Dynamic T Cell Receptor Clonotype Changes in Synovial Tissue of Patients with Early Rheumatoid Arthritis: Effects of Treatment With Cyclosporin A (Neoral®)

ANN VANDERBORGHT, FILIP DE KEYSER, PIET GEUSENS, MARC DE BACKER, MICHEL MALAISE, DOMINIQUE BAETEN, FILIP VAN DEN BOSCH, ERIC M. VEYS, JEF RAUS, and PIET STINISSEN

ABSTRACT. ***Objective.*** To study T cell receptor (TCR) repertoire changes in synovial membrane over a 16 week period in patients with early rheumatoid arthritis (RA); and to study the influence of cyclosporin A (CSA) on TCR repertoire in a subgroup of these patients.

Methods. Synovial tissue biopsies and paired blood samples were obtained from 12 patients with early RA at 2 time points. Seven patients were treated with CSA (Neoral-Sandimmun®, 3 mg/kg/day) and 5 patients with placebo for 16 weeks. TCR V gene repertoires were analyzed by semiquantitative PCR-ELISA. CDR3 spectratyping and sequence analysis was used to compare TCR clonotype distributions.

Results. TCR-specific mRNA was detected in all synovial tissue biopsies at the first sampling, but in only 8/12 biopsies 16 weeks later (4/7 CSA group, 4/5 placebo group). Overrepresented TCR BV genes were found in biopsies of 10/12 patients at the first time point, and in 7/12 patients after 16 weeks (3/7 CSA, 4/5 placebo). CDR3 sequence analysis revealed dynamic repertoire changes with only a few persisting clonotypes in the synovial tissue of placebo controls. Persisting T cell clonotypes were more frequently found in the synovial tissue of CSA treated patients compared to the placebo group.

Conclusion. These data suggest a dynamic process of T cell recruitment in the joints of RA patients. This process, possibly due to activation and subsequent infiltration of new T cell clones, apparently is influenced by CSA treatment. Synovial tissue T cells were no longer detected after 16 weeks' CSA treatment in 3 patients. In the other CSA treated patients, new T cell clones infiltrated, while other clones were persistently represented in the joints. These data may have important consequences for the design of T cell targeted therapies for RA. (J Rheumatol 2002;29:416–26)

Key Indexing Terms:
RHEUMATOID ARTHRITIS | SYNOVIUM | AUTOIMMUNITY
T CELL RECEPTOR REPERTOIRE | CYCLOSPORIN A

Rheumatoid arthritis (RA) is a chronic inflammatory disease affecting synovial membranes of multiple joints. Although RA is considered to be a typical autoimmune disease, its etiology is still unknown[1]. Several lines of evidence suggest that T lymphocytes are involved in the initiation and perpetuation of RA, although the disease-mediating lymphocytes have not been identified yet. Since little information is available on candidate autoantigens in RA, many groups have compared the T cell receptor (TCR) repertoire of synovial T cells and blood derived T cells, to identify disease-relevant TCR V genes or T cell subtypes[2-4]. An important variable complicating the interpretation of these data is the variable disease duration of RA. We and others have shown that the TCR repertoire in synovium becomes more diverse with progression of the disease[5-8]. This process may be caused by the infiltration of irrelevant "bystander" T cells, or by the influx of T cells newly activated by locally sequestered antigens, a process termed determinant spreading[9]. In addition, it has been hypothesized that T cells play a prominent role in the early stages of the disease process only, while T cell independent mechanisms are involved in later pathogenic

From the Biomedisch Onderzoeksinstituut DWI, Limburgs Universitair Centrum, Diepenbeek; Rheumatology Department, University of Ghent, Ghent; Novartis Pharma, Brussels; and Rheumatology Department, University of Liege, Liege, Belgium.

Supported by Novartis Pharma Brussels, Belgium and the Belgian FWO (grant G.0093.97).

A. VanderBorght, PhD, Postdoctoral Fellow, Vlaams Instituut voor de bevordering van het Wetenschappelijk-Technologisch onderzoek in de industrie (IWT), Biomedisch Onderzoeksinstituut DWI; P. Geusens, MD, PhD; J. Raus, MD, PhD; P. Stinissen, PhD, Biomedisch Onderzoeksinstituut DWI; F. De Keyser, MD, PhD; D. Baeten, MD; F. Van den Bosch, MD; E.M. Veys, MD, PhD, Rheumatology Department, University of Ghent; M. De Backer, MD, Novartis Pharma; M. Malaise, MD, PhD, Rheumatology Department, University of Liege.

Address reprint requests to Dr. P. Stinissen, Biomedisch Onderzoeksinstituut DWI, Limburgs Universitair Centrum, Universitaire Campus, B-3590 Diepenbeek, Belgium. E-mail: piet.stinissen@ luc.ac.be

Submitted March 20, 2001; revision accepted October 16, 2001.

steps of RA[3,10]. Studies to define the role of T cells as possible initiators of the disease process should therefore be focused on patients at an early phase of the disease.

We evaluated TCR V gene repertoire changes and TCR clonotype persistence in the synovial tissue and paired blood of patients with early RA. In addition, we examined whether treatment with cyclosporin A microemulsion (CSA; Neoral-Sandimmun®) influences these TCR changes. We studied 12 patients with early RA treated with either CSA (n = 7) or placebo (n = 5) to evaluate the effects of CSA treatment on the TCR profile in the joints. All patients were sampled for blood and synovial tissue at entry and after 16 weeks of treatment. TCR V gene expression was studied by polymerase chain reaction-ELISA[11], while persistence of TCR clonotypes was analyzed by CDR3 spectratyping and CDR3 sequence analysis[12]. CSA is effective in improving clinical measures in RA[13,14]. The microemulsion based formulation of CSA (Neoral®) has an increased bioavailability, and has been shown to have a similar safety, tolerability, and efficacy profile as the original preparation (Sandimmun®)[15,16]. We studied TCR repertoire changes in the synovial tissue of patients during a 16 week followup period, while a subgroup of patients was treated with CSA.

MATERIALS AND METHODS

Patient population and study design. Eighteen patients with early RA with disease duration less than one year from 3 centers were included in a double blinded, placebo controlled study (Table 1). Patients either met the 1987 revised American College of Rheumatology criteria (17 patients)[17], or presented with synovitis and/or inflammatory polyarthralgia. The patients were HLA typed and tested for the RA related haplotypes HLA-DR4 or HLA-DR1 (Table 1). All patients were positive for rheumatoid factor. All patients had oral piroxicam as the only treatment before entering the study, and this treatment was continued during the study. After giving signed informed consent, patients were randomized; 12 patients were treated with a CSA microemulsion (Neoral-Sandimmun) and 6 with placebo for 16 weeks. Peripheral blood and synovial membrane tissue was sampled at entry and at Week 16. Synovial tissue was obtained by fine needle arthroscopy[18]. Roughly 15 small biopsies were obtained at each arthroscopy and samples were preferentially taken in macroscopically inflamed areas of the synovial membrane. Patients were clinically examined by blinded rheumatologists at Week –3, –1 (baseline), and at Weeks 2, 4, 6, 8, 10, 12, and 16. Three patients taking CSA therapy were withdrawn from the study before Week 16 because of an increase of serum creatinine (n = 1), gastric complaints (n = 1), and flare of disease (n = 1). Two other patients who were followed until Week 16 discontinued the study medication prematurely because of an increase of serum creatinine (one taking CSA) and thrombocytopenia (one placebo). One placebo treated patient violated the study protocol because of administration of an intraarticular betamethasone injection.

