mixed lymphocyte culture responses, suppression of the immunoglobulin synthesis of mitogen-stimulated B cells, and suppression of the generation of erythrocytoid progenitors in patients with pure red blood cell aplasia.15-17

Because of the largely chronic and indolent course of the disease and in light of the preserved phenotype and functions, LGL proliferations were for many years believed not to be monoclonal leukemias. However, this belief was challenged in multiple reports18-23 where the LGL proliferation were shown to be monoclonal by Southern blot analysis using probes for the T-cell receptor (TCR) β and γ subunits. The results of these studies can be summarized as follows: in general, the T-cell type proliferation (CD2+, CD3+, CD8+CD4−) shows a monoclonal pattern when assessed using a TCR constant-region β probe. The less common NK type proliferation (CD2−, CD3−, CD8−) shows neither a monoclonal pattern of TCR gene rearrangement nor even the pattern of polyclonal rearrangement seen in peripheral blood T cells suggesting that these cells do not rearrange their TCR genes.22

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mon clinical features, granulocytopenia, or anemia, we considered the possibility that the hematologic abnormalities might represent a T-cell-mediated autoimmune disorder that involves recognition by the TCR of the clonal T cells of an antigen on a protein required for granulopoiesis or erythropoiesis. If this were the case, there might be preferential use of specific TCR variable region genes. Therefore, in an effort to characterize the usage of the TCR \( \alpha \) and \( \beta \) genes in patients with LGL proliferation, we cloned and sequenced TCR \( \alpha \) and \( \beta \)-chain mRNAs derived from the peripheral blood LGLs of five patients with T-LGL leukemia. In addition, we present the unusual findings observed in one patient where the presence of a single V-D-J rearrangement of the \( \beta \) chain confirms the monoclonal origin previously determined by Southern blot analysis. Furthermore, in this case, many of the cells studied had a common V\( \alpha \) usage. However, this common V\( \alpha \) was associated with a multiplicity of \( \alpha \) chain N region additions and J segment rearrangements. This pattern of common V\( \alpha \) usage associated with different N and J segments suggests an immune-mediated selection process affecting V\( \alpha \) that occurred after the transformation event that established the clone in this case.

MATERIALS AND METHODS

Patients

Five patients with T-cell-type LGL proliferation were studied for TCR rearrangement. All five patients had a long-standing diagnosis of T-LGL proliferation. The main clinical characteristics are summarized in Table 1 and the phenotypes of peripheral blood mononuclear cells (PBMC) at the onset of the disease are shown in Table 2. The onset of symptoms preceded by several years the present TCR studies. Three patients were severely neutropenic (absolute neutrophil count: \(<500/mm^3\)) with recurrent, life-threatening infectious episodes (leading to one patient’s death). Three patients had chronic anemia (requiring chronic blood transfusion in one patient). Arthritic manifestations were present in two patients. The monoclonality of the LGL proliferation was shown by Southern blot analysis in each case (Fig 1).

Patient no. 5 (the primary focus of this report). This patient was first seen in the Biological Response Modifiers Program of the National Institutes of Health in May 1986. He had a history of T-LGL proliferation with pure red blood cell aplasia for several years. He is the sole patient with pure red blood cell aplasia, although two of the other four patients were anemic. The patient was treated with interferon alpha (\( \alpha \)) (3 million units at first, and then 1.5 million units subcutaneously per day) from May 1986 until September 1987 with periods of interruption necessitated by interferon-related fatigue. This treatment resulted in an increase in bone marrow erythroid precursors that allowed transfusion independence but did not lead to a change in the lymphocytosis. In August 1987, the patient developed moderately severe arthritis requiring treatment with prednisone, 10 mg daily, a nonsteroidal anti-inflammatory agent, and hydroxychloroquine. Interferon therapy was discontinued in September 1987, and the patient has remained transfusion independent (with a hematocrit of 40%) until 1988 when he was lost to follow-up. The RNA used in the present studies was isolated from a sample of PBMC and the TCR usage was studied only once, in December 1987.

