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Lack of T cell oligoclonality in enzyme-digested synovial tissue and in synovial fluid in most patients with rheumatoid arthritis

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SUMMARY

The dominant presence of specific T-cell populations in the rheumatoid joint as detected by Southern blot analysis of T cell receptor (TCR) gene rearrangements would indicate local antigen recognition and T cell proliferation. We therefore studied TCR β chain gene rearrangements using a Cβ2 probe in paired samples of T cell populations from synovial tissue and peripheral blood ($n=6$) as well as synovial fluid ($n=16$) and peripheral blood ($n=18$) of patients with rheumatoid arthritis (RA). Peripheral blood mononuclear cells from healthy donors ($n=7$) served as a control. T cells were studied directly after isolation or after non-specific expansion with OKT3 monoclonal antibody (MoAb) and T cell growth factor (TCGF). DNA samples were digested with *Eco*RI and *Hin*dIII to detect rearrangements to Cβ1 and Cβ2, respectively. Extra bands were detected in all *Eco*RI-digested DNA samples prepared from both freshly isolated and non-specifically expanded T cell populations of patients and healthy donors, possibly representing 'common' (V-) D-J rearrangements. Dominant rearrangements were found in only two out of 16 synovial fluid T cell populations (one freshly isolated and one expanded) and not in peripheral blood or synovial tissue derived T cell populations. No extra bands were detected in *Hin*dIII-digested DNA samples. To investigate the effect of *in vitro* culture techniques on rearrangement patterns we studied DNA samples prepared from synovial tissue T cells obtained both by outgrowth from tissue with TCGF or by enzyme digestion and subsequent expansion either with TCGF or with OKT3 MoAb and TCGF. Whereas the latter T cell population yielded 'common' rearrangements, the former T cell populations yielded different dominant rearrangements. These data indicate that oligoclonality of the T cell populations in synovial tissue and synovial fluid of patients with RA is a rare event. The data also show the influence of *in vitro* culture techniques on the result of TCR gene rearrangement analysis.

Keywords T cells rheumatoid arthritis oligoclonality

INTRODUCTION

Synovial T lymphocytes that react against autoantigens may be important in the pathogenesis of rheumatoid arthritis (RA). Increased expression of HLA class II antigens (Meijer *et al.*, 1982; Duke *et al.*, 1982; De Vries *et al.*, 1984; Cush & Lipsky, 1988), as well as the adhesion molecules LFA-1 (Cush & Lipsky, 1988) and VLA-1 (Laffon *et al.*, 1989), on T lymphocytes infiltrating rheumatoid synovial tissue and in synovial fluid indicate activation *in vivo*, possibly caused by chronic antigenic stimulation. Although their pathogenetic relevance for chronic arthritis is unknown, several autoantigens have been shown to trigger proliferation of synovial T cells in RA patients (Stuart *et al.*, 1980; Holoshitz *et al.*, 1986; Res *et al.*, 1988; Gaston *et al.*, 1989; Pope *et al.*, 1989). If indeed the synovial T cell response is triggered by autoantigens, one might expect a predominance of a limited number of T cell clones in the T cell population. A restricted T cell receptor (TCR) usage in the response to an autoantigen has been demonstrated in animal models of autoimmune disease, e.g. experimental allergic encephalomyelitis (Acha-Orbea, Steinman & McDevitt, 1989). Oligoclonality can be detected by Southern blot analysis of DNA extracted from T cells (Miceli & Finn, 1989). Several investigators have used this technique to investigate rearrangement patterns in RA. While dominant rearrangements were found in T cells obtained by *in vitro* outgrowth from synovial tissue with interleukin-2 (IL-2) or T cell growth factor (TCGF) (Stamenkovic *et al.*, 1988a, 1988b; Miltenburg *et al.*, 1990), few dominant rearrangements were found in freshly isolated synovial fluid T cells (Savill *et al.*, 1987; Keystone *et al.*, 1988). In one

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Table 1. Clinical data of the patients and investigated T cell populations of the patients with rheumatoid arthritis