Blood and synovial tissue were available for TCR analysis from 7 CSA treated and 5 placebo patients at both time points (Table 1). Two of these patients (Patients 7 and 17) discontinued the study medication at Weeks 9 and 12, respectively, but were still included in the TCR analysis. Patient 7 was treated with CSA and Patient 17 with placebo. A reduced number of swollen and tender joints was observed in 11 of 17 patients (8/11 CSA group, 3/6 placebo) (Table 1). This reduction was more pronounced in the CSA compared to the placebo group. Detailed clinical data from this limited trial will be published in a separate report (in preparation). The small size of this explorative study does not allow any conclusions on the clinical efficacy of the medication.

The ethical committees of all participating centers approved the study protocol.

RNA extraction and cDNA synthesis. Analysis of samples and interpretation of primary data were done in a blinded manner. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood and pelleted in ice cold phosphate buffered saline in fractions of 2×10^6 cells. Lavage fluid was transported on ice and the cells were immediately pelleted by centrifugation at 4°C for 15 min. Synovial tissue biopsies were immediately frozen at –70°C. RNA was extracted from 5 randomly selected

Table 1. Patient characteristics.

Patient	Age/Sex	Group	HLA	Sampling		Swollen joints		Tender Joints		Included in TCR Analysis		Comments
			DR1/DR4	Start PB/ST	End PB/ST	Start	End	Start	End	Start	End	
1	54 M	CSA	+/+	+/+	+/+	15	9	10	0	Yes	Yes	
2	67 F	CSA	–/–	+/+	+/+	6	4	10	7	Yes	Yes	
3	46 F	CSA	–/+	+/+	+/+	19	6	26	5	Yes	Yes	
4	46 M	CSA	–/+	+/+	+/+	7	7	6	9	Yes	Yes	
5	40 F	CSA	+/–	+/+	+/+	7	3	11	5	Yes	Yes	
6	37 F	CSA	–/–	+/+	+/+	10	8	13	4	Yes	Yes	
7	60 F	CSA	–/+	+/+	+/+	0	0	2	2	Yes	Yes	Stopped medication after Wk 9*
8	62 M	CSA	–/–	+/+	–/–	1	0	6	4	Yes	No	
9	46 F	CSA	ND	+/+	–/–	11	4	15	13	Yes	No	Stopped medication after Wk 8*
10	18 F	CSA	ND	+/+	–/–	14	NT	16	NT	Yes	No	Stopped medication after Wk 11*
11	65 F	CSA	–/–	+/+	+/–	7	7	28	24	No	No	Stopped medication after Wk 1*
12	37 F	CSA	–/–	–/–	+/+	2	0	2	0	No	No	
13	59 M	Placebo	–/–	+/+	+/+	11	3	17	7	Yes	Yes	
14	35 M	Placebo	ND	+/+	+/+	13	13	23	23	Yes	Yes	
15	68 F	Placebo	–/+	+/+	+/+	7	8	6	1	Yes	Yes	
16	64 F	Placebo	–/–	+/+	+/+	4	4	3	2	Yes	Yes	
17	70 M	Placebo	–/–	+/+	+/+	3	2	3	2	Yes	Yes	Stopped medication after Wk 12*
18	43 F	Placebo	+/+	+/+	+/–	12	7	13	6	Yes	No	Extra medication **

* Summarized in Results. ** Intraarticular betamethasone injection. NT: not tested, due to dropout, PB: peripheral blood, ST: synovial tissue, ND: not done.

biopsies out of 15 biopsies taken during the needle arthroscopy. The biopsies were manually crushed following the manufacturer's homogenization and extraction procedures (Qiagen, Westburg). Total RNA was extracted from 2×10^6 PBMC or lavage fluid cells with the Rn-easy method and reverse transcribed into first-strand cDNA using oligo-dT as described[11].

To check the integrity of the isolated cDNA, a control PCR amplification was performed with primers specific for the $ß_2$-microglobulin gene. An additional control amplification was performed with primers specific for the constant region of the TCR ß-chain gene to check whether sufficient amount of T cell mRNA was present in the synovial tissue biopsies.

TCR repertoire screening using PCR-ELISA. PCR-ELISA was performed as described[7,11]. Relative expressions of TCR BV genes in the total TCR V gene repertoire were presented as fractions of the total TCR V gene expression using the formula

$$\% \, BV_x = (OD_{450} \, (BV_x) \times 100) / \sum OD_{450} \, (BV_n)^{11}$$

Overrepresented TCR BV genes were defined as exceeding an arbitrarily defined cutoff value based on the mean TCR BV gene expression levels in the blood of 10 healthy subjects + 3 standard deviations[7,11].

