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Discrepancy in T Cell Clonal Expansions in Synovial Fluid and Peripheral Blood from Rheumatoid Arthritis Patients

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Rheumatoid arthritis (RA) is an autoimmune disease involving the synovial membrane of peripheral joints. T cells specific for self antigens may play a critical role. Identification of T cell receptors (TCR) of such specific T cell clones is very important for treatment, prevention and identification of relevant autoantigens. To identify specific T cells, TCR Vβ family repertoire and the clonal expansion of T cells were analyzed in this study. The percentage of Vβ 5$^+$ or Vβ 8$^+$ cells in the synovial fluid mononuclear cells (SFMCs) was similar to that in the peripheral blood mononuclear cells (PBMCs). However, the percentage of DR$^+$ T cells in the SFMCs was higher ($p<0.01$). Analyzing the clonality of T cells in 8 Vβ families (Vβ1, Vβ5, Vβ8, Vβ14, Vβ16, Vβ17, Vβ18, Vβ20), clonal expansions in CD8$^+$ T cells from the SFMCs were found more frequently than in the PBMCs. The patterns of clonal expansions were discrepant between the SFMCs and the PBMCs even in the same patient, which suggests several inflamed tissue specific T cell clonal expansions in the SFMCs. These T cell clones might be activated by autoantigens which are not identified yet and responsible for the RA pathogenesis.

Key Words: Rheumatoid arthritis, synovial fluid T cells, T cell receptor, CDR3, clonal expansions, DR$^+$ T cells

Rheumatoid arthritis (RA) is an autoimmune disease characterized by a chronic inflammatory process, primarily involving the synovial membrane of peripheral joints (Harris 1990). The majority of RA patients carries HLA-DR1, HLA-DR4 or both antigens in Caucasians (Stastny 1978, Nepom *et al.* 1989), but other genetic components are also clearly involved in the susceptibility to the disease. Some evidences suggest that T cells specific for self antigens may play a critical role in the initiation of RA. Activated T cells are found in the inflamed synovium (van Boxel and Paget 1975, Howel *et al.* 1991, Bucht *et al.* 1992, Cooper *et al.* 1994) and treatment with immunosuppressive drugs, like cyclosporine, shows clinical efficacy (Shirosky *et al.* 1989).

Cultivated T cells from RA synovial tissues often display dominant T cell receptors (TCR), Vβ14, 16, and 17, which are characteristic of clonal T cell expansion (Chatila *et al.* 1990). These findings are indicative of *in situ* activation of specific T cell populations by synovial antigens and are supportive of the role for these T cells in the pathogenesis of RA. Analysis of animal models of autoimmune

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diseases has provided some evidence for TCR oligoclonality. Oligoclonal TCR repertoire is involved in the induction and maintenance of experimental allergic encephalomyelitis in rats (Urban *et al.* 1988) and antibodies directed against the expressed β chain variable regions (Vβ), Vβ8.2, can be used to prevent and treat the disease in B 10 PL mice (Zaller *et al.* 1990). Identification of TCR Vβ family of such specific T cell clone is very important, because those TCR may provide a highly selective target for treatment as well as vaccine and may be used to identify relevant autoantigens.

In this study we used a simple PCR assay to study the clonality of T cells. The CDR3 region of the TCR β chain is formed by the joining of V, D and J gene segments (Candeias *et al.* 1991) (Fig. 1), accompanied by the random addition of N or P nucleotides.

Thus this CDR3 of the TCR β chain varies in length as well as in sequences. This length variation forms the basis of the CDR3 length assay.

To investigate the oligoclonality of T cells in RA, TCR Vβ family repertoire for Vβ5 and Vβ8 were analyzed by flow cytometry and the clonal expansions of single gene families (Vβ1, Vβ14, Vβ16, Vβ17, Vβ18, Vβ20) were analyzed by two step PCR in T cells from peripheral blood or synovial fluid of RA patients.

MATERIALS AND METHODS

RA patients

Synovial fluid and peripheral blood were collected from 12 RA patients at the same time. Ten patients were female and 2 patients were male. Disease duration varies from 6 months to 3 years.