Patient	Sex	Age (years)	Disease duration (years)	STL	STL exp.	SFL	SFL exp.	PBL	PBL exp.
A	f	80	2			+	+	+	+
B	m	71	7			+	+	+	+
C	m	71	52			+/+*	−/+	+	+
D	m	46	14			+	+	+	+
E	f	20	17			+/+	+/+	+	+
F	f	80	4			+	+	+	+
G	m	58	2·5			+	+	+	+
H	f	41	7			+	+	+	+
I	m	66	7			+	+	+	+
J	f	71	10			+	+		
K	f	69	18				+		+
L	f	68	7				+/+		
M	f	68	14				+		+
N	f	70	25				+		+
O	m	45	8	+	+		+	+	+
P	f	67	29		+		+	+	+
Q	m	64	7	+				+	
R	m	58	10	+				+	
S	m	66	23		+			+	+
T	m	72	12	+	+			+	+

STL, synovial tissue lymphocytes; SFL, synovial fluid lymphocytes; PBL, peripheral blood lymphocytes; exp., expanded.

* T cell populations isolated from two joints (left knee/right knee).

study, dominant rearrangements were also found in peripheral blood T cells of healthy donors and RA patients (Duby *et al.*, 1989), suggesting the presence of 'common' rearrangements among T lymphocytes in peripheral blood. This stresses the importance of searching for rearrangements uniquely present in synovial tissue and synovial fluid. Dominant rearrangements of T cells grown from synovial tissue that differ from those found in peripheral blood T cells of the same patient may indicate local proliferation or homing of T cells *in vivo*, or culture selection *in vitro*. Enzyme digestion of synovial tissue yields suspensions of cells that can be used to study TCR rearrangements directly, thus avoiding possible tissue culture artefacts. Accordingly, we studied freshly isolated T cell populations obtained from paired samples of enzymatically digested synovial tissue and peripheral blood, as well as paired samples of synovial fluid and peripheral blood of RA patients. In order to investigate the effect of *in vitro* propagation of T cells on the TCR rearrangement patterns, DNA of freshly isolated cell populations was compared with DNA of cell populations expanded *in vitro* with TCGF and OKT3 monoclonal antibody (MoAb). In addition, different culture methods used to obtain T cells from synovial tissue were compared with respect to their influence on TCR rearrangement patterns.

MATERIALS AND METHODS

Patient data

Paired samples of peripheral blood/synovial fluid and peripheral blood/synovial tissue were taken from classical RA patients (ARA criteria). Clinical data of the patients are given in Table 1. Control samples of peripheral blood were obtained from healthy volunteers.

Cell culture procedures

Synovial tissue. Synovial tissue was obtained at the time of synovectomy or total joint replacement. Synovial tissue specimens were digested by collagenase type IA (Sigma) (3 mg/ml), DNAse (Sigma) (0·1 mg/ml) and hyaluronidase (Serva) (1 mg/ml) in Iscove's modified Dulbecco's medium (IMDM) containing 1% fetal calf serum (FCS) and incubated at 37°C for 4 h. After incubation, the tissue was pipetted through a nylon gauze into a sterile tube and washed in IMDM supplemented with 10% FCS. Mononuclear cells were obtained by Ficoll/Isopaque density centrifugation and counted. Non-specifically expanded T cells were obtained by placing at least 4×10^6 cells in 24-well tissue culture plates (Costar 3524, Cambridge, MA). These were cultured in IMDM supplemented with antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml) (Boehringer Mannheim, Mannheim, Germany), 10% FCS, 5% v/v TCGF (Miltenburg *et al.*, 1987) and OKT3 MoAb (10^{-5} dilution of ascites) at a concentration of 1×10^6 cells/ml. After approximately 1 week, cells were harvested and restimulated at a concentration of 2×10^5 cells/well in 24-well tissue culture plates in the presence of irradiated (30 Gy) allogeneic peripheral blood mononuclear cells (1×10^6 cells/well), OKT3 MoAb (10^{-5} dilution of ascites) and 5% TCGF. Cells were harvested for DNA analysis after 3–6 weeks of non-specific expansion. Freshly isolated T cells were obtained by incubating the remaining synovial tissue mononuclear cells overnight in plastic flasks at 37°C at a concentration of 1×10^6/ml, in order to deplete adherent cells. The non-adherent cell population was rosetted with AET-