DNA and RNA Isolation

PBMC were obtained from the patients by cytophoresis and were flash frozen. Genomic DNA used for Southern blot analysis and

---

Table 1. Summary of Patients’ Clinical Manifestations

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Severe Fluctuating Neutropenia</th>
<th>Life Threatening Infections</th>
<th>Anemia</th>
<th>Arthritis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+ M. meningitis + sepsis</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+ Recurrent infections</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>+ Mouth ulcers, polymicrobial</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 2. Phenotype of Patients at the Time of Diagnosis (expressed in percentage of positive cells)

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Pt No.</th>
<th>Pt No.</th>
<th>Pt No.</th>
<th>Pt No.</th>
<th>Pt No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>T11 (CD2)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Leu4 (CD3)</td>
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<td></td>
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<tr>
<td>Leu2a (CD8)</td>
<td>83</td>
<td>87</td>
<td>95</td>
<td>96</td>
<td>83</td>
</tr>
<tr>
<td>Leu3a (CD4)</td>
<td>58</td>
<td>72</td>
<td>86</td>
<td>90</td>
<td>79</td>
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<tr>
<td>Mo1 (CD11b)</td>
<td>24</td>
<td>8</td>
<td>14</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Leu1 (CD5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu7 (MNKG1/CD57)</td>
<td>19</td>
<td>64</td>
<td>3</td>
<td>77</td>
<td>64</td>
</tr>
<tr>
<td>NKH1 (CD56)</td>
<td>3</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Leu11 (CD16)</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>36</td>
</tr>
<tr>
<td>HLA DR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu9 (3A1/CD7)</td>
<td>72</td>
<td>19</td>
<td>-</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>Tac (CD25)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

(---): not determined.
"937" 5′ ACA TTT GTT TGA GAA TCA 3′
"645" 5′ ACA CCA TGG CCT TTT 3′
"933" 5′ GAT GAA  TTC  TGT  GAG  TCT  CCT  TTT  3′
"646" 5′ AAT GAA TCT  TGT  GAG  TCT  CCT  TTT  3′
"934" 5′ GTA  ACG  ATC  ACG  GAT  CCG  CCC  CCC  CCC  CCC  CC  3′
"933" 5′ GAT GAA  TTC  GGT  GAA  TAG  GCA  GAC  AGA  CTT  G
"649" 5′ CTG GAT TTA  GAG  TCT  CTC  AGC  TGG  TAC  ACG  GCA  GGG  TCA  GGG TTC  TGG  ATA  3′
"934" 5′ GTA  ACG  ATC  ACG  GAT  CCG  CCC  CCC  CCC  CCC  CC  3′
"645" 5′ ACA TTT GTT TGA GAA TCA 3′
"647" 5′ CTG ATG GCT CAA ACA CAG CAA CTT CCT GTG AAT  3′

Fig 2. Sequences of oligonucleotide primers and probes. "937," "645": constant-region primers for α and β chain, respectively, used in first strand cDNA synthesis. "933," "646": constant-region nested primers for α and β chain, respectively, used in PCR amplification of both chains; these primers contain a BamHI site. "645" and "647": constant-region "negative" strand probes of TCR α and β chains, respectively.

Southern Blots

Genomic DNA Southern blot. Ten micrograms of genomic DNA was digested with 20 U of restriction enzyme (EcoRI, BamHI, or HindIII) for 4 hours at 37°C then run on a 0.65% agarose gel for 18 hours at 35 V. The gel was washed twice for 15 minutes, first in 1 mol/L NaCl and 0.5% NaOH, then in 1 mol/L Tris and 1 mol/L NaCl. The gel was exposed to UV light for 45 seconds before transfer onto nitrocellulose for 24 hours in the presence of 20X SSC (3 mol/L NaCl, 0.3 mol/L sodium citrate). The blot was then heated for 1 hour at 80°C and then incubated (prehybridized) for 3 hours at 60°C in 15 mL of a solution of 10% (wt/vol) nonfat dry milk, 10% formamide, 1% sodium dodecyl sulfate (SDS), 0.5 mg/mL Na heparin, 0.2 mg/mL salmon sperm DNA, 0.9 mol/L NaCl, 2 mmol/L EDTA, and 50 mmol/L sodium phosphate. After the solution was then removed and 50 X 10^6 cpm 32P-labeled, heat-denatured probe (random priming) were added and incubated at 60°C for 18 hours. The blot was washed for 30 minutes at 65°C in 0.5 mol/L NaCl, 0.1 mol/L Tris (pH 6), and 1% SDS then in 0.1X SSC, 0.1% SDS at 65°C. The blot was autoradiographed with an intensifying screen for 72 hours at -70°C.

