Molecular Diagnosis of Cutaneous T-Cell Lymphoma: Polymerase Chain Reaction Amplification of T-Cell Antigen Receptor β-Chain Gene Rearrangements

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The goal of our study was to molecularly diagnose CTCL, by cloning the T-cell antigen receptor beta chain (TCR-β) gene rearrangement from the malignant T cells of a patient with Sézary syndrome, in order to generate a specific oligonucleotide probe capable of detecting CTCL cells through polymerase chain reaction (PCR) amplification.

Total RNA isolated from peripheral blood lymphocytes was reverse transcribed and resultant first strand cDNA was PCR amplified utilizing a concensus primer to the TCR-β variable region (V_β) and a 3′ primer to the TCR-β constant region (C_β). PCR reaction products were subcloned into a plasmid vector and sequenced.

Sequence analysis revealed that the patient's in-frame TCR-β gene rearrangement utilized V_β6.4, D_β1.1, J_β2.2, and C_β2.1 gene segments. Oligo-primers to V_β6.4 and J_β2.2 were utilized to PCR amplify genomic DNA taken from the patient's blood and involved skin. Screening the amplified DNA with an oligo-probe specific for the patient's V-D-J junctional sequences resulted in the detection of the patient-specific sequences. No sequences were detected from DNA from other malignant or benign infiltrates. Thus, we have defined a "molecular fingerprint" specific for a patient's malignant T-cells and can molecularly diagnose CTCL through PCR amplification. *J Invest Dermatol 96:299–302, 1991*

Cutaneous T-cell lymphoma (CTCL) constitutes a malignant proliferation of the thymic-derived lymphocytes (T cells) that are initially detected in the skin and subsequently disseminate to lymph nodes, blood, and other visceral organs [1].

Definitive diagnosis of CTCL, especially in cases of early cutaneous involvement and occult extra-cutaneous involvement in lymph nodes, remains difficult. There are no unique and specific morphologic [2], immunophenotypic [3], or cytogenetic [4] characteristics that can be defined as the *sine qua non* of the malignant cell of CTCL. This represents a critical issue, because the prognosis of the CTCL patient and the selection of modalities used to treat the disease are greatly influenced by the extent of the disease at presentation [5].

Recent advances in the knowledge of T-cell antigen receptor (TCR) genes [which consist of variable (V), diversity (D), joining (J), and constant (C) segments] has greatly enhanced our capacity to detect and diagnose T-cell malignancies [6,7]. We and others have demonstrated a clonal TCR-β chain gene rearrangement on Southern blot analysis of DNA samples derived from cutaneous tumors, peripheral blood lymphocytes, and lymph nodes of patients with CTCL [8–10]. In this communication, we describe our technique of molecularly cloning the TCR-β gene rearrangement from malignant T cells of a patient with CTCL (Sézary syndrome) and generating a patient-specific oligonucleotide probe capable of detecting the patient's CTCL cells through polymerase chain reaction (PCR) amplification [11].

MATERIALS AND METHODS

Clinical Samples Informed consent was obtained from a patient with clinical and laboratory findings of Sézary syndrome [1] prior to aquisition of blood samples and skin biopsies. Peripheral blood lymphocytes (PBL) were isolated from heparinized whole blood by Ficoll-Hypaque centrifugation and aliquots were snap frozen in liquid nitrogen. Four-millimeter punch biopsies of clinically involved skin (erythroderma) were snap frozen in liquid nitrogen. PBL from a healthy volunteer and lymphocytes from the Jurkat T-cell leukemia cell line [12], as well as punch biopsies from a patient with atopic dermatitis and cutaneous lupus, were used as controls.