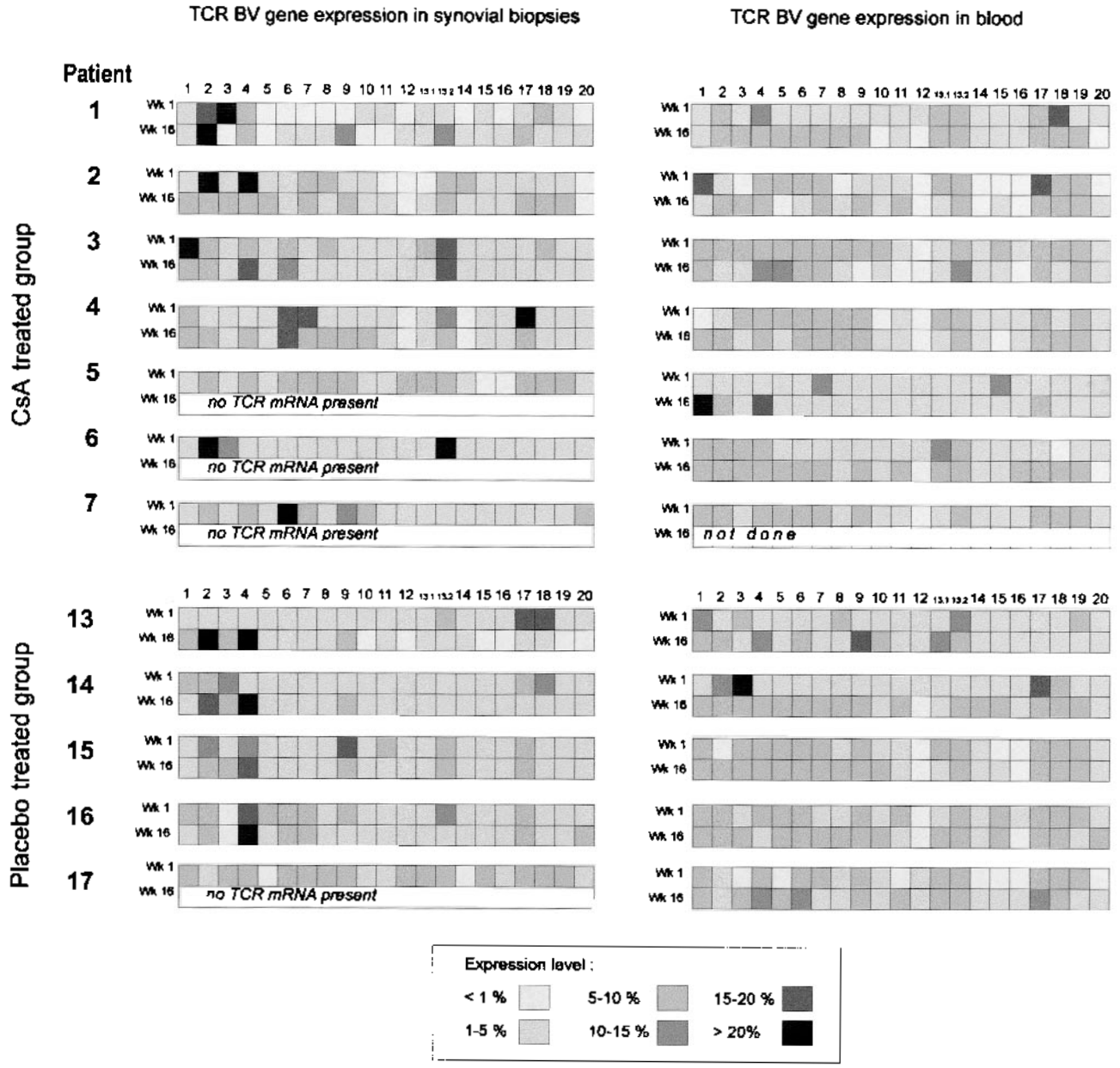

Figure 1. T cell receptor V gene expression profile at 2 time points in synovial tissue and peripheral blood of patients with early RA treated with CSA or placebo. RNA was extracted from blood lymphocytes or synovial tissue biopsies and reverse transcribed to cDNA. This cDNA was used as a template for individual PCR amplifications with primers specific for each BV gene element. The PCR products were quantified by semiquantitative PCR-ELISA. To determine the quality of extracted cDNA (using primers specific for a housekeeping gene) and to determine the TCR mRNA content of the samples (using primers specific for the TCR ß chain constant region) 2 control PCR amplifications were performed on each sample. All control reactions were successful for the housekeeping gene, while some amplifications were not successful for the TCR BV-specific PCR, indicating that no or small amounts of TCR mRNA were present (no TCR mRNA present). Week 1 = entry.

CDR3 spectratype analysis and sequencing of TCR rearrangements. CDR3 region spectratype analysis was performed as described[11]. CDR3 region sequences were determined by subcloning the TCR BV gene amplification products; plasmid DNA was isolated from a representative number of recombinant plasmids and analyzed as described[7].

RESULTS

TCR BV expression in blood and synovial tissue biopsies at first time point. RNA was extracted from PB and synovial tissue biopsies of 16 patients with early RA and reverse transcribed into cDNA. Successful control amplifications ($ß_2$ microglobulin and TCR BC region) were obtained for all PBMC and biopsy samples (data not shown). Using PCR-ELISA the TCR BV gene repertoire was found to be highly diverse in PB and synovial tissue of all patients (Figure 1). To study possible T cell expansions, we subsequently identified the BV genes that were overrepresented in PB or synovial tissue. Overrepresented BV genes were identified in biopsies of 14/16 and in PB of 7/16 patients (Table 2). The mean number of overrepresented BV genes was higher in biopsies (2.1 ± 0.3) than in PB (0.7 ± 0.2; $p < 0.05$), and some BV genes were found to be overrepresented in the synovial tissues of a high proportion of the patients (BV2 in 41%, BV4 in 35% of patients) (Table 2, Figure 1). The overrepresented BV genes in PB differed from those in the biopsies of all patients, except BV18 for Patient 10 and BV3 for Patient 14 (Table 2, Figures 1 and 2). Thus, the patients' TCR repertoire was diverse in both PB and synovial tissue, although overrepresentation of some TCR BV genes was more often detected in the synovial tissue, suggesting a preferential accumulation of some T cell subtypes at the disease site.

Comparison of TCR BV expression in synovial biopsies and lavage in 4 patients. To asses whether TCR BV gene repertoires are similar in synovial tissue and paired lavage fluid, TCR profiles of paired samples were analyzed for 4 patients. In one patient, synovial tissue was analyzed from 2 different biopsies of the same joint. As illustrated in Figure 2, the TCR BV expression profile was similar in the lavage fluid and the synovial tissue for Patients 8, 10, and 18, but was slightly different for Patient 9. The TCR BV gene repertoires in the 2 biopsies of Patient 10 were highly comparable. Overrepresentation of the BV4 and BV18 genes was seen in both samples. Thus, although the TCR expression profile of the biopsies may reflect the T cell composition at one site in the joint only, it seems to correlate with the profile at another site in the joint and with the profile in the lavage fluid. The data suggest that analysis of synovial tissue biopsies provides a reasonable estimate of the TCR expression profile in the joint.

TCR BV gene expression in blood and synovial biopsies after 4 months. To study the fluctuations in the TCR profile over time, PBMC and synovial tissue biopsies of 12 patients were analyzed after 4 months. The $ß_2$-microglobulin control amplification was successful for all PBMC and biopsy samples. The TCR ß chain-specific control was successful for all PBMC samples, but only for 8/12 biopsy samples, suggesting that 4 biopsy samples did not contain sufficient TCR-specific mRNA for further analysis by PCR-ELISA. In one patient (Patient 7), 2 independent synovial biopsies from the same joint were negative for the TCR-specific control PCR. TCR mRNA negative biopsies were more frequently observed in the CSA treated patients (3/7) than in the placebo group (1/5) (not significant).

As for the first sampling, most TCR expression profiles were heterogeneous at the second time point, in both blood and tissue. We then examined possible changes in the

Table 2A. Overview of overrepresented TCR BV genes before and after treatment.

A. Patients with early RA examined at 2 time points.

CsA Treated Group				Placebo Group			
Patient	Sample	BV Genes Overrepresented in the Sample		Patient	Sample	BV Genes Overepresented	
		At Entry	After 4 mo			At Entry	After 4 mo
1	PBMC	None	None	13	PBMC	1	9,11
	Biopsy	2,3	2		Biopsy	17,18	2,9,4
2	PBMC	1,17	1	14	PBMC	2,3,17	None
	Biopsy	2,4	None		Biopsy	3	2,4
3	PBMC	None	5,13.2	15	PBMC	None	None
	Biopsy	1,13.2	6,13.2		Biopsy	2,9	4
4	PBMC	None	None	16	PBMC	None	None
	Biopsy	6,7,13.2,17	6		Biopsy	13.2	4
5	PBMC	None	1,4	17	PBMC	None	None
	Biopsy	None	No T cell mRNA**		Biopsy	None	No T cell mRNA
6	PBMC	None	None				
	Biopsy	2,13.2	No T cell mRNA				
7	PBMC	None	ND				
	Biopsy 1	6,9	No T cell mRNA				
	Biopsy 2	ND	No T cell mRNA				

Table 2B. Control patients with early RA examined at a single time point.

