Frequent Clonal Expansion of Peripheral T Cells in Patients With Autoimmune Diseases: A Novel Detecting System Possibly Applicable to Laboratory Examination

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To investigate T cell involvement in antigen-specific immune responses, it is important to detect accumulating T cells at a clonal level in vivo. However, thus far the clinical application of such analyses has been limited. Here we have established novel primers to anneal with T cell receptor (TCR) β genes of multiple V β families and applied them to reverse transcription-polymerase chain reaction-single strand conformation polymorphism (RT-PCR-SSCP) analysis to evaluate peripheral T cell clonality of autoimmune disease patients. As a result, the new V β primers could detect accumulating T cell

clones in the periphery of healthy individuals and patients. It was revealed that patients with autoimmune diseases such as systemic lupus erythematosus (SLE) had a larger number of clonal accumulations of peripheral T cells compared with normal individuals. Thus, the RT-PCR-SSCP system using the new multifamily V β primers is the first such laboratory examination to detect T cell clonal expansion, and will provide a simple and sensitive tool to aid in the diagnosis and also in the investigation of the pathogenesis of autoimmune diseases. *J. Clin. Lab. Anal.* 12:162–167, 1998. © 1998 Wiley-Liss, Inc.

Key words: T cell clonality; T cell receptor; RT-PCR-SSCP; autoimmunity

INTRODUCTION

Antigen-specific immune response(s) are suggested to play a key role in the pathogenesis of autoimmune diseases. Involvement of T cells at a clonal level in the autoimmune response has been investigated mainly through establishing autoantigen-specific T cell clones or lines from autoimmune patients in vitro. However, detection of such T cell clones in vivo has presented difficulty because of the lack of an appropriate technique. Thus, the precise clonality of the peripheral T cell pool in autoimmune patients has not been fully clarified.

Antigen-specificity of each T cell has been thought to be principally determined by the third complementarity-determining region (CDR3) of T cell receptor (TCR). Thus, analyses of this CDR3 gene usage would be important in understanding T cell responses in autoimmunity. In this regard, we have reported a clonality-analyzing system(1–7), which is composed of reverse transcription (RT), polymerase chain reaction (PCR) with multiple primers specific for each V β family (from V β 1 to V β 20, including V β 5.1/5.2 and

V β 13.1/13.2 subfamilies), and single strand conformation polymorphism (SSCP). Using this method, accumulating T cell clonotypes could be directly detected both in vivo and in vitro(1–7). For example, we found remarkable clonal accumulations of T cells in samples from autoimmune patients, such as in synovium of rheumatoid arthritis patients(1,4). Also,

Abbreviations: TCR: T cell receptor; CDR3: third complementarity-determining region; RT-PCR-SSCP: reverse transcription-polymerase chain reaction-single strand conformation polymorphism; SLE: systemic lupus erythematosus; RA: rheumatoid arthritis; PM/DM: polymyositis/dermatomyositis; MCTD: mixed connective tissue disease; SSC: systemic sclerosis.

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some immunologically predisposed or manipulated mice were revealed to have T cell clonal accumulations(6,7). These findings strongly suggest that antigenic stimuli in patients or hosts drive T cells to clonally expand and accumulate *in vivo*. Also, we have found restricted sequences of TCR CDR3 genes in such accumulating clones (Kurokawa et al., submitted), which indicate antigen-driven accumulation of clones. Thus, such analysis would help in the understanding of T cell-oriented pathogenesis of the autoimmune diseases, and, moreover, would potentially contribute to clinical diagnosis of immunological diseases. However, since more than twenty primers are used in the conventional RT-PCR-SSCP system(1-5), each analysis is extremely costly and laborious. Thus, a simpler and more cost-effective method is needed for processing many specimens for laboratory examination.

For this purpose, we have established a set of novel PCR primers, each of which covers several TCR V β families, to facilitate RT-PCR-SSCP analysis. Using these primers, we have investigated peripheral T cell clonality in patients with a broad range of autoimmune diseases. Our results could be the first to demonstrate the high frequency of T cell clonal accumulations in the periphery of autoimmune patients. This method would be useful for clinical investigation of autoimmunity.

MATERIALS AND METHODS

Primer Design

The novel TCR V β primers (2579-1 and 4550-1) were designed to have homology among multiple V β gene families according to the OligoProbe DesignStation™ program (Advanced Gene Computing Technologies Inc., Irvine, CA)(8) (Table 1). Nucleotide sequences of conventional V β (V β 1 to 20) and C β primers were according to Choi et al.(9). Sequence of a biotinylated C β internal probe for hybridization was: 5'-A(AC)AA(GC)GTGTTCCACCCGAGGTCGCTGTGTT-3'. All of the primers and the probe were synthesized by a DNA synthesizer (Perkins Elmer, Applied Biosystems Division, Foster City, CA) and then purified through OPC columns (Applied Biosystems) before use.