RNA Isolation Snap-frozen specimens were pulverized with sterile RNase-free mortar and pestle and undegraded total cellular RNA was isolated by the RNazol method (Cinna/Biotex Labs, Friendwood, Texas), a modification of the standard guanidium thiocynate methods [13]. Five to 15 micrograms total RNA from 10^7 cells or a 4-mm punch biopsy was obtained in approximately 4 h utilizing RNazol (guanidium thiocyanate, 2-mercaptoethanol, and phenol). After homogenization in RNAzol, chloroform extraction, isopropanol and ethanol precepitation, pellets were resuspended in

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Abbreviations:
bp: basepair
CTCL: cutaneous T-cell lymphoma
MoMLV: Moloney murine leukemia virus
PBL: peripheral blood lymphocytes
PCR: polymerase chain reaction
SS: Sézary syndrome
TCR: T-cell antigen receptor

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1 M LiCl and seven volumes of absolute ethanol and stored at −70°C.

DNA Isolation Snap-frozen specimens were pulverized with sterile mortar and pestle and processed by standard techniques of cell lysis, proteinase-K digestion, phenol extraction, and ethanol precipitation [14].

Cloning TCR-β Gene Rearrangements—PCR Amplification of cDNA Our cloning strategy for TCR-β gene rearrangements utilizes total RNA as the starting material. One to three micrograms of total RNA from PBL (isolated as above and precipitated in 1 M LiCl) was reverse transcribed (RT) with an oligonucleotide primer (C_β-N′: 5′-ACGTCGACCTCGGGTGGGAA-3′) to the TCR-β constant region (C_β) that recognizes sequences near the 5′ end of the first exon of C_β (amino acid 7–13). Total RNA was resuspended in 10 μl of RT buffer [50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT] and mixed with 2.5 mM dNTPs and 100 ng of the C_β-N′ primer. The mixture was heated to 85°C for 3 min and then cooled to 55°C. Forty units of RNasin (Promega) was added and the mixture was annealed at 55°C for 1 h. Four hundred units of Moloney murine leukemia virus (MoMLV) reverse transcriptase (BRL, Gaithersburg, MD) was added to bring the total volume to 20 μl. The C_β-N′ primer was extended at 37°C for 1 h.

The resultant first-strand cDNA product was immediately PCR amplified utilizing the C_β-N′ primer as the 3′ primer and a 5′ primer consisting of consensus sequences to a conserved region [amino acid (aa) 32–37] within the V_β gene region. Because this region represents 70–100% homology among 18 V_β gene families [15,16], our primer is a mixture of nucleotides at six of 23 positions [V_β-37: 5′-CGGATCCT (GT) T (AT) (CT) TGGTA (TC) C (GA) (TA)-CA-3′]. Amplification was performed in a total volume of 100 μl. Five hundred nanograms of additional C_β-N′ primer was added along with 600 ng of V_β-37 primer. Standard buffer [11], 1.25 mM dNTPs and 5 units Taq polymerase (Perkin Elmer Cetus), was added to the reaction mixture and amplified on a DNA thermal cycler (Ericomp). The first five cycles utilized a 50°C annealing temperature for 2 min. Subsequent cycles were annealed at 55°C. The extension step was at 72°C for 2.5 min and denaturation at 94°C for 1 min. Amplification was performed for 30 cycles.

Fifteen microliters of the PCR products were run on a 4% agarose gel (NuSieve) and blotted onto a nylon filter (Zetabind, Cuno, Meriden, CT) and screened with an oligonucleotide probe that recognizes C_β sequences (aa 1–6) that are internal to, or "nested" to, the C_β-N′ primer (aa 7–13). Our C_β-nested probe is a 50% mixture of a probe recognizing C_β1 sequences (5′-GAGGACCTGAA-CAAGGTG-3′) and a probe to C_β2 sequences (5′-GAGGACCT-GAAAAACGTG-3′).