Patient	Sample	BV Genes Overrepresented
8	PBMC	8
	Biopsy	2,3,4,18
18	PBMC	12
	Biopsy	2,4,13.1,18
9	PBMC	9
	Biopsy	2,4,12,18
10	PBMC	18
	Biopsy 1	4,18
	Biopsy 2	4

** Control PCR amplifications were performed with primers specific for β_2 microglobulin to check the integrity of the isolated RNA, and with primers specific for the constant region of the TCR β–chain (TCR BC) to evaluate the TCR mRNA content. All β_2 microglobulin PCR amplifications were successful. TCR BC PCR were not successful for some samples, indicating that no TCR mRNA is present. ND: not done.

number of BV genes that were overrepresented in the patient groups. Overrepresented BV genes were found in 4/12 PBMC samples (3/7 in the CSA group, 1/5 placebo group), which is comparable with the data at entry. Overrepresented BV genes were identified in most biopsy samples that contained TCR-specific mRNA. A significant reduction in the number of overrepresented TCR BV genes was observed in synovial biopsies of CSA treated patients ($p < 0.05$), but not in the synovial tissues of placebo controls (Table 3).

Thus, some patients treated with CSA no longer had TCR mRNA present in the synovial biopsies, suggesting either an inhibition of TCR mRNA expression in the synovial tissue, or the absence (or reduction below the detection limit of the PCR-ELISA) of T cells in these tissues.

Persistence of overrepresented TCR BV genes in synovium and blood. Persistence of overrepresented BV gene families at the disease site is a possible indication that the corre-

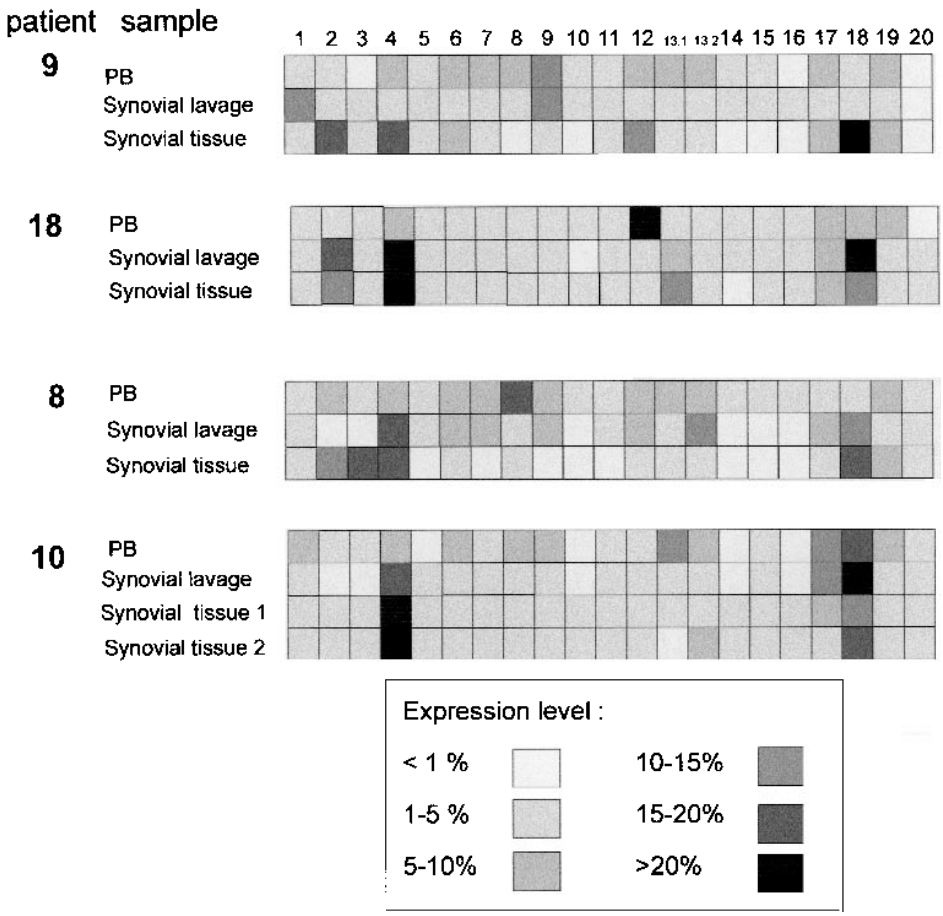

Figure 2. T cell receptor V gene expression profiles in blood, synovial tissue, and lavage fluid of 4 control patients with early RA. Semiquantitative analysis of TCR V gene expression was performed as described in the legend of Figure 1. In one patient (Patient 10) 2 synovial tissue biopsies taken from 2 locations at the same time in the same joint were analyzed.

Table 3. Mean number of overrepresented TCR V genes at entry and at Week 16 after treatment with CSA or placebo. Patients with no TCR-specific mRNA as tested by a control PCR amplification with BC-specific primers were scored as having no overrepresented BV genes.

Sample Group	Mean No. of Overrepresented TCR ßV Genes, ± SE		p
	At Entry	At Week 16	
Peripheral Blood			
Total group (n = 12)	0.50 ± 0.29	0.58 ± 0.27	
CSA treated (n = 7)	0.29 ± 0.29	0.71 ± 0.36	
Placebo treated (n = 5)	0.80 ± 0.58	0.40 ± 0.40	
Synovial biopsy			
Total group (n = 12)	1.67 ± 0.31	0.92 ± 0.29	< 0.05
CSA treated (n = 7)	2.00 ± 0.40	0.57 ± 0.28	< 0.05
Placebo treated (n = 5)	1.20 ± 0.37	1.40 ± 0.51	

sponding T cells may be involved in the disease process. We used CDR3 spectratype analysis to test whether BV clonotypes persisted in the joints of patients with early RA during a 4 month followup period. Since it is not feasible to analyze all BV gene families with this technique, we focused on BV gene families that were found to be overrepresented at one time point in the synovial tissue. CDR3 spectratype analysis provides information on the clonal distribution of individual T cell populations based on the analysis of the lengths of amplified CDR3 regions, which are depicted as peaks in the profile. In addition, the heights of the peaks correspond to the intensity of the bands. When bands of the same CDR3 length are shared between T cell subsets with identical BV (or BV-BJ) expression, this is a strong indication that these are identical T cell clonotypes[12]. CDR3 spectratype analysis was performed for 21 BV families overrepresented in the synovial tissue at either time point (Table 4, Figure 3). These BV gene-specific CDR3 spectratypes were determined in PB and synovial tissue at both time points.