First Strand Synthesis and 3′ Tailing

The method used was a modification of the method by Frohman et al.25 The reactions for the α-chain and β-chain analysis were performed in separate tubes along with separate control tubes. Ten micrograms of total RNA were treated at room temperature with 10 mmol/L methyl mercaptoethanol (140 mmol/L) and RNasin were added and left for 2 minutes at room temperature. Reverse transcription was then performed in the presence 2 mmol/L of high-performance liquid chromatography (HPLC) purified synthetic oligonucleotides complementary to the constant region of the α ("937") or β ("645") chains (sequences shown in Fig 2) and Avian Myeloblastosis Virus Reverse Transcriptase (Life Sciences, St Petersburg, FL) at room temperature for 10 minutes then at 42°C for 1 hour in 50 μL final volume of 50 mmol Tris (pH 8.3 at 4°C), 5 mmol/L MgCl2, 75 mmol/L KCl, 10 mmol/L dithiothreitol, and 1 mmol/L of each deoxynucleotide triphosphate. Free oligonucleotides were then removed by spermine precipitation at 4°C.24 3′ Tailing was then performed with deoxyguanosine triphosphate for 1 hour at 37°C using the Boehringer Mannheim 3′ end labeling kit and following the manufacturer's recommendations. The 25-μL reaction mixture was then heated at 70°C for 5 minutes and then kept on ice. Twenty-five microliters of sodium acetate (10 mol/L, 2 μL EDTA (50 mmol/L), and 73 μL of water were added before ethanol precipitation. After centrifugation and 70% ethanol rinse, the pellets were dissolved in 10 μL of water and used for tail PCR amplification.

Tail PCR Reactions

Primers. Primer sequences are shown in Fig 2. The 3′ end (negative strand) nested primers were chosen from the constant-region sequences, immediately 5′ to the primers used for the first strand synthesis and contained an EcoRI restriction site used in the subcloning of the amplified material. Primers "934" and "916" were positive-strand primers used in both α and β amplification and contained a BamHI restriction site for subcloning purposes.

Reaction. The PCR was performed in separate tubes for α and β chains in 100 μL of 2 mmol/L DTT, 50 mmol/L KCl, 10 mmol/L Tris HCl (pH 8.3), 3 mmol/L MgCl2, 0.01% gelatin, and 1 mmol/L of primer "933" (α chain) and "646" (β chain). 5′ end primers "934" and "916" were used at a final concentration of 0.1 and 1 μmol/L, respectively. Two microliters of AmpliTaq polymerase (Cetus, Emoryville, CA) were used for each reaction. Twenty microliters of mineral oil was then added and the mixture was kept at 4°C. The 10 μL of template were added last and tubes were placed in the Thermocycler (Perkin Elmer-Cetus, Norwalk, CT).

Cycles. The initial cycle was for 5 minutes at 94°C and then five of the following cycles: 94°C for 1 minute, 45°C for 1.5 minutes, and 72°C for 2.5 minutes; only then was primer "916" added to each tube and 25 of the following cycles were performed: 94°C for 1 minute, 55°C for 1.5 minutes, 72°C for 2.5 minutes.