Samples

Heparinized blood samples were obtained with informed consent from patients with autoimmune diseases: 27 systemic lupus erythematosus (SLE), 14 polymyositis (PM)/dermatomyositis (DM), 7 mixed connective tissue disease (MCTD), 36 rheumatoid arthritis (RA), and 12 systemic sclerosis (SSc)

as well as 12 healthy volunteers (Table 2). Patients were considered to be in a stable phase of the disease and were followed at outpatient clinics either of the Department of Rheumatology, Sagamihara National Hospital, or of the Institute of Medical Science, St. Marianna University. All patients included in this study satisfied classification criteria for the respective diseases(10-14). No sign or symptom of infection was observed. Numbers of patients and their diseases, respectively, are indicated in Figure 2.

Mononuclear cells were separated by standard gradient centrifugation. Cells were immediately suspended in denaturing guanidium solution, followed by RNA isolation and cDNA synthesis as previously described (1-5).

PCR-SSCP

The RT-PCR-SSCP analysis was performed as previously reported (1-5) with minor modification. Briefly, PCR mixture was made from template cDNA, dNTP (TaKaRa Shuzo Co, Ltd., Tokyo, Japan), Taq DNA polymerase with 10 $\times$ Taq buffer (Boehringer-Mannheim, Mannheim, Germany), and one of the multifamily V β primers (2579-1 or 4550-1) together with a common C β primer. PCR reaction was performed under the following conditions: denaturation at 94°C for 1 min, amplification by 32 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by final extension at 72°C for 1 min. Amplified PCR products were mixed with loading dye, heat-denatured at 90°C for 2 min, and then electrophoresed to a 4% nondenatured polyacrylamide gel. After electrophoresis, DNA was transferred to a nylon membrane (Immobilon-S, Millipore Co., Bedford, MA) and hybridized with a biotinylated C β internal probe. DNA was visualized by subsequent incubation with streptavidin, biotinylated alkaline phosphatase, and a chemiluminescent system (Plex Luminescence Kit, Millipore Co., Bedford, MA).

The number of accumulating clones was determined by the number of clear bands detected from the background smear as previously described (5). Statistical significance between groups was calculated using the Mann-Whitney test.

DNA Sequencing

To obtain DNA sequences of the amplified DNA, PCR products were subjected to a second PCR using a 4550-1 primer with EcoRI site and an antisense C β -specific primer with HindIII site. After digestion, the amplified DNAs were

TABLE 1. Novel Multifamily V β Primers

Primer no.	Nucleotide sequences	Covered V β families
2579-1	5'-CAGCGACAAGACCCAGG-3'	1,2,3,13,14,15
4550-1	5'-GTCATTGACAAGTTTCCCATC-3'	2,4

TABLE 2. Study Populations

	Male	Female	Age range	Mean
SLE	2	25	29-63	43.2
PM/DM	2	12	33-81	53.8
MCTD	0	7	29-64	46.3
RA	4	32	28-80	50.6
SSc	0	12	32-63	44.5
Healthy	5	7	25-55	40.8

subcloned into a plasmid vector (pBluescript II, Stratagene, La Jolla, CA) and clones were subjected to a di-deoxy DNA sequencing (373A DNA Sequencing System, Perkin Elmer/Applied Biosystems, Foster, CA).

RESULTS

Amplification of Accumulated T Cell Clonotypes With Novel Multifamily V β Primers

The novel multifamily V β primers (2579-1, 4550-1) were designed to have a homology sequence of nucleotides among genes of TCR V β subfamilies (Table 1). First, we aimed to confirm the usability of these primers to amplify TCR V-D-J genes. We performed RT-PCR-SSCP with the primers and a conventional C β primer using cloned V β 2+ T cells and other cDNA of T cell samples. As a result, genes could be amplified by PCR, and DNA could be detected by subsequent hybridization with a specific C β internal probe (data not shown). Thus, the primers were proved to be usable in the RT-PCR-SSCP analyses of TCR β genes.

Our previous experiments with conventional RT-PCR-SSCP(1) demonstrated that T cells with diverse clonality are shown as a smear by SSCP. In contrast, a single T cell clone appears as a single, sharp band. Also, the accumulation of a T cell clone within a heterogeneous T cell population is revealed as a 'sharp band on smear' pattern on

SSCP films. Next, we questioned whether the new V β primers could reproduce such findings.