Separation of $CD4^+$ or $CD8^+$ T cells

Synovial fluid mononuclear cells (SFMCs) or peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque (Lymphoprep, Nyegaard, Oslo, Norway) density gradient centrifugation. The cells were washed three times in RPMI 1640 and resuspended in RPMI 1640-10% fetal calf serum at the concentration of $2\sim3\times10^6$ cells/ml. Positive selection for $CD8^+$ cells was carried out by incubating mononuclear cells with anti-CD8 immunomagnetic beads (Dynal, Great Neck, NY) for 30 min at 4℃ on a rotating shaker. The unbound cells were then incubated with anti-CD4 immunomagnetic beads (Dynal, Great Neck, NY) for the selection of $CD4^+$ T cells.

Surface marker study

Fluorescein isothiocyanate (FITC) or phycoerythrin conjugated monoclonal antibodies of anti-CD19, anti-CD45, anti-CD4, anti-CD8 or anti-DR (Becton Dickson, San Jose, CA) were used. For TCR β chain, FITC conjugated anti-Vβ5 or anti-Vβ8 monoclonal antibodies (T Cell Scientific, Cambridge, MA) were used. Cells ($0.5\sim1\times10^6$ cells/ml) in PBS-0.1% bovine serum albumin (BSA) were incubated for 30 min at 4℃ and washed once with PBS-0.1% BSA. Propidium iodide (Sigma, St. Louis, MO) at final concentration of 25 μg/ml was used to discriminate dead cells from live cells. A total of 10,000 to 20,000 cells were analyzed by FACStar II (Becton Dickson, San Jose, CA).

Analysis of CDR3 size

Total RNA was isolated by acid guanidium isothiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). Total RNA (1 $\sim$5 μg) was used for the first strand cDNA synthesis using random hexamer (:pd(N)$_6$, Pharmacia, Oslo, Sweden) 2 O.D. units. The cDNA synthesis was performed with 600 U of MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD) at 42℃ for 1 hour in a total volume of 100 μl.

The CDR3 length analysis was carried out using a two step PCR. Vβ family specific sense primer combined with a antisense primer specific for the C region (Cβ) was used in the first round amplification (Table 1). A total of 8$\sim$10 μl of the cDNA was used for the amplification of each Vβ family. The conditions for the PCR on the cycle sequencer (Perkin-Elmer Corp., Norwalk, CT, model 2600) was as follows : denaturation at 94℃ for 1 min, annealing at 55℃ for 1 min and extension at 72℃ for 1 min. PCR buffer condition was 10 mM Tris-HCl (pH8.3), 2.0 mM $MgCl_2$, 50 mM

Table 1. Sequences of primers for the first PCR

Primers	5′ ---------- 3′
Vβ1	GCA CAA CAG TTC CCT GAC TTG CAC
Vβ5	ATA CTT CAG TGA GAC ACA GAG AAA C
Vβ8	ATT TAC TTT AAC AAC AAC GTT CCG
Vβ14	GTC TCT CGA AAA GAG AAG AGG AAT T
Vβ16	AAG AGT CTA AAC AGG A GA GTC C
Vβ17	CAG ATA GTA AAT GAC TTT CAG
Vβ18	GAT GAG TCA GGA ATG CCA AAG GAA
Vβ20	AGC TCT GAG GTG CCC AGA ATC TC
Cβ	TTC TGA TGG CTC AAA CAC

Table 2. Sequences of primers for the second PCR

Primers	5′ ---------- 3′
Vβ1	C TCA GCT TTG TAT TTC TGT G
Vβ5	T CGG CCC TTT ATC TTT GCG C
Vβ8	C TCA GCT GTG TAC TTC TGT G
Vβ14	G ACC TCT CTG TAG TTC TGT G
Vβ16	T TCT GGA GTT TAT TTC TGT G
Vβ17	G ACA GCT TTC TAT CTC TGT G
Vβ18	T TCG GCA G(GorC) T TAT TTC TGT G
Vβ20	C TCT GGC TTC TAT CTC TGT G

KCl, with 20 pmol of each primer in 50 μl reaction volume with 2.5 U of *Taq* polymerase (Korea Biotec. Co., Taejon, Korea). After 35 cycles, an additional extension at 72℃ for 10 min was carried out. The PCR products were visualized on 1 % agarose gel and 1~3 μl of these products served as the template for the next round of PCR.