Subsequent subcloning of PCR products was made possible due to the introduction of restriction sites into the 5′ end of our primers. The remaining 85 μl of reaction mixture was phenol/chloroform extracted, ethanol precipitated, double digested with BamHI (V_β-37) and SalI (C_β-N′) (BRL), and sized on 4% low melt agarose gel (NuSieve, FMC, Rockland, ME) [17]. Two hundred to 400 bp bands were ligated into Bluescript SK^+ phagemid vector (Stratagene, LaJolla, CA) and the ligation mixture was used to transform DH5α cells (BRL) [17]. Ampicillin-resistant colonies were lifted onto nitrocellulose filters (Millipore, Bedford, MA) and screened with γ^{32}P-end-labeled C_β-nested probes [17]. Positive colonies were selected for double-strand sequencing by the dideoxynucleotide chain termination method [18]. Sequence identity of specific V, D, J, and C families were analyzed with Wisconsin Genetics Computer Group software [19].

Generation of a Patient-Specific Probe and Detection of the Patient's Malignant T-Cells—PCR of Genomic DNA Oligonucleotide primers with homologous sequences to the V_β and J_β segments utilized in the patient's TCR-β gene rearrangement were generated based on sequence analysis and used as 5′ and 3′ (antisense) primers, respectively, in PCR amplification of genomic DNA. J_β sequences internal to the J_β primer were used to generate a J_β-nested probe. The unique sequences of the V-D-J junctional region were used to generate a patient-specific probe. Primers and probes are shown in *Results*. The oligonucleotides were synthesized by phosphoramidite methodology [20] on an Applied Biosystems 380A DNA synthesizer and purified using HPLC. Genomic DNA (1 μg) was amplified for 30 cycles: denaturing at 94°C for 1 min, annealing at 55°C for 1 min, and extending at 70°C for 1 min. Amplified material was run on a 4% agarose gel, blotted, and probed (as above) with the patient-specific V-D-J probe.

All DNA (and RNA) isolation was performed in areas dedicated to such procedures and free of any plasmid or phage work. In addition, we utilized reagents, water, supplies, and equipment dedicated only to PCR. Amplification and non-amplified samples were handled separately.

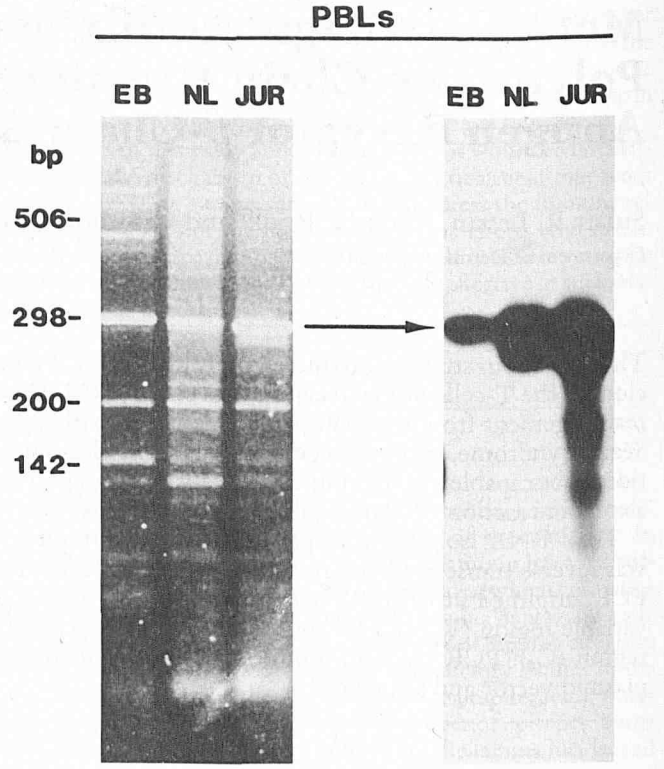

Figure 1. Amplification products of TCR-PCR. *Left:* ethidium bromide stained 4% agarose gel with *lanes* containing PCR products from PBL of patient EB (EB); PBL of a normal control (NL) and Jurkat T-cell line (JUR). Fragment sizes in bp indicated on the *left*. *Right:* Southern blot of gel in *left* probed with a mixture of C_β1 and C_β2 –nested probe (Table I). *Arrow,* amplification bands just below 298 bp contains Cβ sequences.