We analyzed PBLs from twenty autoimmune patients using conventional and new primers (2579-1 and 4550-1). After SSCP, we counted the number of bands detected by respective primers and calculated correlations between them. The total number of bands from all V β families tested (V β 1 to 20) was used for conventional primers. As a result, although the total number of detected bands differs almost ten-fold between conventional (sum of V β 1 to 20) and new (covering only two to six V β families) primers, high correlation indices were obtained (0.67 for primer 2579-1 and 0.78 for 4550-1, respectively)(data not shown).

Thus, the new primers were shown to correlate with conventional analysis at a significant confidence level.

Comparison of Peripheral T Cell Clonality Between Healthy Individuals and Autoimmune Disease Patients

We investigated whether there were characteristic features of peripheral T cell clonality in autoimmune patients compared with healthy individuals using the new primers in the RT-PCR-SSCP system.

Representative results are shown in Figure 1. It was found that healthy individuals (Fig. 1, H1 to H8) had a heteroge-

Fig. 1. Analyses of peripheral T cell clonality by RT-PCR-SSCP with multifamily V β primers. T cell clonality of PBL from healthy individuals and autoimmune patients was analyzed by RT-PCR-SSCP method using novel

multifamily V β primers. **H:** healthy individuals. Numbers indicate sample numbers of respective subjects. **Left:** primer 2579-1; **Right:** primer 4550-1.

neous T cell population in the periphery, with a few distinct clonal accumulations. This result was consistent with our previous observations (2,3). In contrast, many of the autoimmune disease patients were revealed to have an increased number of accumulating T cell clones in PBL (Fig. 1, SLE1 to MCTD2).

To verify that the primer could reflect T cell clonal expansion in PBL, we determined TCR V-D(N)-J sequences in the amplified samples. H-6 and SLE-6 were chosen to be representative, i.e., with or without a distinct band in SSCP as shown in Figure 1. As a result of the sequencing study of V β 4 family (Table 3), H-6 was confirmed to be a heterogenous population. In contrast, a dominant clone was detected in the SLE-6 sample, which showed a distinct band in SSCP analysis using the novel 4550-1 primer (Fig. 1). Thus, the findings of SSCP using the novel primer were shown to reflect the results of conventional analysis and of sequencing studies.

Next, distinct bands were counted for each primer and the numbers were compared. It was demonstrated that the SLE patients had a significantly larger number of accumulating clones than healthy individuals ($P < 0.01$) (Fig. 2). Also, numbers of detected bands in patients with PM/DM and MCTD were relatively high (Fig. 2). Patients with SSc and RA showed no significant difference from healthy controls in numbers of accumulating clones (Fig. 2). When SLE and RA patients were compared, SLE patients had a greater number of T cell clonal accumulations than RA patients ($P < 0.01$) (Fig. 2).

DISCUSSION

Several studies, including ours, have suggested the involvement of T cells at a clonal level in the pathogenesis of autoimmune diseases (1,4,15–18). Among several investigative

methods, our RT-PCR-SSCP is thought to be one of the most sensitive techniques to detect expanding or accumulating T cell clones in vivo and in vitro (1–7). However, its high cost and laboriousness may limit its availability for efficient analyses of various clinical samples. To overcome this problem, we designed new primers according to homology search of nucleotide sequences of published TCR V β genes.

As described in Results, the set of new primers could be used in RT-PCR-SSCP analyses of T cell clonality. This method was relatively simple and inexpensive, and there was a significant correlation with results obtained by the conventional method. Thus, the new primers are expected to be useful for efficient evaluation of the overall degree of T cell clonality in various samples. Sensitivity and specificity are, however, thought to be theoretically lower than with conventional primers. Naturally, it should be noted that the consensus primers may miss a dominant clone (or clones) that exclusively bears a specific V β family which is not covered by the primer. Nevertheless, our previous observations by the conventional method (1,4 and unpublished observation) demonstrated that accumulating T cell clones in autoimmune patients, at least in PBL from RA and SLE patients, used multiple V β families without any distinct restriction. Thus, it would be sufficient to analyze several V β families by the new primers to estimate the degree of T cell clonal expansion.

The results presented here demonstrate the frequent existence of distinct accumulations of T cell clones in the periphery of autoimmune patients. The presence of clonal predominance itself has been reported in some situations, e.g., in RA patients (15–18). However, our present study suggests that such findings may not be limited to a restricted category of diseases, but, rather, are common phenomena among healthy individuals and those with autoimmune disorders.

