Selective Accumulation of Related CD4+ T Cell Clones in the Synovial Fluid of Patients with Rheumatoid Arthritis

Christopher C. Striebich, Michael T. Falta, Yi Wang, Jerry Bill and Brian L. Kotzin

*J Immunol* 1998; 161:4428-4436;
http://www.jimmunol.org/content/161/8/4428

---

**References**

This article cites 53 articles, 16 of which you can access for free at:
http://www.jimmunol.org/content/161/8/4428.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Selective Accumulation of Related CD4⁺ T Cell Clones in the Synovial Fluid of Patients with Rheumatoid Arthritis¹,²

Christopher C. Striebich,*† Michael T. Falta,* Yi Wang,³* Jerry Bill,¹ and Brian L. Kotzin⁴*†‡

The role of T cells in the pathogenesis of rheumatoid arthritis (RA), especially in the perpetuation of advanced disease, remains unclear. Previous studies have focused on the TCR repertoire of synovial T cells in an attempt to determine whether the pattern of expression is characteristic of Ag-stimulated populations. However, the results of past studies have been conflicting. In the present work, we have undertaken an extensive analysis of the TCRs expressed by CD4⁺ T cells freshly isolated from synovial fluid of different joints and blood in three patients with established RA. Despite marked heterogeneity of synovial TCR expression, the results showed that 20 to 30% of the TCR β-chain gene (TCRB) sequences found in one joint were also expressed in a second joint, but not in peripheral blood T cells of the same individual. Analysis of expressed TCRB complementarity-determining region 3 sequences showed the presence of multiple expanded clonal populations that were not predicted by quantitation of β-chain variable region (Vb) expression by immunofluorescence staining. These studies also demonstrated sets of related, but different, complementarity-determining region 3 nucleotide sequences that encoded identical or highly homologous β-chain amino acid sequences. Analysis of matching T cell clones derived from the joint by limiting dilution culture confirmed coexpression of highly homologous TCR α-chain gene (TCRA) and TCRB sequences. Together, these studies suggest that a significant proportion of synovial CD4⁺ T cells has been selected and expanded by conventional Ag(s) in this disease. The Journal of Immunology, 1998, 161: 4428–4436.

Rheumatoid arthritis (RA)³ is a disease of unknown etiology characterized by chronic inflammation in multiple joints. In a significant fraction of patients, this chronic synovitis leads to destruction of the articular cartilage and surrounding structures and is a cause of considerable morbidity (1–3). The presence of large numbers of activated CD4⁺ T cells in synovial tissue has supported the hypothesis that CD4⁺ T cells are important in the pathogenesis of disease (4–8). This hypothesis is further supported by studies demonstrating the association of disease susceptibility and/or severity with inheritance of particular class II HLA alleles (9–14). In the most favored model of disease induction, disease-associated HLA-DR molecules present disease-relevant (synovial) Ags and cause stimulation and expansion of synovial T cells, which then drive the inflammatory process.

Concerns have been expressed with regard to the importance of CD4⁺ T cells in the pathogenesis of RA, especially the role of this T cell subset in the perpetuation of advanced disease (reviewed in Refs. 7 and 15). These arguments are based primarily on studies showing that T cell-derived cytokines are much less abundant than other proinflammatory cytokines such as IL-1 and TNF-α in chronically involved joints (15, 16). Furthermore, therapies directed against T cells and T cell products have not been remarkably effective (17–21), especially when compared with therapies directed against TNF-α (16, 22, 23). Finally, studies have yet to identify the stimulating synovial Ags in patients’ joints.

Since the stimulating synovial Ags in RA are unknown, studies have focused on whether the TCRs expressed by synovial T cells show a pattern characteristic of Ag-stimulated populations. The results to date, however, have not been clear-cut. Without question, the repertoire of synovial T cells is markedly heterogeneous (reviewed in Ref. 24). Several studies have shown an increased expression of particular TCR variable (V) regions. However, there has been little consistency between reports, and different TCR V genes have shown increased utilization in different studies. Other studies have noted particular TCR β-chain gene (TCRB) clonotypes, suggesting clonal expansions within the synovial T cell population. Certain amino acid motifs within the TCR complementarity-determining region 3 (CDR3) have been described in individual patients and among different patients, and these motifs have been used to suggest selection by a similar Ag (24–26). However, these studies have not found highly homologous receptors as found after peptide immunization in animals, and the significance of these motifs in terms of reflecting recognition of the same Ag remains unclear. Finally, despite a paradigm that involves the same presenting HLA-DR4 molecules, no single TCR or clearly related TCR has been identified in different patients.

