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Variable gene usage of T cell receptor γ- and δ-chain transcripts expressed in synovia and peripheral blood of patients with rheumatoid arthritis

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SUMMARY

The synovial tissue and fluid of patients with rheumatoid arthritis (RA) contain activated T cells that probably have a central role in the disease process which leads to joint destruction. A subset of T cells, $\gamma\delta$ T cells detected at the site of inflammation, may be important in the pathogenesis of the disease. This study investigated variable (V) gene usage of $\gamma\delta$ T cell receptors (TcRs) expressed in synovia and peripheral blood of patients with RA by using the polymerase chain reaction (PCR) to amplify TcR γ- and δ-chain transcripts. Most patients showed no restriction in Vγ gene usage since synovial mononuclear cells (SMC) expressed TcR γ-chain transcripts which used the same set of Vγ genes as peripheral blood mononuclear cells (PBMC). In contrast, the majority of patients expressed a restricted SMC Vδ-chain repertoire biased towards Vδ1, but Vδ2 mRNA transcripts were also detected, albeit at low levels in some patients. The TcR δ-chain repertoires of PBMC from healthy control subjects were also characterized. There was variation in the TcR δ-chain repertoires of PBMC from patients when compared with controls, particularly with respect to expression of Vδ4. Vδ4 mRNA transcripts were expressed in PBMC of only two of seven RA patients in contrast with eight of the nine controls ($P=0{\cdot}03$). These findings are compatible with reports that $\gamma\delta$ T cells in the rheumatoid synovium are reactive to *Mycobacterium tuberculosis* and that response to *M. tuberculosis* is restricted to Vγ9/Vδ2-bearing T cells, if a superantigen is involved in the pathogenesis of RA.

Keywords $\gamma\delta$ T cell receptors variable gene usage rheumatoid arthritis synovia

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disorder of unknown aetiology, although both genetic and environmental factors probably contribute to its pathogenesis. As with many autoimmune diseases, RA has a genetic predisposition associated with class II genes of the MHC; susceptibility to seropositive RA is associated with HLA-DR4 (Dw4 and Dw14) and with DR1 [1,2]. The disease leads to joint erosion as a result of chronic inflammation of the synovium which lines the joint cavity. A characteristic feature of the rheumatoid synovium is an intense lymphocytic infiltration [3] containing predominantly activated T cells, as defined by the expression of class II MHC antigens and IL-2 receptor (IL-2R) [4]. These T cells may be involved in eliciting a local inflammatory response against joint-specific antigens causing tissue damage and eventually joint destruction. However, the inciting antigen(s) which triggers T cell activation and proliferation and the mechanisms which lead to tissue injury remain an enigma.

The majority of mature CD3$^+$ T cells express the $\alpha\beta$ T cell receptor (TcR) and are of the helper (CD4$^+$CD8$^-$) or cytotoxic (CD4$^-$CD8$^+$) subsets [5]. These T cells recognize processed foreign or self antigens in association with MHC restriction elements [6]. However, a fraction of peripheral blood T cells (0·5–10%) predominantly of the double negative (CD3$^+$CD4$^-$CD8$^-$) phenotype express an alternative heterodimer composed of a γ and a δ polypeptide chain [7]. $\gamma\delta$ T cells detected in the synovial fluid [8] and synovial membrane [9] of RA patients have been shown to express predominantly variable (V) δ1, when phenotyped using MoAbs to detect Vδ1- and Vδ2-bearing T cells, but a more sensitive and informative approach, as provided by polymerase chain reaction (PCR) amplification of TcR transcripts, is required to characterize fully Vδ gene usage in RA synovium. Proliferative responses of RA synovial T cells (CD4$^-$CD8$^-$) bearing $\gamma\delta$ TcRs have been shown against

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Table 1. Nucleotide sequence of 5′-variable region gene primers used for PCR amplification of TcR γ- and δ-chain mRNA transcripts