The sense primers that were nested 3′ to the Vβ specific primer (Vβ-N) (Table 2) and the antisense primer, Cβ were used in the second round of PCR. For the second amplification, the Cβ primer was end labeled with γ-^{32}P-ATP using T4 kinase (Gibco BRL, Gaithersburg, MD). PCR condition were identical to those of first round PCR, except that primer concentration was reduced to 3 pmol per 50 μl reaction volume. After 15 cycles, each 3~5 μl of the radioactive PCR product was loaded on 6% poly acrylamide sequencing gel. Bands were visualized after overnight exposure to Kodak AR film.

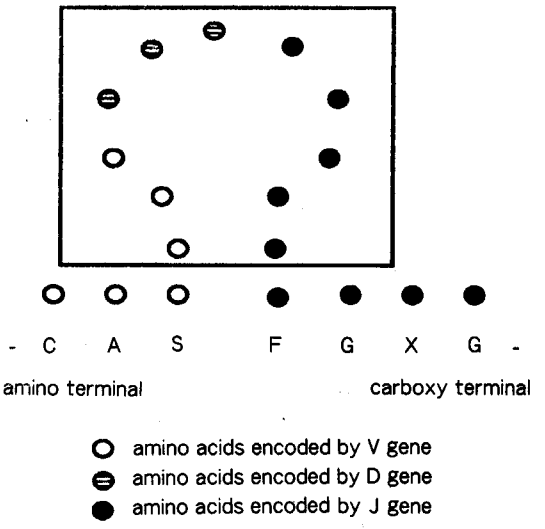

Fig. 1. *Schematic organization of CDR3 in TCR Vβ chain. CDR3 loop is expressed as a box. Junctional diversity can be occurred between V and D or D and J joining sites.*

Usually CDR3 is defined as a region from the consensus amino acid sequence of CASS (C; cysteine, A; alanine, S; serine) at the 3′ end of most Vβ segment to the J region encoded FGXG (F; phenylalanine, G; glycine, X; any amino acid). In this study CDR3 length was defined as the distance from the second S in V region to any amino acid before FGXG in the J region (Fig. 1). Thus, the CDR3 included in the sequence Vβ-CASSLNWSQDTQFGPG-Jβ would count as nine amino acids.

Statistics

Statistical analysis employed the Mann-Witney test or Wilcoxon Signed Rank test.

RESULTS

Expression of CD3, CD19, CD4, CD8 and DR molecules in the SFMCs or PBMCs

$CD3^+$, $CD19^+$, $CD4^+$ and $CD8^+$ cells among the PBMCs or SFMCs were analyzed by mono-clonal antibodies. The mean percentage

Table 3. Percentages of $CD3^+$ and $CD19^+$ cells in rheumatoid arthritis

group \ Phenotype	$CD3^+$ cells (n=12)	$CD19^+$ cells (n=12)
synovial fluid mononuclear cells	75.1±11.9*	1.6±1.8**
peripheral blood mononuclear cells	65.5±20.6	9.4±7.2

Mononuclear cells were stained with anti-CD3 or anti-CD19 monoclonal antibodies and total 10,000~20,000 cells were analyzed. Values are mean±S.D.. *p >0.01. **p=0.007.

Table 4. Percentages of $CD4^+$ and $CD8^+$ T cells in rheumatoid arthritis

group \ Phenotype	$CD4^+$ T cells (n=12)	$CD8^+$ T cells (n=12)
synovial fluid mononuclear cells	43.3±17.7*	30.3±15.5*
peripheral blood mononuclear cells	45.2±16.2	19.9±11.9

Mononuclear cells were stained with anti-CD4 or anti-CD8 monoclonal antibodies and total 10,000~20,000 cells were analyzed. Values are mean±S.D.. *p >0.01.

(75.1±1.9%) of $CD3^+$ cells in the SFMCs was higher than that (65.5±20.6%) of the PBMCs and the mean percentage (1.6±1.8%) of $CD19^+$ cells in the SFMCs was lower than that (9.4±7.2%) of the PBMCs (Table 3). But only the difference in $CD19^+$ cells was significant (Table 3, p=0.007). The mean percentage (30.3±15.5%) of $CD8^+$ cells in the SFMCs was higher than that (19.9±11.9%) of the PBMCs but there was no significance (Table 4, p>0.1).