It is noteworthy that most of the SLE patients tested bear significant accumulations of T cell clones in the periphery, even in a stable phase of the disease. Although the T cell mediated pathogenesis of SLE is not fully understood, the accumulating T cell clones in the systemic circulation may be involved in the disease's perpetuation of this systemic autoimmunity. Using conventional PCR primers, we have observed that the disease activity and degree of T cell clonal accumulations are correlated in SLE (19). Detailed characterization of accumulating clones is now in progress in our laboratory (Ueda et al, manuscript in preparation).

We have also found a significant difference of peripheral T cell clonality between SLE and RA patients (Fig. 2). This may reflect a difference of pathological milieu in which T cells are implicated at a clonal level between the systemic and the organ-specific autoimmune diseases. In fact, our previous observations revealed that RA patients had more accumulating clones in their arthritic joints than in the peripheral T cell population (submitted). Thus, SLE and RA may be distinguished by studies of peripheral T cell accumulations using our system.

TABLE 3. Sequence Analysis of Amplified V β 4+ DNAs From a Healthy (H-6) and an SLE (SLE-6) Subject^a

	V β 4	N	J β		
H-6	YLCS	VKGLAGGD	SYNEQ	(2.1)	1/12
	IYLC	KRVQH	EQYFG	(2.7)	1/12
	YLCS	VETSGN	YEQYF	(2.7)	1/12
	YLCS	VELTGTHH	EQYFG	(2.7)	1/12
	YLCS	VPFGVS	SYNEQ	(2.1)	1/12
	YLCS	VARANVGAR	YEQYE	(2.7)	1/12
	IYLC	RDRGT	YNEQF	(2.1)	1/12
	YLCS	VEDRGS	YNEQF	(2.1)	1/12
	YLCS	VVEDLDGSGRS	YEQYF	(2.7)	1/12
	YLCS	VLAGEV	TGELF	(2.2)	1/12
	YLCS	ARTSSI	YNEQF	(2.1)	1/12
	YLCS	ALREG	ETQYF	(2.5)	1/12
	SLE-6	CSVE	TGTVF	YEQYF	(2.7) 4/9
	LCS	VGLFQ	NTEAFF	(1.1)	1/9
SLE-6	LCS	ARSGFS	SYEQY	(2.7)	1/9
	CS	VGTSTTG	EQYF	(2.7)	1/9
	CS	VEDLRLAEP	YEQY	(2.7)	1/9
	LCS	VVLSGV	NTGELF	(2.2)	1/9

^aH-6 and SLE-6 correspond to the patients shown in Figure 1.

A) primer 2579-1

B) primer 4550-1

Fig. 2. Frequencies of accumulating T cell clones in autoimmune patients and healthy controls. Subjects were grouped according to the number of

bands detected by RT-PCR-SSCP with novel V β primers (A: primer 2579-1, B: primer 4550-1) and are shown by dots.

The mechanism by which T cells form distinct clonal accumulations is not known. In this regard, we have found that the recipient of allogeneic bone marrow may exhibit the appearance of clonal T cell accumulations in the periphery soon after transplantation (3). This suggests that accumulating clones may originate from a drastic antigenic challenge in vivo. Thus, the accumulating clones detected here may be working as a 'reservoir' of immunological memory after a strong and chronic antigenic stimulation.

It is suggested that the degree of clonal accumulation would distinguish autoimmune patients, especially those with SLE, from healthy individuals. Our RT-PCR-SSCP analysis with the consensus primers is the first to demonstrate that this method may be used to test a number of samples at minimal cost. Since there has been no laboratory test thus far to detect accumulating T cell clones directly from clinical specimens, our method would be useful in the clinical field, such as for the diagnosis of autoimmune diseases.

T cell clonal analysis also would be helpful to elucidate T cell involvement in an antigen-specific manner in many kinds of immune responses. In this regard, the system presented here would be a useful method which does not require cloning or sequencing procedures to efficiently survey T cell clonal expansion in a number of samples. For example, we had shown that T cell expansion would extend according to tumor progression in vivo (20). More specifically, T cell clonal expansion was found only within tumor-infiltrating lymphocytes in early-stage patients, whereas in patients with advanced tumors, expanding T cell clones were observed also in lymph nodes with tumor metastasis. This approach would provide a clue to understanding the pathogenesis of many diseases. In this regard, the novel primer presented here rep-

resents a simpler method compared with conventional RT-PCR-SSCP analysis.

In conclusion, we have observed a common phenomenon of clonal accumulations of peripheral T cells among autoimmune patients. Such analysis may provide valuable information about T cell involvement in the pathogenesis of autoimmunity and would be applicable to laboratory examination.

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