Precautions and controls. The manipulations before the PCR step (preparation of all stock solutions and their aliquots, first strand synthesis, tailing, and PCR preparation) were never done in a room in which PCR-amplified products were manipulated. All pipetting equipment was exclusively used for the PCR preparation steps. Pipetting tips were plugged and used only once, so that aliquot tubes were always entered with a new, sterile pipet tip. Aliquots prepared from each stock solution were discarded after a single day's use. Sterile gloves were worn at all times and changed frequently. Two negative control tubes (one with α-chain mixture and one with β-chain mixture) containing yeast tRNA only were carried from the beginning of first strand synthesis and amplified with specific Va and Vβ primers rather than the anchor and anchor-tail primers. Each patient's
RNA was processed on a separate day to further minimize the risk of cross contamination. After PCR, the unopened tubes were carried to another room for the subcloning and sequencing steps.

**Subcloning and Sequencing**

The specificity of amplification of fragments of expected length and the absence of contamination were confirmed by Southern blot analysis (see above) of an aliquot of the PCR products with α- and β-chain constant region probes ("649" and "647," respectively; see Fig 2). The remainder of the PCR products were extracted once with phenol/chloroform-isomyl alcohol and once with chloroform-isomyl alcohol, precipitated with spermine, digested with BamHI and EcoRI for 1.5 hours, and run on 1% LMP agarose gel (Seaplaque) in 1× TBE for 3 hours. Faint bands of expected size were cut out and DNA was extracted several times with phenol and phenol/chloroform-isomyl alcohol, and then chloroform-isomyl alcohol, and then ethanol precipitated. One third of the prepared inserts were ligated overnight at 14°C into 50 ng of Puc18 previously linearized and phosphatased (Cip, Boehringer Mannheim, Indianapolis, IN). DH5α (competent cells (BRL GIBCO, Gaithersburg, MD) were transformed and plated under ampicillin selection. Plasmid DNA was prepared from amplified colonies according to the method of Del Sal et al., digested with BamHI and EcoRI, and run on 1% agarose gel. Greater than 95% of the clones contained an insert of expected size. Sequencing of independent clones was performed in Terasaki plates with a "Sequenase" kit (US Biochemical, Cleveland, OH).

**Computer Analysis of Sequences**

All sequence analyses and sequence homology searches were done using PC-GENE software (Intelligenetics, Mountain View, CA) and using EMBL and GENBANK databases.

In a first step, all the subclones with identical V-segment sequence were analyzed simultaneously, first for amino acid then for nucleotide sequence homology using the multiple sequence analogy program CLUSTAL of the PC-GENE software package. To minimize the illustrations of sequence data, only data not representing 100% homology among the subclones is presented in a figure.

In a second step, a subclone containing the identical V segment for the TCR chain under study was compared with the most recent sequence data from both the GENBANK and EMBL databases. The EMBL 34 database (CDM34HU) contains all primate sequence data and the Unique GENBANK 76-34 database (CDUG-BANK) contains sequences included in GENBANK that are not present in EMBL. This study was performed for each of the α and β chains but is illustrated in a figure only in the case of the β chain in patient no. 5.

**RESULTS**

The TCR Vα and Vβ usage were defined for five patients with T-cell-type LGL leukemia associated with neutropenia or anemia. Using a DNA probe of the human TCR β-chain constant region, the PBMC of these five patients with T-LGL leukemia were shown to contain a population of cells that were monoclonal, because they exhibited a monoclonal pattern of TCR β rearrangement. Specifically, DNA derived from the PBMC from each patient manifested a nongermline band on Southern blot analysis (BamHI digests shown in Fig 1A). PCR amplification, cloning, and sequencing were then used to define the sequence of the TCR α chain in all five patients and of the β chain in two patients. The PBMC preparations for the patients contained polyclonal normal T cells in addition to the T-LGL expansion. To identify the TCR α- and β-chain sequences of the monoclonal population, a series of independent sequences (subclones) were obtained from the PBMC DNA of each patient and compared with the other sequences for the same patient. The sequences for a given patient that were identical to that of other subclones of the same patient were deemed to be the sequence of the V-(D)-J segment of the LGL clone for that patient (Table 3). Different TCR Vα segments were involved in the LGL clone of the five patients. One patient each used Vα16, Vα8.1, Vα31, Vα "FR.1," and Vα19.1. The α-segments used in association with the Vα segment were IGRJA04, IGRJA14, TCRADC04, and JaG, respectively, for patients 1 to 4. The Ja usage for patient no. 5 is detailed in Table 4. The two Vβ segments cloned and sequenced were then used to define the sequence of the β chain in patient no. 5 (see below). Therefore, no pattern of preferential use of Vα or Vβ could be shown in these five patients from an analysis combining Southern blot of genomic DNA and cloning and sequencing of TCR messages. This observation may reflect the fact that the patients were not HLA identical and that they had different clinical manifestations, with granulocytopenia being the hematologic abnormality in three cases and anemia in three cases (in one case, both anemia and granulocytopenia). However, the findings in patient no. 5 merit detailed presentation.