To know the proportion of activated T cells, DR^+ T cells were evaluated. The mean percentage (32.7±15.2%) of DR^+ T cells in the SFMCs was significantly higher than that (2.2±1.0%) of the PBMCs (Fig. 2, p<0.01).

Table 5. Percentages of $V\beta5^+$ and $V\beta8^+$ cells in rheumatoid arthritis

group \ Vβ family	$V\beta5^+$ T cells (n=12)	$V\beta8^+$ T cells (n=12)
synovial fluid mononuclear cells	4.5±1.4*	4.1±1.3*
peripheral blood mononuclear cells	2.8±2.2	2.5±0.8

Mononuclear cells were stained with anti-Vβ8 monoclonal antibodies and total 10,000~20,000 cells were analyzed. Values are mean±S.D.. *p>0.01.

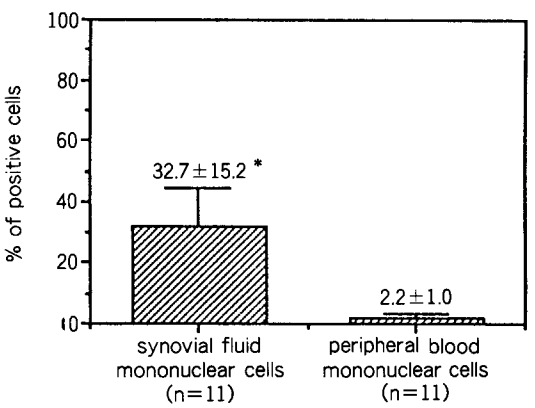

Fig. 2. *DR expression in rheumatoid arthritis patients. Mononuclear cells were stained with anti-DR or anti-CD3 monoclonal antibodies and total 10,000~20,000 cells were analyzed. Values are mean±S.D* p<0.01.*

Comparison of the Vβ gene usage of the SFMCs and PBMCs

To analyze TCR Vβ repertoire, $V\beta5^+$ or $V\beta8^+$ cells were evaluated (Table 5) by monoclonal antibodies. The mean percentage of $V\beta5^+$ cells (4.5±1.4%) and $V\beta8^+$ cells (4.1±1.3%) in the SFMCs were higher than that of the PBMCs (2.8±2.2% and 2.5±0.8%, respectively). But the differences were not significant (Table 5, p>0.1).

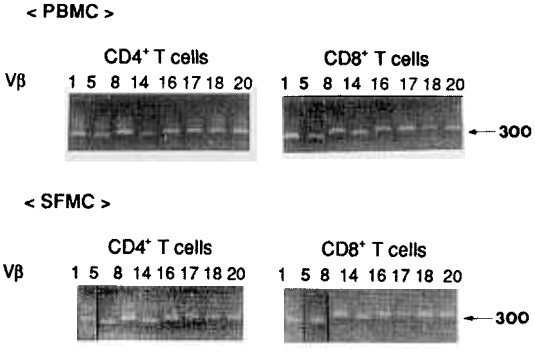

Fig. 3. *First PCR products (patient #1). Ten μl of each PCR products were analyzed on 1.5% agarose. The size marker (←300) indicates 300 bp. PBMC=peripheral blood mononuclear cells. SFMC=synovial fluid mononuclear cells.*

Comparison of the clonality of the SFMCs and PBMCs

Among 3 RA patient, the clonal expansions were evaluated by two step PCR. All the 8 Vβ genes tested in this study were detected in both the SFMCs and PBMCs samples (Fig. 3).