RESULTS

Results of the reverse transcription and PCR amplification utilizing the C_β-N′ and V_β-37 primers demonstrated that the amplification products just below the 298 bp marker on a 4% agarose gel Fig 1, *left*) contained C_β sequences in our Sézary syndrome (SS) patient (EB), normal PBL, and the Jurkat T-cell leukemia cell line (Fig 1, *right*). The amplification band containing C_β sequences (*arrow* in Fig 1), was isolated, subcloned into Bluescript SK^+ phagemid, and transformed into bacteria (as described in *Materials and Methods*). The identification of the TCR-β gene rearrangement representing a monoclonal T-cell population was accomplished by quantitative screening of bacterial colonies containing subcloned PCR products. Bacterial colonies were screened with the C_β-nested probe and multiple colonies containing C_β sequences were selected and sequenced [17]. Our results are presented in Table I and illustrate that when a monoclonal population of T cells (i.e., Jurkat) is present, the num-

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      LeuPheTrpTyrHisGlnThrLeuGlyGlnGlyProGluPheLeuThrTyrPheG
1     GGATCCTTTTTTGGTACCATCAGACCCTGGGGCAGGGCCCAGAGTTTCTGACTTACTTCC   60

      lnAsnGluAlaGlnLeuGluLysSerArgLeuLeuSerAspArgPheSerAlaGluArgP
61    AGAATGAAGCTCAACTAGAAAAATCAAGGCTGCTCAGTGATCGGTTCTCTGCAGAGAGGC  120

      roLysGlySerPheSerThrLeuGluIleGlnArgThrGluGlnGlyAspSerAlaMetT
121   CTAAGGGATCTTTCTCCACCTTGGAGATCCAGCGCACAGAGCAGGGGGACTCGGCCATGT  180

      yrLeuCysAlaArgThrGlnGlyAlaAspThrGlyGluLeuPhePheGlyGluGlySerA
181   ATCTCTGTGCCAGGACACAGGGGGCGGACACCGGGGAGCTGTTTTTTGGAGAAGGCTCTA  240
            V ──▶|N|◀── D ──▶|N|◀── J

      rgLeuThrValLeuGluAspLeuLysAsnValPheProProGluVal
241   GGCTGACCGTACTGGAGGACCTGAAAAACGTGTTCCCACCCGAGGTCGAC            290
            J ──▶|◀── C
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Figure 2. Sequence analysis of patient EB's TCR-β gene rearrangement. V, N, D, J, and C regions are indicated below the sequence: V_β6.4 (6-194); D_β1.1 (197–205); J_β2.2 (208–254); C_β2.1 (255–287); N regions (195–196 and 206–207). Amino acid translation is indicated above the sequence (6–287). *Underline,* primer sequences.

ber of colonies containing the identical clonal TCR-β rearrangement will be overrepresented or "dominate." In contrast, normal PBL do not contain a dominant clone(s). All colonies (nine of nine) selected and sequenced from our SS patient contained the identical clone.

Sequence analysis from patient EB revealed an in-frame TCR-β rearrangement utilizing V_β6.4, D_β1.1, J_β2.1, and C_β2.1 (Fig 2). In addition, several nucleotides were present in between the V_β, D_β, and J_β segments. These additional nucleotides, known as N-regions (Fig 2), increase the diversity within the TCR-β gene and confer additional specificity to each TCR rearrangement [6].