Here we report our analysis of the CD4⁺ T cell repertoire in synovial fluid from different joints and peripheral blood in patients with advanced RA. The results show that despite the marked heterogeneity of synovial CD4⁺ T cells, a large fraction of the TCR

---

¹Departments of Medicine and Pediatrics, National Jewish Medical and Research Center, Denver, CO 80206; NeXstar Pharmaceuticals, Inc., Boulder, CO 80301; and Departments of ²Medicine and ³Immunology, University of Colorado Health Sciences Center, Denver, CO 80262.

Received for publication April 3, 1998. Accepted for publication June 10, 1998.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported in part by a research grant from NeXstar Pharmaceuticals (Boulder, CO), General Clinical Research Center Grant RR00591 from the National Institutes of Health, and in part by the Charlie Smyth Fellowship from the Rocky Mountain Chapter of the Arthritis Foundation (to C.C.S.).

The following sequences discussed in this report have been deposited in GenBank: accession numbers AF043747–AF043878, AF043884–AF043895, and AF043982–AF043995.

Current address: Alexion Pharmaceuticals, Inc., New Haven, CT 06511.

Address correspondence and reprint requests to Dr. Brian L. Kotzin, Division of Allergy and Clinical Immunology (B-164), University of Colorado Health Sciences Center, 4200 East Ninth Ave., Denver, CO 80262. E-mail address: brian.kotzin@uchsc.edu.

Abbreviations used in this paper: RA, rheumatoid arthritis; TCRB, TCR β-chain gene; CDR, complementarity-determining region; TCRA, TCR α-chain gene; TCRBV or TCRBV, TCR α- or β-chain variable region gene segment.

---

Copyright © 1998 by The American Association of Immunologists

0022-1767/98/$02.00
Clonotypes was found in synovial fluid, which is indicative of in vivo clonal expansions. Furthermore, we found separate synovial CD4+ T cell clones that use highly homologous TCR α-chain (TCRA) and TCR β genes. In several instances, these clones used different CDR3 nucleotide sequences to encode identical TCRs. This coselection of both TCRα and TCRβ genes expressed by particular T cell clones in the sy-novial fluid of individual patients strongly suggests that these cells have been selectively stimulated and expanded by a conventional Ag(s) in this disease.

Materials and Methods

Patients and HLA-DR typing

All patients met the American College of Rheumatology criteria for the classification of RA (27) and were studied under the guidelines of the human subjects institutional review board at the National Jewish Medical and Research Center (Denver, CO). Various characteristics of these patients are shown in Table I. All patients had active inflammatory disease at the time of participation. Arthrocentesis of two joints (both knees) and phlebotomy were performed on the same day. Mononuclear cells from the peripheral blood and synovial fluid were isolated by Ficoll-Hyapogen density gradient centrifugation (Pharmacia, Piscataway, NJ). EBV-transformed B cell lines were prepared by infecting PBL with supernatant from the EBV-secreting marmoset cell line B95-8 (28) in the presence of 0.5 μg/ml cyclosporin A (Sandoz, East Hanover, NJ). These lines were used for HLA-DR typing, which was performed using standard serologic and molecular techniques at the Clinical Laboratories of Denver Immunologic Associates (Denver, CO).

Immunofluorescence analysis of T cell Vβ expression

The percentages of CD4+ T cells in peripheral blood and synovial fluid that express different TCR Vβ chains were determined by two-color immunofluorescence staining and cytofluorographic analysis. CD4 expression was determined using a fluorescein-conjugated mAb to CD4 (Becton Dickinson, San Jose, CA). TCR Vβ expression was determined with biotinylated mAbs directed against the Vβ2 (clone E22E7.2, Immunotech, Westbrook, ME) (29), Vβ3.1 (clone 8F10, T Cell Diagnostics, Cambridge, MA) (30, 31), Vβ8.1/8.2 (clone MX-6, T Cell Diagnostics) (32), Vβ14 (clone CAS1.1.3, Immunotech) (33, 34), and Vβ17 (clone E17.5F3, Immunotech) (29). The biotinylated anti-Vβ mAbs were detected with either CyChrome-streptavidin (PharMingen, San Diego, CA) or phycoerythrin-streptavidin (Southern Biotechnology Associates, Birmingham, AL). Fluorescence intensity was analyzed on a FACSscan (Becton Dickinson), and the percentage of CD4+ T cells bearing each Vβ was determined.