Among $CD4^+$ T cells in the SFMCs of 3 RA patients, 5 clonal expansions were detected (Fig. 4 & Fig. 5). In the SFMCs from patient #1, there was one clonal expansion in $V\beta18^+$ T cells (CDR3 length: 8 amino acids). In the SFMCs from patient #2, there were 3 clonal expansions; 2 in $V\beta5^+$ T cells (CDR3 length: 8 and 10 amino acids) and one in $V\beta14^+$ T cells

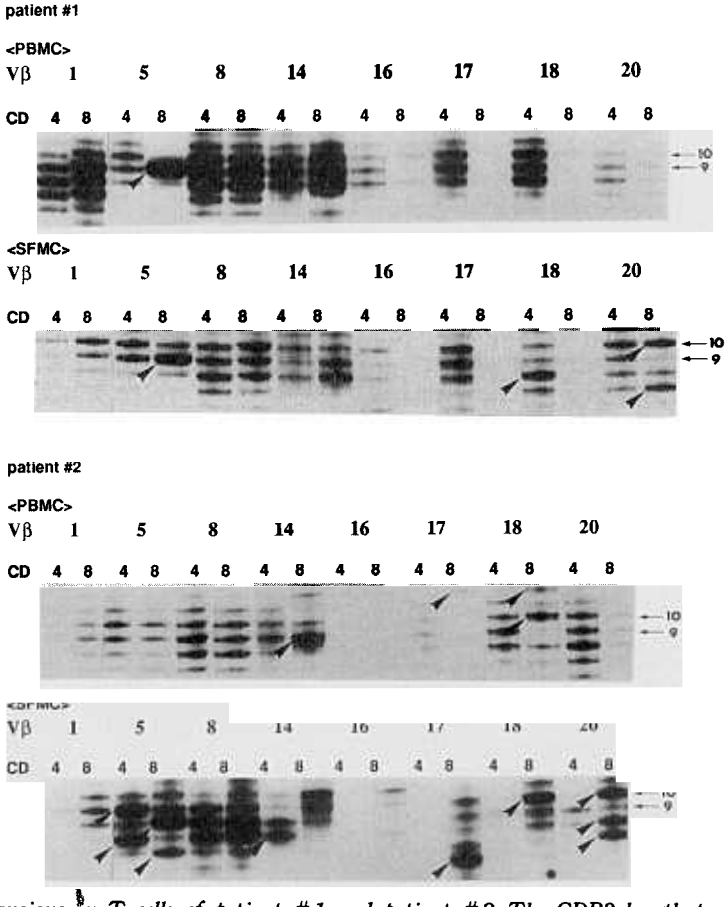

Fig. 4. *Oligoclonal expansions in T cells of patient #1 and patient #2. The CDR3 length profile was analyzed by two step PCR. Each 3~5 μl of radiolabelled second PCR products were separated on 6% denaturing poly. acrylamide gel. Bands were visualized after overnight exposure. The size marker (←9) indicates a CDR3 with 9 amino acids and the other size marker (←10) indicates a CDR3 with 10 amino acids.*

Vβ / patient	1		5		8		14		16		17		18		20	
	PBMC	SFMC	PBMC	SFMC	PBMC	SFMC	PBMC	SFMC	PBMC	SFMC	PBMC	SFMC	PBMC	SFMC	PBMC	SFMC
1														8		
2				8 10				8								
3						8										

Fig. 5. *Oligoclonal expansions in CD4⁺ T cells from peripheral blood or synovial fluid among rheumatoid arthritis patients. CDR3 length profile was analyzed by two sep PCR. The value is the amino acid number of CDR3 which shows the dominant clone. PBMC= peripheral blood mononuclear cells. SFMC=synovial fluid mononuclear cells. □/□=No dominant band was shown.*

Vβ / patient	1		5		8		14		16		17		18		20	
	PBMC	SFMC	PBMC	SFMC	PBMC	SFMC	PBMC	SFMC	PBMC	SFMC	PBMC	SFMC	PBMC	SFMC	PBMC	SFMC
1			9	9												7 10
2				7 9			9				12	6	10 12	10		7, 8 10
3			11		10											

Fig. 6. *Oligoclonal expansions in CD8⁺ T cells from peripheral blood or synovial fluid among rheumatoid arthritis patients. CDR3 length profile was analyzed by two step PCR. The value is the amino acid number of CDR3 which shows the dominant clone. PBMC= peripheral blood mononuclear cells. SFMC=synovial fluid mononuclear cells. □/□= No dominant band was shown.*

(CDR3 length: 8 amino acids). In the SFMCs from patient #3, there was one clonal expansion in Vβ8⁺ T cells (CDR3 length: 8 amino acids). There was no clonal expansion of CD4⁺ T cells from the PBMCs of 3 RA patients (Fig. 4 & Fig. 5).