The first type of information from this analysis is whether the T cells of a particular BV family are polyclonal, oligoclonal, or monoclonal. This was defined by a Gaussian distributed profile with at least 4 bands (polyclonal), a less heterogeneous profile with 2–4 bands (oligoclonal), or a single band that represented the majority of the peak area (monoclonal). Studies have shown that dominant peaks represent clonal expansions within a particular BV family[12]. All CDR3 profiles in the blood were poly- or oligoclonal at both time points, and in both patient groups (Table 4). Some polyclonal spectratypes did not show a typical Gaussian distribution, but contained bands at increased frequency, suggesting that they correspond to T cell populations that are expressed at increased levels in the blood (BV9 gene spectratype of Patient 15 at entry) (Figure 3). At the second time point the CDR3 profiles in blood were either less, more, or equally heterogeneous, and no obvious differences were found between the 2 patient groups (Table 4). As all CDR3 profiles in PB were highly complex, it is difficult to draw conclusions about the persistence of T cell clonotypes. Different T cell clonotypes with the same CDR3 length can be present in these heterogeneous samples.

In the synovial tissue biopsies, the majority of analyzed BV genes were of oligoclonal or monoclonal origin. TCR BV families that were overrepresented in biopsies were predominantly mono- or oligoclonal, indicating that only a limited number of T cell clonotypes were represented in these expanded T cell populations. The diversity of about half of the synovial CDR3 spectratypes remained unchanged at the second time point, while the remaining profiles became either broader or more skewed, irrespective of the treatment. Interestingly, for the 3 CSA treated patients who had sufficient TCR mRNA in the biopsies (TCR B chain amplification positive), CDR3 bands with identical fragment length were observed in BV gene-specific synovial spectratypes tested at 2 time points (Figure 3, Table 4). Out of 7 mono- or oligoclonal BV gene families tested from 3 CSA treated patients, 6 BV genes were found to contain identical CDR3 fragments at both time points. These results suggest a persistence of T cell clonotypes in the synovial tissue of these patients. For the placebo control patients, on the other hand, identical CDR3 bands observed at 2 time points were found at a much lower frequency (Figure 3, Table 4). Out of 7 mono- or oligoclonal BV gene families tested from 4 placebo controls, only one BV gene family was found to contain CDR3 fragments that were identified at both time points. It should be noted that spectratype analysis might not be sensitive enough to detect low frequency clonotype expansions in oligo- or polyclonal profiles.

In conclusion, dynamic CDR3 profile changes with few persisting clonotypes were found in the synovial tissue of placebo controls. These dynamic fluctuations were also observed in the synovial tissue of the CSA treated patients, but in these patients synovial T cell clonotype persistence was found more frequently in the 4 month followup period.

CDR3 region sequence analysis. It could be argued that a single peak obtained with the spectratype analysis (one CDR3 length) might not necessarily mean that only one T cell clone is present. It is possible that 2 or more independent T cell clones with different TCR sequences share a TCR BV gene segment of identical CDR3 length and thus will give rise to a single peak in the spectratype analysis. To test whether the spectratype data could be further proven by DNA sequence information, CDR3 DNA sequences of selected BV families from 3 patients were determined. CDR3 region sequences were represented in Table 5, which can be located at: http//alpha.luc.ac.be/~lucp1248/

The BV2 gene family of Patient 2 was found to be polyclonal by spectratype analysis in blood and synovial tissue at both time points (Table 4). CDR3 sequence analysis confirmed these findings, since a high degree of clonal diversity was observed (Table 5A). Some unique CDR3

Table 4. CDR3 spectratype analysis of selected BV genes in synovial tissue and blood.

Patient	BV Gene	Sample	BV Expression (%) Entry	BV Expression (%) Wk 16	CDR3 Spectratype Analysis CDR3 Profile* Entry	CDR3 Profile* Wk 16	Profile Changes	Persistence in** Synovial Tissue?
CSA Treated Group								
1	BV2	PBMC	5–10	5–10	Oligo	Poly	Broadening	
		Biopsy	> 15	> 15	Mono	Mono	No change	Yes
2	BV2	PBMC	1–5	5–10	Poly	Poly	No change	
		Biopsy	> 15	5–10	Poly	Poly	No change	—†
	BV4	PBMC	5–10	5–10	Poly	Poly	No change	
		Biopsy	> 15	5–10	Oligo	Poly	Broadening	—
3	BV1	PBMC	5–10	5–10	Poly	Poly	No change	
		Biopsy	> 15	5–10	Mono	Mono	No change	No
	BV6	PBMC	5–10	5–10	Poly	Poly	No change	
		Biopsy	5–10	10–15	Mono	Oligo	Broadening	Yes
	BV13.2	PBMC	5–10	10–15	Poly	Poly	No change	
		Biopsy	> 15	> 15	Oligo	Oligo	No change	Yes
4	BV6	PBMC	5–10	5–10	Poly	Oligo	Skewing	
		Biopsy	> 15	> 15	Oligo	Oligo	No change	Yes
	BV7	PBMC	5–10	5–10	Poly	Poly	Broadening	
		Biopsy	>15	5–10	Poly	Oligo	Skewing	Yes
	BV13.2	PBMC	5–10	5–10	Oligo	Oligo	Skewing	
		Biopsy	10–15	5–10	Oligo	Oligo	No change	Yes
	BV17	PBMC	5–10	5–10	Poly	Oligo	Skewing	
		Biopsy	> 15	5–10	Oligo	Mono	Skewing	Yes
7	BV6	PBMC	5–10	5–10	Poly	NT	NA	
		Biopsy	> 15	—††	Mono	NT	NA	NA
	BV9	PBMC	5–10	5–10	Poly	NT	NA	
		Biopsy	10–15	—††	Oligo	NT	NA	NA
Placebo Group								
13	BV2	PBMC	1–5	5–10	Poly	NT	NA	
		Biopsy	1–5	> 15	Mono	Oligo	Broadening	No
	BV9	PBMC	1–5	> 15	Poly	Poly	No change	
		Biopsy	1–5	10–15	Mono	Oligo	Broadening	Yes
	BV17	PBMC	1–5	1–5	Poly	Poly	No change	
		Biopsy	> 15	1–5	Oligo	Poly	Broadening	—†
	BV18	PBMC	1–5	1–5	NT	Poly	NA	
		Biopsy	> 15	1–5	Oligo	Oligo	No change	No
14	BV2	PBMC	10–15	5–10	Poly	Poly	No change	
		Biopsy	5–10	> 15	Oligo	Oligo	No change	No
15	BV2	PBMC	< 1	5–10	NT	Poly	NA	
		Biopsy	10–15	5–10	Oligo	Oligo	Broadening	No
	BV4	PBMC	5–10	5–10	NT	NT	NA	
		Biopsy	10–15	> 15	NT	Mono	NA	NA
	BV9	PBMC	5–10	5–10	Oligo	Poly	Broadening	
		Biopsy	> 15	5–10	Oligo	Mono	Skewing	No
16	BV13.2	PBMC	5–10	5–10	Poly	Poly	No change	
		Biopsy	10–15	1–5	Mono	Oligo	Broadening	No

CDR3 spectratypes were determined by amplification of CDR3 regions of selected BV genes using fluorescently labeled PCR primers. The amplified products were separated on high resolution polyacrylamide gels and analyzed by an automated DNA sequence analyzer. The fragment lengths were calculated using appropriate size standards and Genescan software.