Primer	Sequence (5′-3′)	Primer	Sequence (5′-3′)
Vγ I	GGAGAACGAAGTCAGTCA	Vδ1	TCTGGATCAAGTGTGGC
Vγ II	CCAGTACTAAAACGCTGTC	Vδ2	TCTGGGCAGGAGTCATGT
Vγ III	CAGCTATCCATTTCCACGG	Vδ3	GGCACGCTGTGTGACAAA
Vγ IV	GCAAATAAGAGTGCCCAC	Vδ4	GACACCAGTGATCCAAGTT
		Vδ5	GATGACCAGCAAGTTAAGC
		Vδ6	TATCATGGATTCCCA

Mycobacterium tuberculosis antigens, and in some cases specifically to the 65-kD heat shock protein [10], which represents a candidate antigen involved in the aetiology of RA. γδ T cells reactive against *M. tuberculosis*, shown to be restricted to Vγ9/Vδ2-bearing T cells [11], could initiate an autoimmune response within the joint via cross-reactivity with cartilage components [12] or other self-antigens [13] which may lead to a breakdown of host self-tolerance.

The repertoires of human Vγ and Vδ genes are limited compared with Vα and Vβ genes. Currently, 14 Vγ genes have been identified, 12 of which belong to four subgroups, designated VγI–VγIV. The VγI subgroup contains nine genes, consisting of five functional genes (V2, V3, V4, V5 and V8) and four pseudogenes (V1, V5P, V6 and V7) whereas subgroups II, III and IV each contain a single gene, designated V9, V10 and V11, respectively. Two pseudogenes, VA and VB, located upstream of V9 and V11, respectively, belong to none of these groups [14]. The repertoire of TcR Vδ genes consists of at least six subfamilies of Vδ gene segments, designated Vδ1–Vδ6, each of which contains a single gene [15].

Our aim was to investigate the repertoires of TcR Vγ and Vδ genes expressed in synovial membrane and peripheral blood of patients with RA by using the PCR to amplify rearranged TcR V-C transcripts. To evaluate V gene usage we designed six TcR Vδ gene-specific primers to amplify the six known human Vδ gene segments [15] and four Vγ gene-specific primers to amplify the Vγ genes of subgroups VγI–VγIV [14,16].

PATIENTS AND METHODS

Patients

Peripheral blood and synovial tissue were obtained from seven patients attending the Rachel Forster Hospital for elective joint surgery that involved either synovectomy or replacement. The duration of disease was 8–20 years, but the disease was still active in all patients as adjudged by erythrocyte sedimentation rate (ESR) and measurement of C reactive protein (CRP) just before surgery. All patients met the American Rheumatism Association 1987 revised criteria for RA [17]. The study had appropriate institutional ethical approval.

Cell separation

Peripheral blood was collected into preservative-free heparin and mononuclear cells (PBMC) were separated over a Ficoll–Hypaque gradient. Synovium was collected into HBSS and was mechanically minced, before enzymatic digestion. The sample was then filtered and mononuclear cells (SMC) were separated on a Ficoll gradient, as described elsewhere [18].

Controls

PBMC were isolated from heparinized blood obtained from nine healthy donors, by density gradient centrifugation using Lymphoprep (Nycomed, AS, Norway).

Controls for PCR amplification

cDNA prepared from a pool of PBMC obtained from five healthy donors served as the positive control for PCR amplifications. Lymphocytes ($1–2 \times 10^6$) from each individual were stimulated with concanavalin A (Con A) (10 μg/ml) for 72 h at 37°C/5% CO_2 in RPMI media containing 5% human AB serum and then pooled before cDNA synthesis. Con A was used to stimulate expression of rearranged TcR genes and PBMC from five individuals were pooled to ensure that all TcR V genes were represented in the control PBMC. Negative controls (no cDNA) were included with each pair of primers to test for any possible contamination of reagents used for RNA isolation, cDNA synthesis or PCR amplification.