Analysis of TCRB gene sequences

Peripheral blood and synovial fluid mononuclear cells were double stained with fluorescein-conjugated anti-CD4 and phycoerythrin-conjugated anti-CD8 mAbs (Becton Dickinson). CD4+CD8- cells were sorted using an EPICS 751 or Elite cytofluorograph (Coulter, Hialeah, FL). The cell pellet (≥100,000 cells) was immediately frozen, and total cellular RNA was isolated using a commercially available kit (Ultraspec RNA Isolation System, Biotecx Laboratories, Houston, TX) according to the manufacturer’s directions. This RNA was used for the synthesis of first-strand cDNA using reverse transcriptase (SuperScript RT, Life Technologies, Grand Island, NY) and random hexamers (Pharmacia). One-twentieth of the cDNA from each sample was added to a 50-μl reaction mixture containing forward TCRB primer 5′-ATCTTCTAGAGAAGAAGGCGC-3′ and reverse TCRb constant region gene segment (TCRBc) primers (each at a final concentration of 0.3 μM), and PCR amplification (using AmpliTag or AmpliTaq Gold, Perkin-Elmer, Branchburg, NJ) was performed for 35 cycles under the following conditions: 95°C melting, 55°C annealing, and 72°C extension, each for 1 min, followed by a final extension step at 72°C for 10 min. The primer sequences were: TCRBV2, 5′-TCATCAACATGGAAGCCTGACACT-3′; TCRBV3, 5′-GTCTTCTAGAGAAGAAGGCGC-3′; TCRBV8.1, 5′-ATTACTTCTAAACAACTGTCGG-3′; TCRBV14, 5′-GTCTCTCAGAAAGAGAAGGAAAT-3′; TCRBV17, 5′-CAGATGAAATGACTTTCAGC-3′; and TCRBC, 5′-CTCTAUTOCTGCTAACCAC-3′. Each PCR product was ligated into the pCRI TA cloning vector as specified by the manufacturer (Invitrogen, San Diego, CA), and the ligated product was transformed into Epicurian coli XL-1 Blue supercompetent Escherichia coli cells (Strategene, La Jolla, CA). Colonies containing appropriate inserts were chosen randomly for sequencing. Cycle sequencing was performed using the Ready Reaction DyeDeoxy Terminator cycle sequencing kit (Perkin-Elmer, Foster City, CA) and automated sequencer (Applied Biosystems/Perkin-Elmer). Sequencing primers included an M13 reverse primer, 5′-CAGGAAACAGCTATGAC-3′ (Promega, Madison, WI), and a M13 forward primer, 5′-CAGCAAGCAGGCTTGTGAAGAC-3′ (Life Technologies).

Cloning of synovial fluid CD4+ T cell clones and analysis of expressed TCRB gene sequences

To further analyze the TCRB repertoire and to identify the TCRα coexpressed in particular T cell clones, synovial fluid mononuclear cells were stained for CD4 and the TCR Vβ of interest as described above, and double-positive cells were sorted and cloned by limiting dilution. Variable numbers (1, 3, or 10/well) of sorted T cells and 5 × 10^4 irradiated (9000 rad) TK6 feeder cells/well were cultured in 96-well microtiter plates (Falcon, Becton Dickinson Labware, Bedford, MA) in RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 10% FCS (HyClone, Logan, UT), 20% HL-1 culture medium (BioWhittaker), 20 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (all from Life Technologies), 0.25 μg/ml PHA (Murex Diagnostics, Dartford, U.K.), and 10% T cell growth factor supernatant (a gift from R. J. Albertini, University of Vermont, Burlington, VT) (35). After 12 to 14 days of culture, T cell colonies were transferred to 1-ml cultures. Cells from confluent cultures were harvested, and total cellular RNA was isolated using a commercially available kit (RNAid PLUS, BHI 101, La Jolla, CA), cDNA was prepared, and the TCRB gene fragments were amplified using the TCRBV primer corresponding to the Vβ sorted and TCRBC primer as described above. Each PCR product was purified using a DNA binding membrane spin column (QiAquick PCR Purification Kit, Qiagen, Chatsworth, CA) and sequenced using a TCRBC sequencing primer (5′-CGAC TTCGGGTTGGAACA-3′).