**Table 3. Vα, Ja, and Vβ Usage in the LGL Clone of Each Patient**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Vα Use</th>
<th>Ja Use</th>
<th>Vβ Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vα16*</td>
<td>IGRJA04</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>Vα8.1</td>
<td>IGRJA14</td>
<td>Vβ7</td>
</tr>
<tr>
<td>3</td>
<td>Vα31</td>
<td>TCRADC04</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>Vα FR 1</td>
<td>JaG</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>Vα19.1</td>
<td>Variable</td>
<td>Vβ4</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not done.

* See footnote in text.

---

**Table 4. Ja Usage in the 8 Vα19.1 Subclones in Patient no 5 (see Fig 6)**

<table>
<thead>
<tr>
<th>Subclone</th>
<th>Ja Used</th>
<th>Comments</th>
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<tbody>
<tr>
<td>1</td>
<td>U</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>IGRJA06</td>
<td>Different N region than 3</td>
</tr>
<tr>
<td>3</td>
<td>IGRJA06</td>
<td>Different N region than 2</td>
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<td>4</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>U</td>
<td>J 81% identical to 1</td>
</tr>
<tr>
<td>6</td>
<td>I</td>
<td>VNJ 100% identical to 7</td>
</tr>
<tr>
<td>7</td>
<td>I</td>
<td>VNJ 100% identical to 6</td>
</tr>
<tr>
<td>8</td>
<td>Ja</td>
<td>bp 4170–4230 of HSTCRADC04*</td>
</tr>
</tbody>
</table>

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The denominations Vα15 and Vα16 used by Klein et al. no longer appear in the EMBL database (release 34). Vα16 refers to the Vα segment used in the sequence HSTCAYXY of the EMBL 34. Vα15 now appears as Vα19.1.

Additional note:

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Abbreviation: ND, not done.

* See footnote in text.
TBC04 1- CTAGGCTCTGGTTCCTGCTGTCTAATCTCTCAGCAAAGGCGAGGATAT