Isolation of total RNA and first strand cDNA synthesis

RNA was isolated from PBMC, SMC and control PBMC by the acid guanidinium thiocyanate-phenol-chloroform extraction method [19]. Approximately 10 μg of total RNA was converted to single-stranded cDNA by incubation for 1 h at 37°C in 1 × cDNA synthesis buffer (50 mM Tris-HCL (pH 8·3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT), 1 mM of each dNTP, 50 pmole of constant region gene antisense primer Cδ1 (5′-GACAGCATTGTACTTCCCACTGG-3′) or Cγ1 (5′-GTCGTTAGTCTTCATGGTGTTCCC-3′), 25 units RNasin (Promega, Madison, WI) and 50 units M-MuLV reverse transcriptase (Pharmacia, Uppsala, Sweden). Cδ1 and Cγ1 primers correspond respectively to nucleotide positions 273–295 and 178–201 of the published sequences [20,21]. Reaction products were purified by phenol-chloroform extraction followed by centrifugation in a centricon 100 microconcentrator (Amicon, Danvers, MA).

PCR amplification

Approximately 5% of single-stranded cDNA template was amplified in 50 μl reactions containing 1 × PCR buffer (10 mM Tris-HCl (pH 8·4), 50 mM KCl, 2·5 mM $MgCl_2$, 0·01% gelatin), 0·2 mM of each dNTP, 25 pmole 5′Vδ or Vγ sense primer (Table 1), 25 pmole of 3′nested constant region gene antisense primer Cδ2 (5′-TTCACCAGACAAGCGACA-3′) or Cγ2 (5′-AGGTATGTTCCAGCCTTC-3′) and 1·25 units Taq DNA polymerase (Promega). Cδ2 and Cγ2 primers correspond respectively to nucleotide positions 171–189 and 75–82. Reaction mixtures were overlaid with mineral oil and subjected to 35

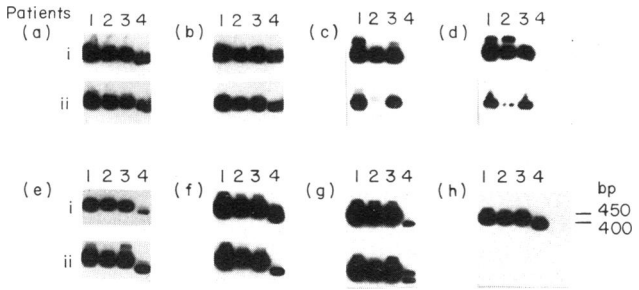

Fig. 1. PCR amplification and Southern blot analysis of rearranged TcR γ-chain transcripts expressed in PBMC (i) and SMC (ii) of seven patients with RA and in Con A stimulated pooled PBMC. Lanes 1–4 correspond respectively to amplifications of cDNA with VγI–VγIV primers each in conjunction with Cγ2. Blots were hybridized with an internal TcR Cγ specific oligonucleotide probe (Cγ3) and exposed for 6 h. Approximate sizes are indicated on the right in bp.

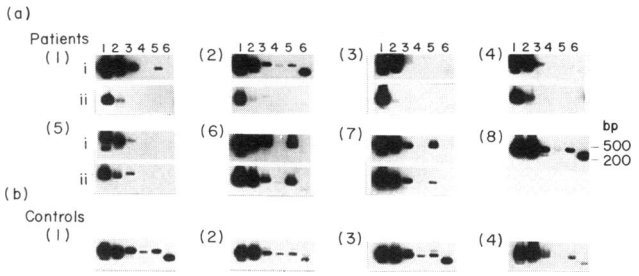

Fig. 2. (a) PCR amplification and Southern blot analysis of rearranged TcR δ-chain transcripts expressed in PBMC (i) and SMC (ii) of seven patients with RA, in Con A stimulated pooled PBMC and (b) in PBMC of four controls. Lanes 1–6 correspond respectively to amplifications of cDNA with Vδ1–Vδ6 primers each in conjunction with Cδ2. Blots were hybridized with an internal probe (Cδ3) and exposed for 6 h.

cycles of amplification using a thermal cycler (Bartelt Instruments). PCR conditions for amplification of TcR γ- and δ-chain cDNA were 95°C for 1 min, 50°C for 1 min and 72°C for 1 min for each cycle.