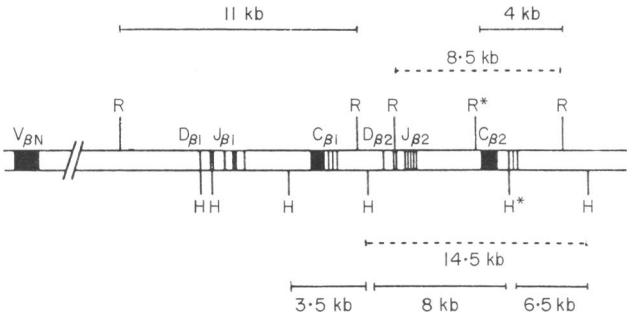

Fig. 1. Diagram of the germline arrangement of the β chain of the TCR, showing the locations of the restriction endonuclease sites for *Hin*dIII (bottom) and *Eco*RI (top) and the regions recognized by the Cβ probe. Fragments caused by partial digestion of the restriction endonuclease site (indicated by asterisks) are indicated by dotted lines.

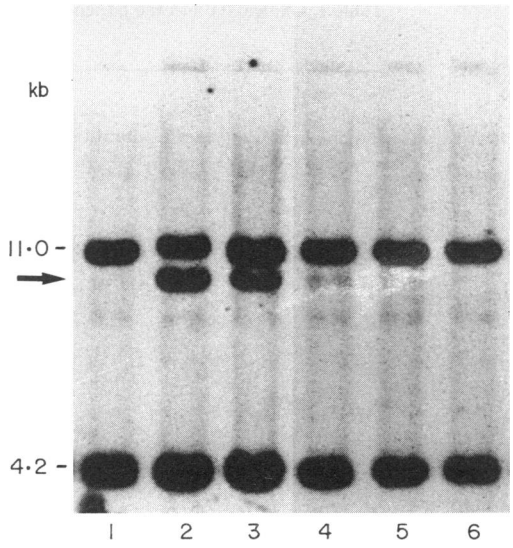

Fig. 2. Sensitivity of the Southern blotting analysis. U937 monocytic DNA (lane 1) and Jurkat T cell DNA (lane 2) were mixed in varying proportions (lanes 3–6, T cell DNA *versus* total DNA: lane 3, 1/3; lane 4, 1/9; lane 5, 1/27; lane 6, 1/81). The intensity of the rearranged β chain fragment (indicated by arrow) was determined densitometrically and compared with the intensity of the germ-line 11·0-kb fragment. The rearranged β chain fragment was detected at 52·3, 28·6, 5·3, 2·3% of total DNA (lanes 2–5, respectively), but not in a DNA preparation containing 1/81 Jurkat T cell DNA (lane 6).

treated sheep erythrocytes to enrich for T lymphocytes (Indiveri *et al.*, 1980). The percentage of T cells, determined by FACS analysis of cells stained with OKT3 MoAb, was 40–60%. DNA of these T cells was extracted after more than 5×10^6 were isolated. Synovial tissue T cells from one patient were expanded in three different ways: enzyme digestion followed by OKT3 and TCGF from the beginning; enzyme digestion followed by TCGF for the first week and subsequent expansion with OKT3 and TCGF; and outgrowth from tissue fragments with TCGF for the first week followed by expansion with OKT3 and TCGF. Briefly, synovial tissue fragments were placed in 24-well tissue culture plates in IMDM supplemented with antibiotics, 10% FCS and 5% TCGF. After approximately 1 week, growing cells were separated from the tissue fragments and expanded using OKT3 and TCGF as described above.

Synovial fluid and peripheral blood. Mononuclear cells were isolated from synovial fluid and peripheral blood by Ficoll/Isopaque density gradient centrifugation. Cells were counted. If fewer than 10×10^6 cells were obtained, they were non-specifically expanded using the OKT3/TCGF protocol. If more than 10×10^6 cells were isolated, 10×10^6 were used for direct DNA analysis and the remaining cells were expanded with OKT3 and TCGF.

DNA analysis

Five to twenty-million cells were harvested for DNA extraction. Southern blot analysis was performed on *Eco*RI- and *Hin*dIII-digested DNA. With these restriction enzymes it is possible to detect TCR rearrangements involving both Cβ1 and Cβ2 gene segments. A rearrangement to Cβ1 can be detected in an *Eco*RI digest by a shift of the 11·0-kb germline band while a rearrangement to Cβ2 can be detected in a *Hin*dIII digest by a shift of the 8·0-kb band (Fig. 1). Extra bands on a Southern blot represent either rearrangements, partial digests or polymorphism of the restriction enzyme recognition site.