Analysis of TCRA genes expressed in T cell clones

RNA from T cell clones that expressed the TCRβ of interest was isolated for anchored PCR amplification of TCRA cDNA using the rapid amplification of 5′ cDNA end system (Life Technologies). Briefly, TCRα first strand cDNA was synthesized using a TCRA-specific primer (5′-TTCAGCTTAAACACAGGAGGTC-3′) and SuperScript II reverse transcriptase (Life Technologies). RNA was then digested with a RNase mixture, and the cDNA was purified using a silica-based membrane column (GlassMax DNA isolation spin cartridge, Life Technologies). A homopolymeric dC tail was then added to the cDNA using dCTP and terminal deoxynucleotidyl transferase. This dC-tailed cDNA was amplified for 35 cycles of PCR, using the abridged anchor primer provided by the manufacturer and a second nested TCRα primer, 5′-GAACCTCAGG TATCTGGAGTA-3′. A second round of 35 cycles of nested PCR was then performed using the abridged universal amplification primer provided, a third nested TCRα primer, 5′-TCAGAGGCTACCACTGGC-3′, and 5 μl of first round product. The anchored PCR product was ligated into pCRII and cloned as described above. The insert was sequenced as described above using a TCRα sequencing primer (5′-TGTACA CCGGCAAGGTCAGG-3′).
The CDR3 sequence of each clone’s expressed TCRA was verified by performing a TCRAV-specific PCR using the appropriate TCRAV primer (TCRAV1, 5’-CTGAGTGACACTCTCA-3’; TCRAV5, 5’-GGCCCTGAAAATTCAGGA-3’; TCRAV8, 5’-GGAGAAXTGTTGGACGAGCATC-3’; TCRAV11, 5’-AGGAAGGAAGGACAAAGTGTTGTT-3’; or TCRAV16, 5’-CATCACAGGGGATAACCTGGT-3’) and the nested TCRAC primer described above. The subsequent PCR products were then sequenced using the TCRAC sequencing primer.

Results

**TCR Vβ expression determined by immunofluorescence staining**

Paired synovial fluid (knee) samples as well as peripheral blood from three individual patients with long-standing, active RA were studied (Table I). We first determined the percentage of CD4+ T cells that expressed particular TCR Vβs using immunofluorescence staining and cyt fluorographic analysis. For these studies, we used a limited panel of anti-Vβ mAbs directed to certain V regions suggested to be important in RA, including Vβ2, 3.1, 8.1/8.2, 14, and 17 (24, 36–42). As shown in Figure 1, the synovial CD4+ Vβ repertoire was generally similar to that of PBL, although there were a few exceptions. For example, in patient CS-2, there was a greater than twofold increase in the percentage of cells expressing Vβ3.1 in the synovial fluid compared with peripheral blood. A similar finding was noted in patient CS-3, who demonstrated an increased percentage of Vβ8.1/8.2+ cells in both joints compared with that in blood. There was one example of a larger Vβ8.1/8.2+ subset in blood compared with synovial fluid lymphocytes in patient CS-2. None of the patients demonstrated a consistent decrease in the synovial fluid Vβ percentages compared with those in blood. The absence of such reciprocal changes suggests that another synovial subset expressing a Vβ not studied in this analysis was unlikely to be greatly expanded and dominate the synovial population.

**Analysis of TCRB CDR3 sequences**

We sorted freshly isolated synovial fluid and peripheral blood CD4+ T cells from each patient to analyze the TCRB gene repertoire expressed in these lymphocyte populations. cDNAs were PCR amplified with TCRBV-specific primers that corresponded to the five Vβ subsets studied above. The PCR products from each sample were then cloned, and randomly selected bacterial isolates containing the appropriate insert were sequenced to determine the nucleotide and amino acid sequences of the CDR3. Sequences of interest for each patient are ordered by TCRBV and TCRBJ gene segment usage in Figure 2. This figure includes identical nucleotide sequences found at least three times, sequences of a clone closely related to or identical with another sequence in the same fluid, and sequences matching in the other fluid or in blood (indicated by bold type). When the same CDR3 amino acid sequence is shown more than once (such as the TCRBV14-LGTEG-BJ1S2 (BJ1.2) sequences in Fig. 2A), a different CDR3 nucleotide sequence (i.e., expressed by a different clone) was used to encode the identical amino acid sequence (see below).