HSTCBXY 8- CTAGGCTCTGGTTCCTGCTGTCTAATCTCTCAGCAAAGGCGAGGATAT

BETA04 51- CTGTCACAGTGGAGCTCCCTGACAGTACAGTGCGCTGACCTG

HSTCBXY 58- CTGTCACAGTGGAGCTCCCTGACAGTACAGTGCGCTGACCTG

BETA04 101- TCACCAGACCTGTTCCCTCTAAGCGACGCTACGTCAGTGATC

HSTCBXY 108- TCACCAGACCTGTTCCCTCTAAGCGACGCTACGTCAGTGATC

BETA04 151- ATCGGAACTGCAATTCGAGGCTAGAGTACTAGTATC

HSTCBXY 158- ATCGGAACTGCAATTCGAGGCTAGAGTACTAGTATC

BETA04 201- CATTGACAGTTTCCATCCGCCCCACACCTTAATATTCTCCACTCAG

HSTCBXY 208- CATTGACAGTTTCCATCCGCCCCACACCTTAATATTCTCCACTCAG

BETA04 251- CTGTCAGACACCTGAGCCTGAGACAGACGCTACAGTGAGCTGAC

HSTCBXY 258- CTGTCAGACACCTGAGCCTGAGACAGACGCTACAGTGAGCTGAC

BETA04 301- GAACTCTGCGGAAGCTATCTCGGAGCGAGCGACGCTACAGTGAGCTGAC

HSTCBXY 308- GAACTCTGCGGAAGCTATCTCGGAGCGAGCGACGCTACAGTGAGCTGAC

BETA04 346- ACCGTCTAGCGAGCCTGAAACAC

HSTCBXY 356- ACCGTCTAGCGAGCCTGAAACAC

β Chain of Patient No. 5

Using a TCR β probe, the PBMC of patient no. 5 were shown to contain a population of cells with a monoclonal TCR rearrangement (Fig 1b). Five of 12 TCR β subclones (42%) studied in this patient had a completely identical β-chain sequence. The five subclones showed complete homology with the Vβ4 and Cβ2 sequences of the EMBL database (Fig 3). The D and J segments used in these five subclones were Dβ2.1. The Jβ2.1 segment lacked the six most 5' bases of the germline sequence and the Dβ2.1 lacked the two most 5' and the five most 3' bases of the germline sequence. There is a 3-base N region between D and J. The seven other sequenced subclones from the peripheral blood T cells show V-D-J rearrangements that differed from each other and from the five identical subclones discussed above (data not shown). Presumably, these represent subclones that reflect the Vβ usage of the polyclonal T cell that contaminated the monoclonal patient’s PBMC. The sequence data also confirm the usage of Cβ2 by the clonal proliferation shown on the genomic Southern blot analysis (presence of a nongermline band on the BamHI and HindIII digests and absence of a new band on the EcoRI digest; Fig 1b). This pattern of variable region nucleotide sequences of 12 subclones is consistent with the hypothesis that the five identical subclones represent TCR β-chain mRNAs from a T-cell lineage LGL monoclonal proliferation, whereas the seven remaining subclones represent mRNAs from normal circulating polyclonal lymphocytes.

α Chain in Patient No. 5

In analysis of the TCR α-chain usage in patient no. 5, 8 of the 20 TCR α subclones (40%) obtained by cloning of the PCR-amplified material from the patient’s PBMC had a TCR α-chain sequence that showed identity limited to the V and C regions, whereas each of the remaining 12 subclones had unique V-J sequences. In Fig 4, we show the identity in the eight similar subclones that employed the Vα19.1 segment in association with the Cα segment. The amino acid and nucleotide alignments of the sequences with a common Vα (starting at the initiation codon) are shown in Figs 5 and 6, respectively. All but subclone “8” appeared functional, because they manifested an open reading frame from the leader sequence through the constant region. In contrast to the identity in Vα usage in these eight subclones, the J segments used in association with the identical Vα19.1 segments were very diverse (Table 4). All eight subclones used previously published Jα sequences along with various N regions (there were no P nucleotides): specifically, Jα U was used by subclone “1.” Jα P by subclone “4,” and Jα I by subclones “6” and “7.” Subclones “2” and “3” used the same Jα (IGRJA06), but their N region was different. Subclone “5” used a segment 81% identical to Jα U. Subclone “8” used a different Jα segment. Only subclones “6” and “7” manifested 100% identity of the entire L-V-N-J-C coding sequence.

Thus, in summary, although 8 of 20 subclones obtained for the analysis of the PBMC of patient no. 5 manifested identical use of the Vα19.1 segment, they used different Jα sequences and various N region additions.

**DISCUSSION**

We studied the Vα usage in five patients with monoclonal T-cell-type LGL proliferation associated with neutropenia or anemia. The five patients did not use a Vα segment in...
their respective clonal population that was the same as used by the other patients. This lack of common Vα usage is probably a reflection of the human population diversity in terms of major histocompatibility complex (MHC) genotypes that in part determine TCR V segment usage.