Southern blot analysis

Ten microlitres of PCR amplified cDNAs were electrophoresed in 1·25% tris-acetate EDTA agarose gels and blotted onto Gene Screen Plus hybridization transfer membrane (Biotechnology Systems, NEN Research Products) using 0·4 M NaOH as the transfer buffer. Membranes were neutralized in 2 × SSC (0·3 M NaCl, 0·03 M sodium citrate), fixed under ultraviolet light for 3 min and dried at 65°C. Blots were prehybridized for 5 h at 40°C in 5 × SSPE (5 mM EDTA, 50 mM $NaPO_4$ (pH 7·4), 900 mM NaCl), 5 × Denhardt's solution (0·1% bovine serum albumin (BSA), 0·1% Ficoll 400, 0·1% PVP), 0·1% SDS and salmon sperm DNA (100 μg/ml) and hybridized overnight at 40°C with [^{32}P]-kinased oligonucleotide probe Cδ3 (5′-GATGGTTTGGTATGAGGCTG*A*-3′) or Cγ3 (5′-GGAAA-CATCTGCATCAAGTTGT-3′). These internal oligonucleotide probes correspond respectively to nucleotide positions 124–144 and 6–27 and were designed not to overlap with either Cδ1 and Cδ2 or with Cγ1 and Cγ2 primers, respectively. Filters were washed twice in 1 × SSPE/0·1% SDS for 20 min each at room temperature and once in 0·1 × SSPE/0·1% SDS for 10 min at 43°C for TcR γ and 45°C for TcR δ and then exposed to X-ray film (Fuji) with intensifying screens at −70°C.

RESULTS

V gene usage of TcR γ- and δ-chain transcripts expressed in Con A stimulated pooled PBMC

V gene usage in control cDNA was determined by PCR amplification of rearranged TcR mRNA transcripts. Expression of TcR V genes was considered positive when a PCR product of the expected size could be visualized by ethidium bromide staining and its specificity confirmed by hybridization with an internal radiolabelled oligonucleotide probe. In some cases PCR products could not be visualized on ethidium bromide gels but gave positive signals when hybridized. All V gene specific primers successfully amplified rearranged TcR γ-(Fig. 1h) and δ-chain transcripts (Fig. 2, (8)) in Con A stimulated pooled PBMC, although compared to normal unstimulated PBMC, Vδ4 transcripts were less readily detected.

Table 2. Summary of results obtained for the analysis of V gene usage of TcR δ-chain transcripts expressed in PBMC (a) and SMC (b) of seven patients with RA

	TcR Vδ gene subfamilies					
Patient	Vδ1	Vδ2	Vδ3	Vδ4	Vδ5	Vδ6
1 a	+	+	+	−	+	−
b	+	+	−	−	−	−
2 a	+	+	+	+	+	+
b	+	W	W	−	−	W
3 a	+	+	+	−	W	W
b	+	W	W	−	−	−
4 a	+	+	+	−	W	W
b	+	+	W	−	−	−
5 a	+	+	+	−	−	−
b	+	+	+	−	−	W
6 a	+	+	+	−	+	−
b	+	+	+	W	+	W
7 a	+	+	+	W	+	W
b	+	+	+	W	+	W

Presence or absence of V gene expression is denoted as +/− and W, weakly expressed.

V gene usage of TcR γ-chain transcripts expressed in SMC and PBMC of patients with RA

Figure 1 shows that in five of seven patients studied there was no restriction in Vγ gene usage since SMC expressed the same set of Vγ genes as PBMC. We observed a few differences between the SMC and PBMC of patients 3 and 4 in that VγII was barely detectable in SMC compared with PBMC and there was no detectable expression of VγIV in the SMC. In both of these patients VγIV was weakly expressed in the PBMC. Thus, there were no marked differences between the SMC and PBMC TcR γ-chain repertoires, of any of the seven RA patients studied. We observed additional bands in some of the PCR amplifications which may correspond to single and double stranded DNA

products due to unequal amounts of primers, as observed previously [22].

V gene usage of TcR δ-chain transcripts expressed in SMC and PBMC of patients with RA

V gene usage of the expressed TcR δ-chain repertoires of seven RA patients were determined (Table 2, Fig. 2a). Patients 1–4 each expressed a restricted Vδ-chain repertoire in SMC which predominantly used the Vδ1 and Vδ2 genes, as compared with PBMC, whereas patient 5 expressed a restricted Vδ-chain repertoire both in PBMC and SMC. Expression of Vδ3 (except patient 1) and in two of these patients (2 and 5) Vδ6 genes were detected in the SMC. However, no expression of Vδ4 or Vδ5 was observed in the SMC of these patients. In contrast, patients 6 and 7 showed no restriction in Vδ gene usage since all six Vδ genes were amplified in the SMC of each patient.