DNA was prepared from freshly harvested cells after lysis in 2 ml of 100 mM NaCl, 10 mM EDTA, and 25 mM Tris/HCl (pH 8·0), supplemented with 0·6% (w/v) SDS as previously described (Sambrook, Fritsch & Maniatis, 1989). Proteinase K was added to a final concentration of 100 μg/ml, and the samples were incubated for 18 h at 37°C. Samples were extracted twice with phenol/chloroform/isoamylalcohol (25/24/1) and subsequently with chloroform/isoamylalcohol (24/1). Then sodium acetate (pH5·5) was added (final concentration 0·3 M) and DNA was precipitated using two volumes of alcohol. The DNA was washed with 70% ethanol, air dried, and dissolved in TE buffer (10 mM Tris/HCl (pH 7·5), 1 mM EDTA). For each experiment, 15 μg of DNA were digested with 50 U *Eco*RI or *Hin*dIII overnight at 37°C. The DNA fragments were separated on 0·8% agarose gels and blotted to nylon membranes (Biotrace; Gelman Sciences, Ann Arbor, MI) using 0·4 M NaOH/0·6 M NaCl buffer. Membranes were neutralized in 2 × SSC, air dried and vacuum baked (2 h at 80°C). The blots were pre-hybridized using 2 × SSC (5 min at room temperature), 0·1 × SSC/0·5% SDS (60 min at 65°C) and hybridization mix (0·25 M NaPi, 0·25 M NaCl, 7% SDS, 1 mM EDTA, 10% PEG-6000; 60 min at 65°C). Blots were hybridized overnight at 65°C using the ^{32}P-labelled probe in 10–15 ml hybridization mix. Following hybridization the blots were washed twice at 65°C in 2 × SSC/0·1% SDS and twice at 65°C in 0·1 × SSC/0·1% SDS. The blots were exposed to Kodak XAR-5 films at −70°C using intensifying screens. The TCRβ probe used in this study was the HPB β2 probe (Yoshikai *et al.*, 1984).

RESULTS

To determine the sensitivity of Southern blotting analysis, DNA from the human leukaemia T cell line Jurkat with a rearranged β chain (Fig. 2, lane 2) was mixed in varying proportions with germ-line DNA from the monocytic cell line U937 (Fig. 2, lane 1) and cut with *Eco*RI. The intensity of the rearranged β-chain fragment was determined densitometrically using a laser scan, and compared with the intensity of the germ-line 11·0-kb fragment. The rearranged β chain fragment was detected in a DNA preparation containing 1/27 Jurkat T-cell DNA at 2·5%