* The CDR3 profile was considered polyclonal, oligoclonal, or monoclonal when, respectively, more than 4, between 2 and 4, or only a single band was observed.

** If bands of the same length (the same mobility) were present in the synovial tissue samples at entry and Week 16, it was assumed that T cells with corresponding CDR3 regions persisted in the synovium.

† Persistence of bands in polyclonal profiles was not taken into account, since it is likely that these bands contain more than one CDR3 sequence.

†† No T cell mRNA.

NT: not tested, NA: not applicable.

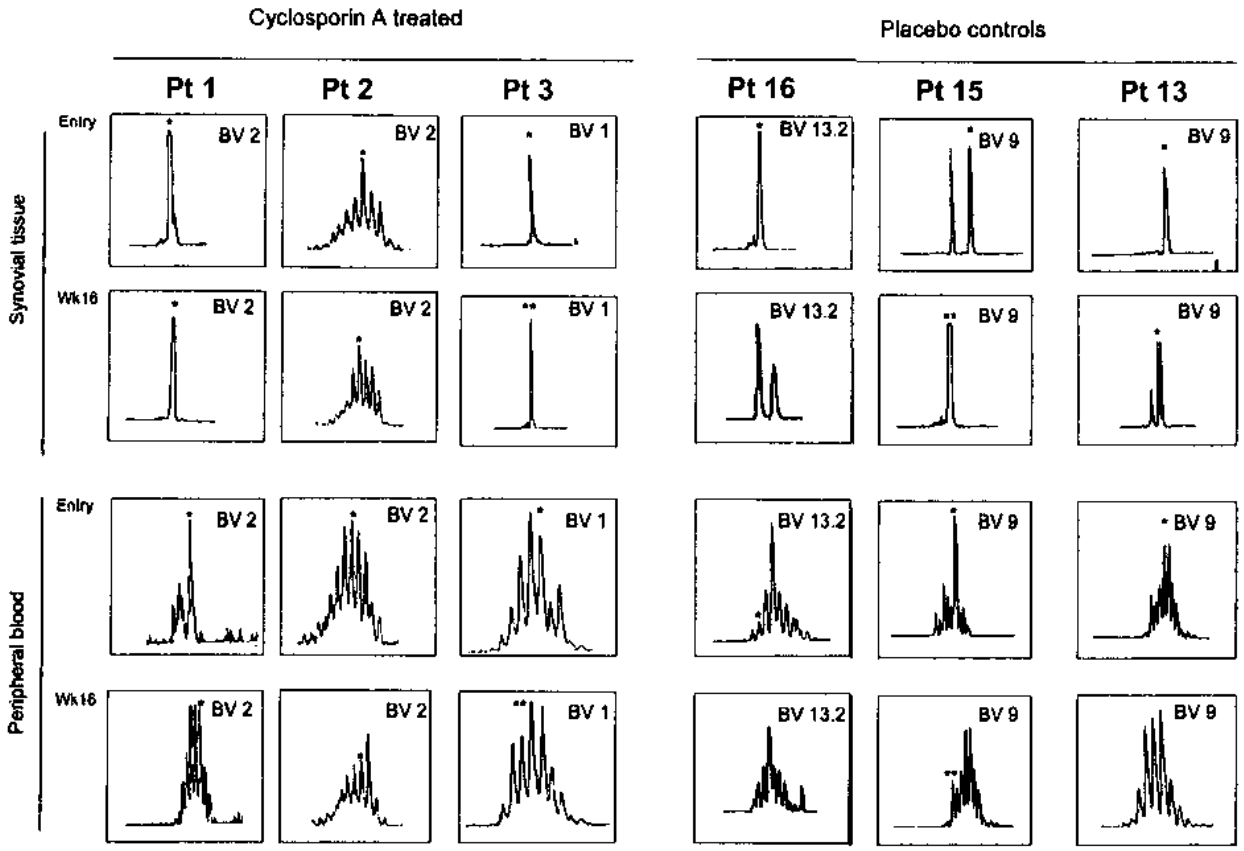

Figure 3. CDR3 spectratype analysis of selected TCR BV gene families overrepresented in blood or synovial tissue at the first or second sampling. PCR products corresponding to a BV gene family that was overrepresented at either time point in blood or synovial tissue were reamplified using a fluorescein labeled BC-specific nested primer and the BV gene-specific primer. Amplified products were analyzed with an automated 373 ABI DNA sequencer and Genescan software (Perkin Elmer). In CSA treated patients, peaks with identical CDR3 length were observed in blood and synovial tissue at 2 time points (peaks marked *). In the placebo controls, CDR3 peaks in the synovial tissue identified at the first time point (entry) were no longer present at the second time point (Week 16), except for Patient 13, where one peak (marked *) was found in synovial tissue at both time points. Some CDR3 peaks were, however, identified in blood and synovial tissue at the same time point (marked with * or **).

sequences were shared between blood and tissue samples, while other CDR3 sequences were found at both time points. These data therefore confirm the observations of the spectratype analysis (Table 4). Spectratype analysis revealed a polyclonal BV1 gene family in the blood of Patient 3, and a monoclonal spectratype profile in the synovial tissue. This polyclonal composition of the BV1 family in the blood was also observed by CDR3 sequence analysis (Table 5B). In the synovial tissue, one specific clonotype represented 75% of the total BV1 family. Unexpectedly, a second clone was found at a frequency of 13%. At the second time point 2 clones of identical CDR3 length were observed and both clones represented 88% of the total BV1 family. The CDR3 sequences of these clones were different from the clones isolated at the first time point. Note that the BV1 peaks on the spectratype analysis also had a different length, indicating that these were different clones. The BV9 family of Patient 13 was polyclonal at both time points as determined by spectratype analysis. This observation was confirmed by the heterogeneous CDR3 sequences found in the 2 PBMC samples. For synovial tissue, the spectratype data showed a monoclonal and oligoclonal profile at the first and second time point. A single synovial T cell clonotype CDR3 sequence was found in 71% of the plasmids sequenced. This T cell clonotype sequence was also found in the paired blood sample and persisted, although at a reduced frequency, in blood and synovial tissue at the second sampling. These data suggest that new T cell clonotypes have infiltrated into the synovium in this patient.

Taken together, the CDR3 DNA sequence data are in agreement with the observations from spectratype analysis. However, the data also show that a single peak in the spectratype analysis may still be composed of more than one T cell clonotype.