Our results showed variation in the PBMC δ-chain repertoires of the seven RA patients tested (Table 2, Fig. 2a). Vδ1, Vδ2 and Vδ3 genes were expressed in PBMC of all patients, whereas Vδ4 was only expressed in patient 2 and was virtually undetectable in patient 7. The Vδ5 gene was absent from only one individual (patient 5) although it was only detected in patients 3 and 4 after prolonged exposure of autoradiograms (48 h). Vδ genes that were only detected after prolonged exposure have been called 'weakly' expressed. Lastly, Vδ6 was absent or weakly expressed in PBMC of all individuals except patient 2. Overall, we found that Vδ gene usage ranged from three Vδ genes (patient 5) to all six Vδ genes (patients 2 and 7).

V gene usage of TcR δ-chain transcripts expressed in PBMC of control individuals

Nine healthy control individuals were used to determine the 'normal' TcR δ-chain repertoire; results are given in Fig. 2b for four of the controls. Expression of all six Vδ genes was found in each control tested, with one exception (control 4) which did not express the Vδ4 gene. Vδ4 gene usage in peripheral blood of controls (8/9) was significantly different from that in RA patients (2/7), with Fisher's exact test, $P=0{\cdot}03$. Vδ5 and Vδ6 genes were detected in PBMC of all controls, whereas in the periphery Vδ5 was absent in one patient ($P=0{\cdot}44$) and Vδ6 was absent in three patients ($P=0{\cdot}06$).

DISCUSSION

Activated T cells which infiltrate the rheumatoid synovium are thought to play a fundamental role in the pathogenesis of RA. A selective expansion of T cells reactive to a limited number of antigenic determinants within the synovium may reflect local expansion of T cells *in situ*, or possibly local trapping of particular families of lymphocytes from the recirculating pool. Such T cells may express, in terms of V gene usage, a TcR repertoire with restricted heterogeneity. Restricted Vα and Vβ gene usage by autoreactive T cells has been clearly demonstrated in experimental allergic encephalomyelitis (EAE), an animal model of human multiple sclerosis, which has allowed specific TcR Vβ chains to be exploited as targets for immune intervention [23]. This may have important implications in human autoimmune disease; therefore it is necessary to demonstrate whether particular receptors are indeed involved in initiating or perpetuating an autoimmune response which may lead to the development of autoimmunity.

In this study we investigated the diversity of the γδ TcR repertoire in RA by using PCR to amplify transcripts isolated from SMC and PBMC. Our results showed that in most patients studied expression of Vδ genes in the synovium was highly restricted; SMC expressed a limited repertoire of Vδ-chain transcripts which predominantly used the Vδ1 and Vδ2 genes, when compared with PBMC. In contrast, we found no evidence for preferential usage of particular Vγ genes since the repertoire of Vγ-chain transcripts expressed in SMC showed only minor differences compared with those expressed in PBMC. Previous reports using MoAbs to detect γδ T cells and their subsets have shown that synovial T cells of RA [8,9] and JRA [24] patients express γδ receptors which predominantly use the Vδ1 gene, as do T cell clones derived from RA synovial fluid [25], in contrast to human PBMC where Vδ2 appears to be predominant [26]. Our PCR-based data also support a restricted δ-chain repertoire skewed towards Vδ1 but were able to identify the other Vδ-chain transcripts present in RA synovium. Some non-Vδ1 transcripts found abundantly in the peripheral blood were scarcely detected in the synovium. However, it should be noted that many factors contribute to the relative efficiencies of PCR reactions, including template-primer binding and efficiency of cDNA synthesis; therefore autoradiographic signal intensities do not necessarily correlate with the abundance of mRNA. Consequently, since Vδ2 was also expressed in SMC of all of our patients, even though scarcely detected in some cases, we conclude that Vδ usage observed in this study was not exclusively restricted towards Vδ1.