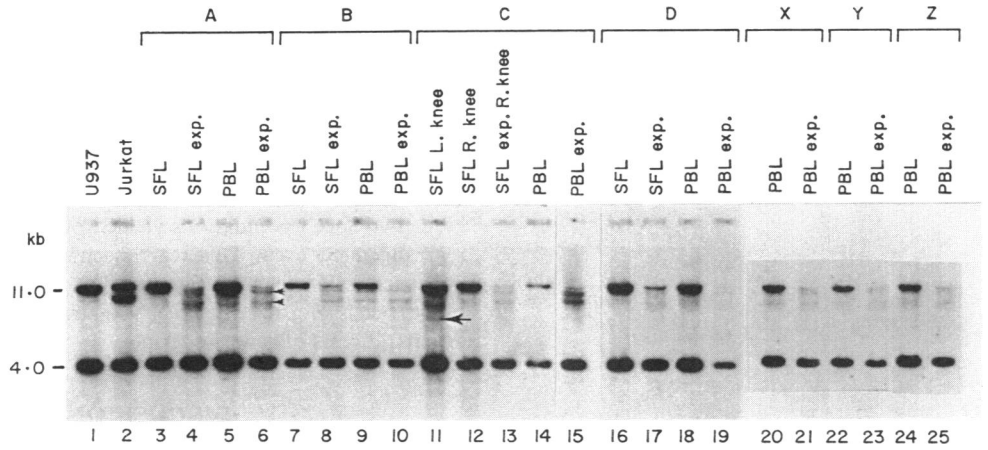

Fig. 3. *Eco*RI-digested DNA samples isolated from T cell populations of peripheral blood (PBL) from three healthy donors (donors X,W,Z) and from T cell populations of synovial fluid (SFL) and peripheral blood (PBL) from four patients (patients A–D). The U937 monocytic cell line (lane 1) and the Jurkat T cell leukaemia cell line (lane 2) served as a control. Both freshly isolated and non-specifically expanded T cell populations (exp.) show extra bands, respectively, representing a 10·5-kb and an 8·5-kb fragment (indicated by arrows in lane 6). The 8·5-kb fragment can be the result of partial digestion. Rearrangement patterns are identical to those found in peripheral blood of normal donors, except in one patient (lanes 11–15, patient C; dominant rearrangement indicated by arrow).

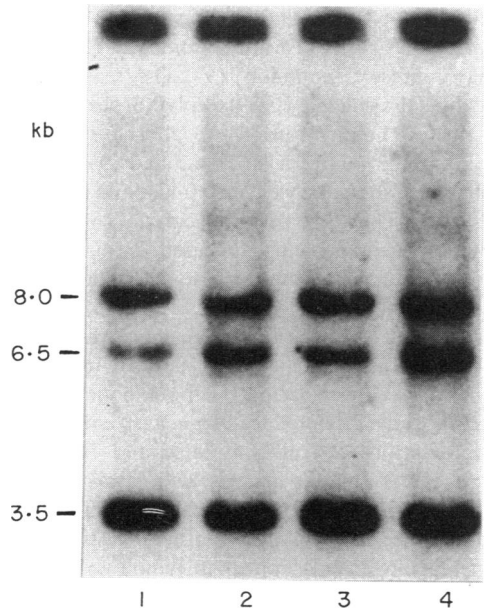

Fig. 4. *Hin*dIII-digested DNA samples isolated from T cell populations of synovial fluid (lanes 1 and 2; lane 2, expanded) and peripheral blood (lanes 3 and 4; lane 4, expanded) of patient I. No extra bands are seen. Results are representative for all *Hin*dIII-digested DNA samples.

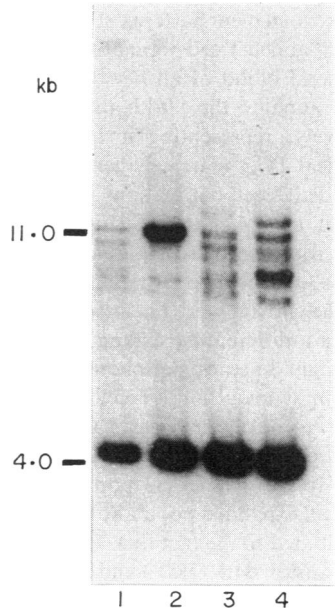

Fig. 5. *Eco*RI-digested DNA samples isolated from T cell populations of synovial tissue (lane 1, expanded), synovial fluid (lane 4, expanded) and peripheral blood (lanes 2 and 3; lane 3, expanded) of patient O. The non-specifically expanded T cell population from synovial fluid shows a unique rearrangement pattern not present in the other T cell populations studied.

(densitometer) or more of total DNA (Fig. 2, lanes 3–6). Thus, any dominant T cell clone detected amongst the synovial fluid or peripheral blood T cell population must be present to at least the 2·5% level.

To investigate whether dominant rearrangements exist in RA we examined both freshly isolated and expanded mononuclear and T cell enriched populations isolated from synovial tissue ($n=6$), synovial fluid ($n=16$) and peripheral blood ($n=18$) for the presence of TCR β chain rearrangements. Peripheral blood mononuclear cells from seven healthy donors served as a control. Both fresh and non-specifically expanded (using the OKT3/TCGF protocol) populations were used in this study for two reasons: first, to enable DNA analysis of those samples where few cells ($< 5 \times 10^6$) were isolated, and second, to determine whether our nonspecific expansion method affects rearrangement patterns. The OKT3/TCGF expansion protocol was used, since almost all T cells will be propagated with this method (Weber *et al.*, 1985).