DISCUSSION

Our aims were to study whether the TCR repertoire of T cells in the synovial tissue of patients with early RA is biased; to study whether the TCR repertoire in synovial tissue changes in the same joint during the course of RA;

and to study whether the TCR repertoire changes in the synovial tissue are influenced by cyclosporin A treatment. These issues may inform our understanding of the T cell mediated pathogenesis of RA. TCR repertoire analysis was performed on synovial tissue because this is the major site of inflammation in RA. T cells that are potentially involved in the disease process are therefore most likely found in synovial tissue samples[19]. We used needle arthroscopy to obtain synovial tissue samples in patients with early arthritis[18]. This is a noninvasive, safe, and well tolerated approach. The samples, however, only contain small amounts of cellular material. To analyze TCR repertoires in these biopsies, highly sensitive PCR techniques are required. We reported that PCR-ELISA is a powerful method to study TCR V gene biopsy samples[11]. A strong bias could be that the cellular composition of these needle biopsies is different when samples are taken from different sites in the joint. However, studies have shown that multiple samples taken by needle arthroscopy provide a good estimate of synovial membrane inflammation[20,21]. In addition, the biopsies were preferentially taken in macroscopically inflamed areas of the membrane, which seem to correlate with microscopic signs of inflammatory activity, as demonstrated by Lindblad and Hedfors[22]. Also, shared amino acid profiles in CDR3 regions have been reported in multiple synovial tissue needle biopsies from the same joint[23]. Our data also showed a similar TCR BV gene profile for synovial tissue and lavage fluid, and similar BV gene profiles at 2 sites in the same joint. Together, these results indicate that needle biopsies are useful to study TCR BV gene expression in the joints of patients with RA.

Our results revealed biased TCR BV gene repertoires in synovial tissue of the majority of patients. Some BV genes were more frequently found to be overrepresented in synovial tissue, including BV2, BV4, and BV18, while no BV gene was uniformly overrepresented in all patients. Our data do not confirm the reported overexpression of the BV3, BV14, and BV17 families in RA[24]. The BV genes that were overrepresented in the synovial tissue were generally not overrepresented in the blood, indicating a preferential accumulation of some T cell populations in synovial tissue. The skewed but variable TCR repertoire we observed in the joints of patients with early RA is consistent with other reports[6,19,25]. Although overrepresented BV genes were found in the blood of some patients, the TCR V gene expression profile was different in most patients at both samplings. In synovial tissue, a biased TCR repertoire was observed in all patients before and after treatment. We tested whether identical clonotypes were present among the overrepresented BV families using spectratype and CDR3 sequence analysis. We found mostly different TCR clonotypes at both time points in the synovial tissue of the untreated patients. Many TCR clonotypes present at the first sampling were no longer observed at the second sampling in the untreated patients, suggesting that the corresponding T cell populations were no longer present in the synovial tissue. This is remarkable, since some of these TCR families represented more than 15% of the total TCR repertoire at the first time point. Together, these data are consistent with the accumulation of predominantly different T cell subsets in the synovial tissue at the 2 time points, suggesting a dynamic process of T cell activation or T cell recruitment in the joints. Our data are in agreement with the report of Khazaei, *et al*[26], who studied BV12 and BV14 TCR clonotypes in 2 RA patients, in CD4 T cells isolated from synovial fluid at 2 time points with an interval of 3 or 9 months. In these patients, with disease duration of one and 4 years, different dominant clones were found at the 2 time points. However, persistence of dominant clones has been described in some patients with chronic RA. Alam and coworkers[27] studied TCR CDR3 sequences in 2 RA patients with longstanding disease. They also found different dominant clones at 2 time points in the synovial tissue of these patients, although some clones persisted over time. Similar observations were made in our previous study, which showed persistence of dominant clones in synovial fluid of a patient with chronic RA[7]. Taken together, the current information may indicate that some T cell clones persist in the joints of patients with severe longstanding RA, while dynamic T cell clonotype changes are taking place in the joints of patients with early RA.

What causes the dynamic TCR repertoire changes in early RA? There is evidence that activated autoimmune T cells may undergo rapid apoptosis at the disease site. For example, it has been shown that freshly isolated synovial T cells are highly susceptible to apoptosis[10]. The antigen-specific apoptosis process is potentially induced by cytokines (e.g., transforming growth factor-ß) or may be induced by the liberation of free, unprocessed antigen[28]. We thus hypothesize that the predominant clones are depleted in the synovial tissue through rapid apoptosis. However, as a result of the local or peripheral activation of other T cells, new T cell clones accumulate in the joints of these patients. These observations are in agreement with the determinant spreading concept that was described in experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis[9]. In this concept, new antigens or epitopes released as a consequence of the ongoing tissue destruction and inflammation may induce activation and expansion of other T cell clonotypes in the synovium, thereby playing a role in perpetuation of the disease. On the other hand it is also possible that these newly activated T cells are bystander cells with no major role in the disease process.

Persistence of some dominant clones was more frequently observed in the patients treated with CSA. These data indicate that the dynamic clonotype changes in synovial tissue, as seen in the control RA patients, are potentially influenced by treatment with CSA. CSA is known to

inhibit early events of T cell activation, most likely by inhibition or downregulation of the expression of interleukin 2 (IL-2), IL-4, interferon-γ, and the IL-2 receptor, but may also suppress apoptosis[29]. It can be speculated that CSA treatment may have suppressed activation of new T cell clones in the blood or synovium of these patients, while clearance through apoptosis may have been reduced[29], resulting in a more stable TCR expression pattern in the joints. In accord with this hypothesis is the observation that no TCR mRNA was detected in synovial tissue of 3 out of 7 RA patients after CSA treatment, although this was also noted in one control patient. Although the expression of TCR mRNA may have been selectively inhibited in these samples, the most likely explanation for this observation is that these tissue samples contain only a few T cells.

Our observations may explain the inconsistent findings of previous studies. If the TCR repertoire changes rapidly over time in the joints of patients with early RA, analysis of TCR repertoires will reveal different information at various phases of the disease course. In addition, these observations have important consequences for the design of immune therapies targeted at potentially pathogenic T cell subsets in RA. To be effective such therapies may need to target T cells in a V gene independent manner, especially in early RA. The optimal scenario would be to follow TCR repertoires in the joints of individual patients, and then to use either V gene-specific or unspecific therapies. However, these patient-specific approaches are probably not realistic for routine clinical application. Alternatively, our data suggest that RA patients may be treated with an agent that prevents activation of new T cells, in combination with a therapy that depletes dominant T cell clonotypes at the same time. T cell vaccination or TCR peptide vaccination can be used as an approach to deplete the dominant T cell populations[24,30], while CSA is an attractive drug to prevent activation of new T cell subsets. Although CSA has been shown to be effective in early RA, the major adverse effects are currently considered an important drawback[31].

Our data show that the TCR repertoire in synovial tissue of patients with early RA is biased, and that this pattern undergoes dynamic changes during a period of 16 weeks. These dynamic repertoire changes are possibly influenced by CSA treatment, suggesting that CSA may affect T cell activation or recruitment of T cells into the joints of patients with early RA. These data provide new information about the possible T cell mediated pathogenesis of RA, and may have important consequences for the design of new T cell targeted therapies for RA.