Despite the lack of common Vα usage among five patients, we present here evidence for a monoclonal proliferation with an unusual pattern of TCR α-chain rearrangements in a patient with T-LGL leukemia (patient no. 5) that might reflect an antigenic selection process. The TCR β-chain rearrangement was identical in 5 of the 12 TCR β subclones. This is highly unlikely to occur by chance alone when one considers the very large number of possible combinations of V-D-J segments and N additions. This view is supported by the work of Rosenberg et al., who used a methodology similar to ours and showed lack of common V-J TCR β usage by polyclonal normal PBMC. Therefore, the five common TCR β transcripts from patient no. 5 presumably represent transcripts from cells of the LGL clone. This observation of clonality at the Vβ level confirmed the preliminary view, based on genomic Southern blot data, that this patient (like the other four) had a circulating monoclonal population of T-LGLs. The demonstration of a common TCR β-chain gene rearrangement pattern on Southern blot analysis has for many years been the standard criterion used to establish monoclonality of a T-cell population at a molecular level. However, the size of the α-chain locus has, in the general case, precluded its clinical use in Southern blot analysis of clonality.

We used an analysis of the Vα locus of patient no. 5 as part of the LGL-Vα usage study and to define the stage of maturation of the T-cell when the transformation occurred in this case. Cell-surface phenotype, cellular functions, and the presence and number of rearranged TCR genes have all been used to determine the stage of maturation at which the determinant oncogenic event occurs in T-cell malignancies. The hallmark of the more mature T cells are cell surface expression of CD2, CD3, along with CD4 or CD8, DNA rearrangement of TCR γ and β genes, or mRNA expression of TCR α and β chains. Clinically, the malignant nature of the LGL proliferations had long been questioned. Indeed, although aggressive forms of the disease do exist, the patients' outcomes are largely dictated by the severity of the neutropenia and its associated infectious complications and not by the lymphoproliferation. Like normal NK, CD2+, and CD3- cells, the rarer NK-LGL proliferations (also CD2+, CD3-) do not rearrange their TCR genes and are not of the classic mature T-cell lineage. However, the LGL in the vast majority of T-LGL proliferations (CD2+, CD3-, CD8-) do rearrange these genes, express TCR α and β mRNAs, and manifest cell-surface proteins showing their mature T-cell lineage. Furthermore, their monoclonal pattern on Southern blot with a TCR β-chain probe has been shown in multiple reports. It had been assumed that
the pattern of α-chain rearrangement also was monoclonal and, therefore, that the oncogenic event occurred after the TCR rearrangement process was completed, particularly because T-LGL express functional CD3 and TCR molecules on their surface. We performed cloning and sequencing of the α-chain mRNA to test this hypothesis.

We undertook these studies to further elucidate the TCR gene usage in this disease. Indeed, by cloning and sequencing of α- and β-chain cDNAs of several patients, we have shown that, in accord with the hypothesis discussed above, there was an identical sequence manifested by the α subclones and an identical sequence in the β-region additions; J, junctional; Cst, constant region.

Clone: L... V...
1 MVKIRQFLAILWLQGLCYSVAAKNEEVQESPQNLTAQEEFTINGCSYSQGISALH 55
2 MVKIRQFLAILWLQGLCYSVAAKNEEVQESPQNLTAQEEFTINGCSYSQGISALH 55
3 MVKIRQFLAILWLQGLCYSVAAKNEEVQESPQNLTAQEEFTINGCSYSQGISALH 55
4 MVKIRQFLAILWLQGLCYSVAAKNEEVQESPQNLTAQEEFTINGCSYSQGISALH 55
5 MVKIRQFLAILWLQGLCYSVAAKNEEVQESPQNLTAQEEFTINGCSYSQGISALH 55
6 MVKIRQFLAILWLQGLCYSVAAKNEEVQESPQNLTAQEEFTINGCSYSQGISALH 55
7 MVKIRQFLAILWLQGLCYSVAAKNEEVQESPQNLTAQEEFTINGCSYSQGISALH 55

Fig 5. Patient no. 5 α-chain subclones: multiple protein sequence alignment. The alignment was done on the seven functionally rearranged, translatable sequences. (*) or (.) show that a position is perfectly or well conserved, respectively.

The the a-chain mRNA to test this hypothesis.