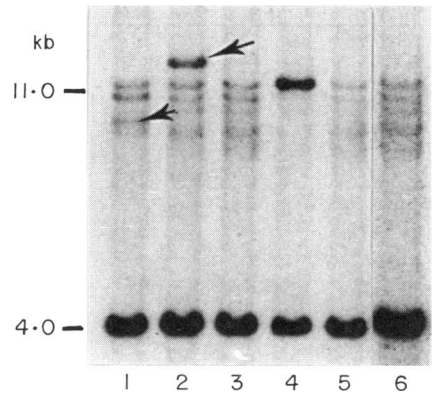

Fig. 6. *Eco*RI-digested DNA samples isolated from T cell populations of synovial tissue (lanes 1–3), peripheral blood (lanes 4 and 5) and synovial fluid (lane 6). Synovial tissue T cell populations, either obtained by enzyme digestion, outgrowth with TCGF for 1 week followed by non-specific expansion (lane 1) or obtained by outgrowth from tissue fragments with TCGF for 1 week followed by non-specific expansion (lane 2), show extra bands (indicated by arrows) not present in the synovial tissue T cell population obtained by enzyme digestion followed by non-specific expansion (lane 3) or in the other T cell populations.

The TCR rearrangement patterns of both fresh (Fig. 3, lanes 20, 22, 24) and expanded T cell populations (Fig. 3, lanes 21, 23, 25) from peripheral blood of all healthy donors showed two extra bands additional to the 4·0-kb and the 11·0-kb germ-line bands, respectively, representing a 10·5-kb and an 8·5-kb fragment. Identical TCR rearrangement patterns were seen in fresh and expanded T cell populations from synovial fluid and peripheral blood of RA patients (Fig. 3, lanes 3–19). No differences were found in paired samples of synovial fluid and peripheral blood, except in one patient (Fig. 3, lanes 11–15, patient C). Fresh synovial fluid T cells from the left knee of this patient exhibited a dominant rearrangement not present in T cells from the right knee or peripheral blood. In two other patients in whom synovial fluid T cells from two knees were studied, there were no differences in rearrangement patterns between the samples (patients E and L, data not shown). As compared with fresh lymphocyte populations, the germ-line 11·0-kb band of all expanded populations stained less intensely. This can be attributed to the fact that T cells predominate after non-specific expansion with OKT3 and TCGF. No extra bands additional to the 8·0-kb, 6·5-kb and 3·5-kb germ-line bands were seen in the *Hin*dIII digests (Fig. 4, patient I) of DNA samples from synovial fluid and peripheral blood T cell populations of RA patients.

Since the synovial tissue is likely to be the primary site of the immune response, T cell populations from this compartment are of particular interest. Synovial tissue from six patients was studied. T cells harvested from enzymatically digested tissue did not show dominant rearrangements when studied freshly or when expanded non-specifically. Rearrangement patterns of one patient are shown in Fig. 5 (patient O). In this patient the expanded T cell population from synovial fluid showed a dominant rearrangement pattern differing from those of expanded T cell populations from synovial tissue or peripheral blood. However, fresh synovial fluid T cells of this patient were not available for comparison with expanded synovial fluid T cells.

To investigate further the effect of *in vitro* growth of T cells, rearrangement patterns of non-specifically expanded synovial tissue T cells were compared with those of synovial tissue T cells obtained by outgrowth from tissue with TCGF and those of synovial tissue T cells obtained by outgrowth from enzyme digested tissue fragments with TCGF. The former T cell population did not show dominant rearrangements, while the latter two T cell populations yielded one (different) dominant rearrangement each. This rearrangement was not present in nonspecifically expanded synovial fluid T cells or in peripheral blood T cells (Fig. 6, patient P). No extra bands were seen in the *Hin*dIII digests of synovial tissue.