Based on the sequence analysis in Fig 2, we generated an oligonucleotide primer to the specific V_β6.4 (V_β-6.4: 5′-CAACTAGAAAAATCAAGGCTGCTCAGTGAT-3′) and two oligo-probes to J_β2.1 utilized in our SS patient's TCR-β rearrangement. Oligoprimer J_β-2.1 contains sequences of the 3′ end of the J_β2.1 gene segment including sequences of 3′ intron [21] and was used as the 3′ or antisense primer in the PCR amplification of genomic DNA (J_β-2.1: 5′-AACCGCCTCCTTACCCAGTACGGT-3′). J_β-2.1–nested probe (J_β-2.1n: 5′-GAGCTGTTTTTTGGAGAAGGCTCT-3′) hybridizes to J_β2.1 sequences internal to the J_β-2.1 primer sequences and was used to detect amplification products containing the J_β2.1 gene segment. We generated a patient-specific V-D-J probe that hybridizes to the 3′ end of V_β6.4, the N-regions, Dβ1.1, and the 5′ end of J_β2.1 (VDJ-EB: 5′-AGGACACAGGGGGCGGACACC-3′) and only detects amplification products that contain the unique TCR-β gene rearrangement of patient EB. Genomic DNA from patient EB's PBL, clinically involved skin, and other T-cell malignancies and benign lymphocytic infiltrates of the skin were used in a PCR reaction using the V_β-6.4 and J_β-2.1 primers. The patient-specific V-D-J probe was then used to identify the patient-specific sequences present in the amplification products (Fig 3). Patient EB's PBL and skin demonstrated the strongest amplification products (Fig 3, *top*). When probed with the patient-specific V-D-J probe, only amplified material from patient EB's PBL and skin hybridized (Fig 3, *bottom*), indicating the presence of the patient's unique TCR-β rearrangement in the blood and skin not present in other malignancies or benign infiltrates.

DISCUSSION

We have identified a "molecular fingerprint" of the malignant T cells from a patient with CTCL (Sézary syndrome) by cloning and characterizing the TCR-β chain gene rearrangement. This was accomplished by initially reverse transcribing total RNA and PCR amplifying the resultant cDNA with the use of an oligonucleotide primer to the C_β region (C_β-N′) and a concensus probe to V_β region (V_β-37).

Our C_β-N′ primer has four distinct advantages. First, it primes only TCR-β transcripts and therefore is more specific than oligo-dT priming. Second, because of its location near the 5′ end of the exon, only a small portion of the C_β gene segment is amplified, which produces relatively short amplification products that can be sequenced rapidly. Third, the 5′ sequences of the C_β exon that are amplified (aa 1–6) provide easy identification of C_β1 or C_β2 gene segments because they differ by only two bases [21]. Lastly, point mutations were added to the 5′ end of the primer to generate a SalI restriction site for subsequent subcloning.

The V_β-37 primer, containing a Bam HI site on the 5′ end, provides a means to amplify TCR-β cDNA with unknown 5′ sequences and eliminates the need for oligo-dT tailing of cDNA, as has been described in other methods of PCR amplifying targets with unknown 5′ sequences [22]. From sequence analysis of 38 independent clones from normal PBL, our V_β consensus primer does not appear to have a selection bias toward a subset of V_β genes. The percentage of V_β genes represented corresponded to the number of genes within each V_β family [15,16] (sequence data not shown). This eliminates the need for a more laborious alternative method to PCR cloning TCR-β gene rearrangements that utilizes 18 V_β primers (one for each V_β family) in 18 different PCR reactions [23].

Oligonucleotide primers were constructed to the specific V_β and J_β segment utilized in the patient's TCR-β chain gene rearrangement. Through sequence analysis we constructed a patient-specific probe that hybridized to sequences of the V-D-J junctional area. We were able to utilize these primers to amplify genomic DNA and identify amplification products utilizing the specific V-D-J probe.