Among CD8⁺ T cells from the SFMCs of 3 RA patients, a total of 10 clonal expansions were detected (Fig. 4 & Fig. 6). In the SFMCs from patient #1, there were 3 clonal expansions; one in Vβ5⁺ T cells (CDR3 length: 9 amino acids) and 2 in Vβ20⁺ T cells (CDR3 length: 7 and 10 amino acids). In the SFMCs from patient #2, there were 7 clonal expansions; two in Vβ5⁺ T cells (CDR3 length: 7 and 9 amino acids), one in Vβ17⁺ T cells (CDR3 length: 6 amino acids), one in Vβ18⁺ T cells (CDR3 length: 10 amino acids) and 3 in Vβ20⁺ T cells (CDR3 length: 7, 8 and 10 amino acids). In the SFMCs from patient #3, there was no clonal expansion.

In contrast to the 10 clonal expansions of CD8⁺ SFMCs, a total of 7 clonal expansions were detected among CD8⁺ T cells from the PBMCs of 3 RA patients (Fig. 4 & Fig. 6). In the PBMCs from patient #1, there was one clonal expansion in Vβ5⁺ T cells (CDR3 length: 9 amino acids). In the PBMCs from patient #2, there were 4 clonal expansions; one in Vβ14⁺ T cells (CDR3 length: 9 amino acids), one in Vβ17⁺ T cells (CDR3 length: 12 amino acids), and 2 in Vβ18⁺ T cells (CDR3 length: 10 and 12 amino acids). In the PBMCs from patient #3, there were 2 clonal expansions; one in Vβ5⁺ T cells (CDR3 length: 11 amino acids) and one in Vβ8⁺ T cells (CDR3 length: 10 amino acids).

DISCUSSION

In this study specific clonal expansions of CD8⁺ T cells in the synovial fluid from RA patients were analyzed. Our study demonstrates consistent T cell clonal expansions in the SFMCs. Previous reports of repeated TCR Vβ14⁺ and Vβ17⁺ T cell expansions in synovial fluid or tissue have suggested that clonal T cell expansions might be an important aspects of RA pathogenesis (Howel *et al.* 1991; Paliard *et al.* 1991, Scottini *et al.* 1992).

There are several critical variables to be considered when comparing studies on the analysis of TCR repertoire. The source of the cells, where they are obtained from synovial fluid or synovial tissue and whether they are obtained from relatively intact joints or from end-stage joints could influence the results. Another variable is whether the cells have or

have not expanded *in vitro* as well as the stimulation protocol that was used. In this study, we collected synovial fluid samples from patients with actively inflamed, but not end-stage, joint. The oligoclonal expansion of T cells were analyzed directly from the aspirated SFMCs and compared with the PBMCs from the same individual without *in vitro* expansions.

There are two possibilities that could account for a relative increase in mRNA amount with specific CDR3. One possibility is an absolute increase in the number of cells expressing specific CDR3 and the other possibility is the disproportional increase of mRNA expression on a T cell because they are selectively activated *in vivo*. In addition it is possible that RNA levels may not correlate well with the cell surface receptor expression. But no distortion was detected between the RNA and protein levels (Singer *et al.* 1990). Further, it is unlikely that the degree of T cell activation results in substantial distortion of TCR expression (Paliard *et al.* 1990), although the possibility of the up-regulation of mRNA could not be ruled out completely.

Analyzing T cell clonality we attemped to speculate on what type of antigen(s) may be involved. Generally, T cell expansions might be induced by a superantigen or a conventional antigen. Superantigens bind to major histocompatibility complex class II molecules of antigen presenting cells and to certain Vβ chains of TCR, irrespective of the antigen binding specificity of TCR. The initial hyperactivation by a superantigen results in the release of several lymphokines (Meithke *et al.* 1993) and the expression of functional IL-2 receptor (Meithke *et al.* 1994). However it caused the down-regulation of TCR complex of the responding Vβ$^+$ T cells after 6~20 hours of stimulation (Heeg *et al.* 1993). Superantigens activate T cells by a Vβ family specific pattern (Choi *et al.* 1991), while conventional antigens activate T cells by a clonotype specific pattern. Therefore, in order to discriminate family specific expansions and clonotype specific expansions of T cells from RA patients, the analysis of CDR3 size was performed in this study.