DISCUSSION

Controversy exists as to whether an oligoclonal T cell response is characteristic of the rheumatoid inflammation. The data presented indicate a lack of dominant rearrangements in the majority of T cell populations from synovial tissue, synovial fluid and peripheral blood of the RA patients studied. Dominant rearrangements were only found in one fresh and one non-specifically expanded T cell population from two patients. However, extra bands were detected in all *Eco*RI-digested DNA samples from T cells of synovial tissue, synovial fluid and peripheral blood of patients as well as in DNA samples from peripheral blood T cells of healthy controls. These rearrangements may represent 'common' (V-) D-J rearrangements as described by Duby *et al.* (1989). Polymorphism and partial digestion of *Eco*RI restriction sites are alternative explanations, but these cannot account for all extra bands. Polymorphism is rare and has been shown to yield an 8·0-kb band only (Waldman *et al.*, 1985), while partial digestion yields an 8·5-kb band. Since no dominant rearrangements appeared or disappeared after propagation we cannot determine to what degree our non-specific expansion protocol with OKT3 and TCGF affects oligoclonal T cell populations. Thus, we cannot conclude whether the unique rearrangement pattern in an expanded synovial fluid T cell population from one patient was initially present or may have been induced by *in vitro* culture. However, where combinations of freshly isolated and expanded T cell populations were tested, no major selection was induced by the non-specific *in vitro* propagation with OKT3 and TCGF. Our present data confirm the findings of other investigators that few dominant rearrangements appear in lymphocytes freshly isolated from synovial fluid and peripheral blood of RA patients (Savill *et al.*, 1987; Keystone *et al.*, 1988; Duby *et al.*, 1989).

The lack of dominant TCR gene rearrangements in lymphocytes of synovial fluid and peripheral blood may simply be a reflection of the aberrant T cell immune response being confined to synovial tissue. Nevertheless, we were unable to find dominant TCR gene rearrangements in freshly isolated or nonspecifically expanded T cell populations obtained from synovial tissue. This supplements previous investigations on freshly isolated synovial tissue T cells using MoAb recognizing Vβ5 and Vβ8 TCR gene families. No preferential or dominant use of these Vβ gene families was found in T cells isolated from the joints of RA patients (Brennan *et al.*, 1988). Our data on synovial tissue T cells differ from previous findings of Stamenkovic *et al.* (1988b) and of our group (Miltenburg *et al.*, 1990), indicating dominant rearrangements in most synovial tissue T cell populations.

It can be concluded, therefore, that the methodology used to obtain T cells plays a crucial role in the results of TCR gene analysis. Accordingly, we compared the methods employed to obtain T cells. Synovial tissue T cells obtained by enzyme digestion of tissue fragments and subsequent non-specific expansion only showed common rearrangements. In contrast, synovial tissue T cells obtained either by enzyme digestion and subsequent expansion with TCGF, or by outgrowth from tissue fragments with TCGF, showed (different) dominant rearrangements. This interesting observation suggests that outgrowth with IL-2 containing TCGF selects for IL-2-sensitive T cells or otherwise T cells rapidly proliferating *in vitro* that were not predominantly present in the original population. Responsiveness of these cells to low doses of IL-2 or TCGF indicates prior *in vivo* activation. However, the relevance to the disease of these cells remains to be eluded. The finding of different dominant rearrangements in T cells obtained by enzyme digestion or outgrowth from different tissue fragments suggests an unequal distribution of T cell populations within the synovial tissue.

Indeed, recent findings in our laboratory showed that separately expanded synovial tissue fragments from a joint of one patient, using the TCGF protocol, yielded several different rearrangement patterns (manuscript in preparation). To circumvent an influence of *in vitro* culture techniques on the results of TCR gene analysis, we studied both fresh and non-specifically expanded synovial tissue T cell populations obtained by enzyme digestion. No dominant rearrangements were seen in these T cell populations. However, this observation does not exclude the possibility of T cell oligoclonality in the synovial tissue. It is conceivable that, although the initial T cell response against putative autoantigens in early disease stages may have been oligoclonal, non-specific T cell recruitment has diluted antigen-reactive clones to undetectable levels. Since the patients studied all had a disease duration of at least 2 years, the absence of 'early onset' disease cases in this study may have contributed to the absence of dominant rearrangement patterns. In addition, in isolating T cells from tissue the T cell enrichment steps can lead to cell loss and significant changes in the various T cell subpopulations (Thoen *et al.*, 1989). Furthermore, Southern blot analysis only reveals oligoclonality if the rearrangement represents more than 2·5% of the TCR genes. Clones present with a lower frequency remain undetected but may be relevant for the disease process. Therefore, the results of this study do not allow a definite conclusion on T cell oligoclonality in RA. Since the analysis of T cell populations that accumulate specifically in rheumatoid synovial tissue may be the key to the pathogenesis of this disease, further studies in this field are warranted.

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