We undertook these studies to further elucidate the TCR gene usage in this disease. Indeed, by cloning and sequencing of α- and β-chain cDNAs of several patients, we have shown that, in accord with the hypothesis discussed above, there was an identical sequence manifested by the α subclones and an identical sequence in the β subclones representing the T-LGL clone in the majority of patients we studied. However, this was not the case in patient no. 5 presented in this report. In this case, the unique β rearrangement confirmed that oncogenic event occurred in the LGL after β-chain DNA rearrangement had been completed, but the variations observed in the pattern of α-chain rearrangement indicate that this transformation event occurred before completion of the α-chain rearrangement. This pattern is consistent with the normal hierarchy of TCR rearrangement with β-chain preceding α-chain rearrangement. Specifically, during thymocyte maturation, the β-chain transcript level remains relatively constant, whereas γ-chain transcripts are abundant early in differentiation and fall to a very low level in mature cells. In contrast, α-chain mRNAs are barely detectable early in thymic T-cell development and then increase during the maturation process. α Chain expression is thought to be the final step in αβ TCR-CD3 expression, which in turn leads to the downregulation of the recombinaque.

If it is clear that in patient no. 5, malignant transformation occurred before α-locus rearrangement, one might have anticipated that Va usage would be different in the different subclones examined, thereby showing the absence of selection in Va usage. In contrast to this possibility, α rearrangement did not take place randomly as this T-LGL clone with a fixed β chain differentiated to maturity. The pattern of Va usage observed, with a predominance of a single Va segment (Va19.1) with different Jα and N-segments in 40% of the subclones examined, is highly suggestive of the selection of a limited number of TCR α-chain molecules in a still differentiating and functional LGL clone. A similar pattern of TCR rearrangement has been observed in i cells responding to a particular antigen. A well-characterized system is the response to pigeon cytochrome c in a mouse model: T-cell clones, proliferating in response to pigeon cytochrome c, were found to use exclusively one Va segment and a limited number of Jα segments, whereas the usage of Vβ was more diverse. Evidence for limited usage of Vα as well as Vβ segments in human autoimmune pathology has been recently presented for multiple sclerosis and rheumatoid arthritis. In addition, variability in the J region of the chain can result in alteration of the reactivity to the same antigen. As in clones of same MHC restriction and antigen specificity has been found to be responsible for the presence of added alloreactivity. In light of the preferential Va usage with distinct Jα- and N-region use in this case, it is tempting to speculate that the LGL proliferation of patient no. 5 was partially under antigenic pressure. In concert with the final TCR specificity contributed by the invariant β chain, this might have led to the formation of some TCR molecules with autoreactivity responsible for the clinical autoimmune phenomena.

Finally, it must be noted that the pattern of TCR α- and β-chain rearrangements in patient no. 5 are reminiscent of what is observed during superantigen selection of TCR molecules. As in rheumatoid arthritis, a role for superantigen-driven selection could be speculated in this case, although it must be noted that, to our knowledge, superantigens have been reported to be involved in the selection of
only the TCR β chain and not the TCR α chain, the chain apparently being under antigenic pressure in our case.

In conclusion, our analyses of the TCR rearrangement of LGL in five patients with T-LGL leukemia indicates that these cells in a given patient have a common clonal origin. Furthermore, these analyses support the view that, at least in one patient, the transformation took place after TCR β but before TCR α-chain rearrangement. However, in this unusual case, the TCR α-chain rearrangement manifested a pattern characterized by preferential Va usage associated with diversity of the N-region addition and the Ja segments used. This pattern of TCR α rearrangements suggests the possibility that a selection process affected the choice of Va used as this clone with a common TCR β rearrangement matured. Antigenic pressure (possibly a superantigen) might have provided such a selection process that might ultimately have led to the development of a malignant clone that might be linked to the patient’s autoimmune phenomen.

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T-cell receptor gene rearrangement in T-cell large granular leukocyte leukemia: preferential V alpha but diverse J alpha usage in one of five patients

C Kasten-Sportes, S Zaknoen, RG Steis, WC Chan, EF Winton and TA Waldmann

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