The ability to rapidly molecularly define TCR-β gene rearrangements has many applications. By identifying specific V, D, J, and C regions that are involved in TCR-β gene rearrangement in CTCL we can generate a new molecular probe specific for the malignant cells. Through PCR amplification it will be possible to molecularly diagnosis CTCL with increased sensitivity and specificity. This will provide a new means by which we can define the natural history and the clonal origin of CTCL in individual patients. It will be possible to retrospectively analyze archival samples [24] of patients with CTCL and identify the earliest biopsies in which the malignant clone is present. Patient-specific T-cell probes can be utilized in in situ hybridization/transcription [25] to further delineate clinicopathologic correlation and distinguish malignant T cells from tumor-infiltrating lymphocytes.

By assessing the TCR repertoire in normal and diseased states, it will be possible to molecularly identify pathogenic T-cell clones by

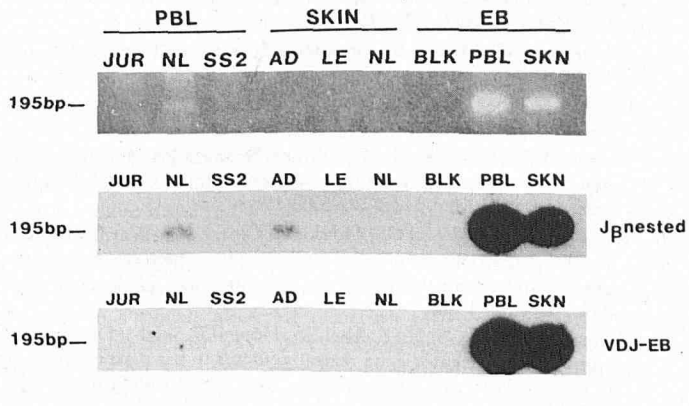

Figure 3. PCR Amplification of genomic DNA with primers specific for patient EB's TCR-β rearrangement. *Top,* ethidium bromide stained 4% agarose gel with *lanes* containing amplification products of genomic DNA from Jurkat T-cell line (JUR); normal control PBL (NL-PBL); PBL of a different Sézary syndrome patient (SS2); skin from a patient with atopic dermatitis (AD); skin from a patient with cutaneous lupus (LE); skin from a normal control (NL-skin); a negative control water blank (BLK); PBL (PBL-EB) and involved skin (SKN-EB) from patient EB. 195-bp size marker is on the *left. Middle,* Southern blot of the gel in the *top* probed with the Jβ-nested probe. *Bottom,* Southern blot of the gel in the *top* probed with patient EB's specific V-D-J oligonucleotide probe (VDJ-EB).

Table I. Quantitative Screening of Subcloned TCR-β Rearrangements

Tissue Type	Number of Colonies with Identical TCR-β Sequences/ Number of Colonies Sequenced
Jurkat T-cell line (JUR, Fig 1)	9/9[a]
SS-PBL (SS, Fig 1)	9/9
Normal PBL (NL, Fig 1)	0/38

[a] Jurkat sequences confirms published TCR-β sequences [12].

detecting the presence of the dominant clones within inflammatory or mixed T-cell populations [23,26]. Analysis of PCR amplification products by quantitative screening (as in Table I) or by the recently reported technique of denaturing gradient gel electrophoresis [27] will enable us to identify a dominant clone in early CTCL skin lesions. Our technique will provide a means to generate a specific molecular T-cell probe and prospectively follow the course of the disease and detect the earliest involvement of extracutaneous sites.

Lastly, because the TCR-β gene product is a protein expressed on the surface of mature T cells, this technique lays the foundation for new therapeutic approaches directed at immunomodulation of pathogenic T-cell clones in T-cell malignancies and T-cell–mediated autoimmune diseases. Possible new modalities include anti-idiotypic monoclonal antibody therapy and TCR peptide vaccination [28,29].

In summary, this is the first demonstration of molecular cloning and sequencing of a TCR-β gene rearrangement from a malignant population of human T cells utilizing the "molecular fingerprint" to detect the presence of the malignant cells through PCR amplification of genomic DNA.

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