The extreme sensitivity of the PCR technique in detecting even small numbers of particular TcR transcripts confirms the earlier findings, based on monoclonal antibody studies, that certain γδ T cells in affected joints have preferentially expanded and proliferated, presumably in response to an antigen(s) which interacts selectively with the variable element, encoded primarily by the Vδ1 gene, of the γδ TcR. Whether these particular T cells are autoreactive and the nature of the antigen/ligand which these potentially arthritogenic T cells recognize, remains elusive. Despite these unanswered questions, the predominance of $V\delta1^+$ T cells within the inflammatory lesion suggests a potentially pathogenic role for these T cells in RA. An alternative explanation could involve selective physiological tissue distribution, or selective retention, of particular γδ T cells, which for some reason have migrated to the synovial compartment. Tissue-specific expression of Vδ genes is well documented in mouse, in a variety of tissues [27], and although there is little evidence of this phenomenon in man [28], a recent report has shown selective localization of $V\delta1^+CD8^+$ T cells in the human epithelium of the large intestine [29].

In the more common TcR, the V-elements of both α and β TcR chains usually contribute to the specificity of TcR for antigen/MHC [30]. In contrast a group of ligands, so-called 'superantigens', stimulate T cells expressing particular Vβ chains almost regardless of other components of the receptor, although they do require interaction with class II molecules [31]. Receptor ligands of this type include self-antigens encoded by the minor lymphocyte stimulating locus (Mls) in mouse [32] and some bacterial enterotoxins which stimulate human [31] as well as mouse T cells [33] in a Vβ-specific fashion. The nature of activation of γδ T cells is not completely clear; some appear to require MHC restriction [34,35] and others do not [10]. A potential contribution of a superantigen to the pathogenesis of

RA may explain our findings of restricted use of Vδ genes but not Vγ genes in RA synovium. A striking feature of human γδ T cells is their frequent reactivity towards mycobacterial antigens [10,34,36–38] including, in some cases, the highly conserved 65-kD heat shock protein [10,34,38]; such reactivity has some analogy with T cell activation by superantigens [11]. However, these studies have shown that reactivity towards heat-killed *M. tuberculosis* is an exclusive property of Vγ9$^+$(VγII)/Vδ2$^+$-bearing T cells and not Vδ1$^+$ T cells. In the present study, Vδ2 and Vδ1 were the predominant Vδ genes expressed in RA synovium but there was no detectable bias in use of Vγ9 (VγII); whether there is preferential pairing of Vδ2 and Vγ9 polypeptide chains within the rheumatoid synovium, is unknown. In two patients there were barely detectable levels of VγII in SMC, compared with PBMC. This may reflect a reduction rather than predominance of VγII/Vδ2 T cells in the synovium, possibly due to previous activation by a superantigen, since in the case of Vβ-specific superantigens, reactivity often leads to the eventual disappearance or inactivation of those peripheral T cells bearing specific Vβ elements; a phenomenon termed programmed cell death [39].

A potential association with a superantigen may also contribute to the observation of variation in the TcR δ-chain repertoires of PBMC from patients when compared with controls. Most strikingly, Vδ4 was expressed significantly less often in RA patients compared with controls. Similarly, deficiency in expression of Vβ14 of the TcR αβ in PBMC of some RA patients has been reported [40], although Vβ14$^+$ T cells were detected in the synovial fluid. Such a complete deletion of Vδ4 as observed here could reflect either superantigen-based negative selection during thymic maturation [41], or superantigen-based programmed cell death [39].

The particular bias in Vδ usage observed in patients with RA of long duration may not necessarily correlate with any bias present early in the pathogenesis of the disease. Further, since only small numbers of autoreactive T cells may be required for the chronic progression of RA, the apparent bias in Vδ1 usage by SMC may be an observation less relevant to the development of RA, than the finding of small numbers of Vδ2 mRNA transcripts in SMC of most patients examined. These findings are compatible with reports that γδ T cells in the rheumatoid synovium are often reactive to *M. tuberculosis* [10,38] and that response to *M. tuberculosis* is restricted to Vγ9/Vδ2-bearing T cells [11], if a superantigen is involved in the pathogenesis of RA.

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