First, we attemped to find out the evidence of T cell activation in the synovial site. As a result, we found there were definite T cell activations. The percentage of DR$^+$ T cells in the SFMCs was increased significantly in comparison to that in the PBMCs.

The TCR Vβ family repertoire was analyzed by a serological method (Posnett *et al.* 1988). Until now serological method for Vβ repertoire had some serious limitations because only a few monoclonal antibodies specific for certain human Vβ regions were available. T cells bearing Vβ5 or Vβ8 were evaluated because Vβ5$^+$ or Vβ8$^+$ T cells are known to be activated by the superantigenic stimulation of staphylococcal exotoxins. We found the percentage of T cells bearing Vβ5 or Vβ8 was not increased in the SFMCs, which suggests no expansions of T cells bearing Vβ5 or Vβ8.

To find out whether the specific Vβ$^+$ T cells in the SFMCs were oligoclonal, a two step PCR was done. In this study, single gene families (Vβ1, Vβ14, Vβ17, Vβ18, Vβ20) and Vβ5 /Vβ8 were analyzed. First of all, all the 8Vβ genes were used in the SFMCs or PBMCs. Although the majority of T cells within the RA joint were polyclonal, the T cells important in the pathogenesis of RA are likely to undergo *in vivo* expansions or activation with exogenous IL-2 because they might have IL-2 receptor. In the SFMCs, clonal expansions in CD8$^+$ T cells were found in all three RA patients tested. There were also clonal expansions in the PBMCs from the same 3 RA patients. However, the patterns of clonal expansions were diverse. Vβ14$^+$ or Vβ17$^+$ T cells increased and were related to superantigen in RA (Paliard *et al.* 1991). But in our study, the evidence for involvement of superantigen activating Vβ14$^+$ or Vβ17$^+$ T cells was not found. The clonal expansion detected in Vβ14$^+$ CD8$^+$ and Vβ17$^+$ CD8$^+$ T cells from patient # 2 suggests that there were clone specific T cell expansions not Vβ family specific expansions. The presence of discrepant TCR repertoire in RA synovial fluid provided good supportive evidence that some type of antigen selection process occurs. Our studies have shown that the oligoclonality was not equally distributed between CD4$^+$ and CD8$^+$ T cells.

The finding that the oligoclonal expansions are primarily confined to $CD8^+$ T cells suggests that cytotoxic T cells or regulatory suppressor T cells were activated. The clonal expansions were found in 5 out of from 10 normal adults and the clones responsible for such dominant bands were identified as $CD45RO^+$, which is the marker for the activated or memory T cells (Hingorani *et al.* 1992). However, studies of the SFMCs from patients with other inflammatory arthritis will be needed to determine whether the $V\beta$ discrepancies that we observed are specific for rheumatoid arthritis.

We found 2 concordant patterns of clonal expansions from the SFMCs and the PBMCs ; one in $CD8^+$ $V\beta5^+$ T cells from patients #1, which are composed of CDR3s with 9 amino acids and one in $CD8^+$ $V\beta18^+$ T cells from patients #2, which are composed of CDR3s with 10 amino acids. The synovial T cell clones might be derived from the dominant pool of the peripheral blood T cells. But gene sequencing of these clones must be performed to determine that these clones in the SFMCs and the PBMCs from the same patient are exactly identical.

In conclusion, we found that there were definite T cell activations in synovial site and clonal expansions of T cells in both the SFMCs and the PBMCs from RA patients. The clonal patterns of T cells were discrepant between the PBMCs and the SFMCs even in the same patient. These findings suggest that the pathogenic T cells might expand in the inflamed synovial site or migrate to the inflamed synovial site. But our study could not demonstrate a common pattern of clonal expansions in the SFMCs from 3 RA patients tested. The findings indicate, however, that T cell subpopulations expressing different TCR variable genes were involved in the pathogenesis in different RA patients. Therefore, different TCR variable gene could be used to gain further knowledge in the identification of relevant antigens and pathogenic T cells.

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