ACKNOWLEDGMENT

We thank L. Philippaerts, J. Bleus, C. Bocken, and E. Smeyers for excellent technical help; N. Hellings, A. Van der Aa, M. Buntinx, L. Linsen, and C. Dekoning for helpful discussions; Dr. A.M. Peretz for including patients; Drs. F. Lorre and L. De Meester for monitoring the study; and Prof. M. Waer for critical reading of the manuscript.

REFERENCES

1. Sewell LK, Trentham DE. Pathogenesis of rheumatoid arthritis. Lancet 1993;341:283-6.
2. Fox DA. The role of T cells in the immunopathogenesis of rheumatoid arthritis: new perspectives. Arthritis Rheum 1997;40:598-609.
3. Panayi GS. T-cell-dependent pathways in rheumatoid arthritis. Curr Opin Rheumatol 1997;9:236-40.
4. Struyck L, Hawes GE, Chatila MK, Breedveld FC, Kurnick JT, van den Elsen PJ. T cell receptors in rheumatoid arthritis. Arthritis Rheum 1995;38:577-89.
5. Bucht A, Oksenberg JR, Linblad S, Gronberg A, Steinman L, Klareskog A. Characterization of T cell receptor alpha beta repertoire in synovial tissue from different temporal phases of rheumatoid arthritis. Scand J Immunol 1992;35:159-65.
6. Fischer DC, Opalka B, Hoffman A, Mayr W, Haubeck HD. Limited heterogeneity of rearranged T cell receptor V alpha and V beta transcripts in synovial fluid T cells in early stages of rheumatoid arthritis. Arthritis Rheum 1996;39:454-62.
7. VanderBorght A, Geusens P, Vandevyver C, Raus J, Stinissen P. Skewed T cell receptor variable gene in the synovium of rheumatoid artritis patients: Clonal expansion and persistence of synovial T cells. Rheumatology 2000;39:1189-201.
8. Elewaut D, De Keyser F, Van den Bosch F, Verbruggen G, Veys EM. Clonal analysis of T cell lines expanded from rheumatoid synovium and subcutaneous nodules. Broadening of the clonal spectrum in rheumatoid synovium in relation to disease duration [abstract]. Arthritis Rheum 1997;40 Suppl:992.
9. Lehman PV, Sercarz EE, Forsthuber T, Dayan CM, Gammon G. Determinant spreading and the dynamics of the autoimmune T cell repertoire. Immunol Today 1993;14:203-8.
10. Hasunuma T, Kato T, Kobata T, Nishioka K. Molecular mechanism of immune response, synovial proliferation and apoptosis in rheumatoid arthritis. Springer Semin Immunopathol 1998;20:41-52.
11. VanderBorght A, Van der Aa A, Geusens P, Vandevyver C, Raus J, Stinissen P. Identification of overrepresented TCR genes in blood and tissue biopsies by PCR-ELISA. J Immunol Methods 1999;223:47-61.
12. Pannetier C, Even J, Kourilsky P. T-cell repertoire diversity and clonal expansions in normal and clinical samples. Immunol Today 1995;4:176-8.
13. Dougados M, Awada H, Amor B. Cyclosporin in RA: a double blind, placebo controlled study in 52 patients. Ann Rheum Dis 1998;47:127-33.
14. Wells G, Tugwell P. Cyclosporin A in rheumatoid arthritis: overview of efficacy. Br J Rheumatol 1993;32 suppl 1:51-6.
15. Wahlberg J, Wilczek HE, Fauchald P, et al. Consistent absorption of cyclosporin from a microemulsion formulation assessed in stable renal transplant patients over a one-year study period. Transplantation 1995;60:648-52.
16. Flipo RM, Emery P, Scott DG, et al. Safety and tolerability of conversion from stable Sandimmun maintenance treatment to Sandimmun Neoral in patients with rheumatoid arthritis. J Rheumatol 1998;25:1263-9.
17. Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315-24.
18. Baeten D, Van den Bosch F, Elewaut D, Stuer A, Veys EM, De Keyser F. Needle arthroscopy of the knee with synovial biopsy sampling: technical experience in 150 patients. Clin Rheumatol 1999;18:434-41.
19. Ramanujam T, Luchi M, Schumacher HR, et al. Detection of T cell receptors in early rheumatoid arthritis synovial tissue. Pathobiol 1995;63:100-8.
20. Youssef PP, Smeets TJ, Bresnihan B, et al. Microscopic

measurement of cellular infiltration in the rheumatoid arthritis synovial membrane: a comparison of semiquantitative and quantitative analysis. Br J Rheumatol 1998;37:1003-7.
21. Bresnihan B, Cunnane G, Youssef P, Yanni G, Fitzgerald O, Mulherin D. Microscopic measurement of synovial membrane inflammation in rheumatoid arthritis: proposals for the evaluation of tissue samples by quantitative analysis. Br J Rheumatol 1998;37:636-42.
22. Linblad S, Hedfors E. Intraarticular variation in synovitis. Artritis Rheum 1985;28:977-86.
23. Struyk L, Hawes GE, Mikkers HM, Tak PP, Breedveld FC, van den Elsen PJ. Molecular analysis of the T-cell receptor beta-chain repertoire in early rheumatoid arthritis: heterogeneous TCRBV gene usage with shared amino acid profiles in CDR3 regions of T lymphocytes in multiple synovial tissue needle biopsies from the same joint. Eur J Clin Invest 1996;26:1092-102.
24. Moreland LW, Morgan EE, Adamson TC, et al. T cell receptor peptide vaccination in rheumatoid arthritis: a placebo-controlled trial using a combination of Vbeta3, Vbeta14, and Vbeta17 peptides. Arthritis Rheum 1998;41:1919-29.
25. Goronzy JJ, Bartz-Bazzanella P, Hu W, Jendro MC, Wlaser-Kuntz DR, Weyand CM. Dominant clonotypes in the repertoire of peripheral CD4+ T cells in rheumatoid arthritis. J Clin Invest 1994;94:2068-76.
26. Khazaei HA, Lunardi C, So AK. CD4 T cells in the rheumatoid joint are oligoclonally activated and change during the course of disease. Ann Rheum Dis 1995;54:314-7.
27. Alam A, Lambert N, Lule J, et al. Persistence of dominant T cell clones in synovial tissues during rheumatoid arthritis. J Immunol 1996;156:3480-5.
28. Bauer J, Wekerle H, Lassmann H. Apoptosis in brain-specific autoimmune disease. Curr Opin Immunol 1995;7:839-43.
29. Sugano N, Ito K, Murai S. Cyclosporin A inhibits H_2O_2-induced apoptosis of human fibroblasts. FEBS Lett 1999;447:274-6.
30. Zhang J, Medaer R, Stinissen P, Hafler D, Raus J. MHC-restricted depletion of human myelin basic protein-reactive T cells by T cell vaccination. Science 1993;261:1451-4.
31. Panayi GS, Tugwell P. The use of cyclosporin A microemulsion in rheumatoid arthritis: conclusions of an international review. Br J Rheumatol 1997;36:808-11.