A markedly heterogeneous repertoire was noted for all synovial fluids analyzed, and a large number of unique sequences was found within each TCRBV subset in each RA patient studied. Clones expressing each of the five TCRBV genes used nearly all 13 TCRBJ gene segments (Fig. 2 and data not shown). However, repeated sequences of individual clonotypes were clearly apparent. In these three subjects studied for five different TCRBV subsets, >40 clones occupied >5% (three or more repeated sequences) of the total sequences within a TCRBV subset in a single joint. The probability for one set of three repeated sequences to be found by chance alone (assuming ~5,000 cells within a particular TCRBV subset from >100,000 CD4+ cells sorted) was calculated to be $p = 1.6 \times 10^{-7}$. A few synovial clones were quite large and comprised nearly 50% of a particular TCRBV family, such as the BV3-SSLQQAGBAJ-2.3, BV14-LFLAGG-BJ2.2, and BV17-SPAG-BJ2.2 clones in patient CS-2. In general, repeated sequences were not present in peripheral blood, although one clonal expansion was found only in the blood BV17 subset of patient CS-2.

We obtained synovial fluid from one patient 6 mo after the initial analysis to determine whether large clones persist in patients with continued evidence of inflammatory arthritis. In patient CS-2, a CD4+ clone that initially occupied 45% of the TCRBV3 subset was noted to actually increase in frequency to 71% (44 of 62 sequences) of the left knee TCRBV3 sequences at the later time point. Similarly, the large Vβ17+ clone in this individual persisted at about 50% (31 of 64 sequences) of the left knee TCRBV17 sequences. These data from one individual suggest that the synovial fluid TCR repertoire may remain relatively constant over time in patients with continued arthritis.

Different TCR sequences were considered to be related if they had identical TCRBV and BJ gene segments and highly homologous CDR3 amino acid sequences. We also required the length of the CDR3 be identical, since this has been shown to be important in TCR recognition of Ag (43, 44). Related sets of sequences were identified within the BV2, BV3, and BV14 subsets of CS-1, within
the BV8 and BV14 subsets of CS-2, and within the BV3, BV8, and BV17 subsets of CS-3 (Fig. 2). More related sets of sequences were also probably present but had other slight variations in BJ usage or length of the CDR3. The ultimate forms of related TCRs were those that expressed different TCRB genes (defined as having at least two nucleotide differences in the CDR3) but identical TCR b-chain amino acid sequences. These are shown in Figure 2 as repeats of the same amino acid sequence. Examples were found in Figure 2.

FIGURE 2. Analysis of TCRB CDR3 nucleotide sequences from synovial fluid lymphocytes and PBL from patients CS-1 (A), CS-2 (B), and CS-3 (C). Sequences found in both joints or found multiple (three or more) times in one anatomic site or that had sequence similarity to another sequence(s) are shown. The same TCR b-chain amino acid sequence is shown more than once when it is encoded by different TCRB gene nucleotide sequences (not shown). The number of identical sequences (at the nucleotide level) are given over the total number of sequences analyzed for a given TCRBV subfamily from a given anatomic site. Sequences present in more than one joint are shown in bold type. The probability for one set of three repeated nucleotide sequences to be found by chance alone (assuming 5,000 cells within a particular TCRBV subset from 100,000 CD4+ cells sorted) was calculated to be \( p = 1.6 \times 10^{-7} \). Based on the potential diversity of the TCRB gene repertoire, the probability for finding by chance alone a match in a separate joint or finding a related sequence was estimated to be very low (\( p < 10^{-6} \)). ND, not determined. These sequences have been submitted to GenBank (accession nos. AF043747–AF043873).
Based on the enormous potential sequence diversity within a TCRBV subset (45, 46), related sequences, as defined above, are predicted to occur very rarely by chance alone ($p < 10^{-6}$) and almost certainly represent selection by Ag. No related clones were found in the peripheral blood of these three patients. The most striking set of related synovial clones was apparent in the TCRBV14 subset of patient CS-1 (Figs. 2A and 3). Overall, there were 10 different related clones that had the BV14-LGTEG-BJ1.2 related motif, and half were found in both joints studied. We identified four different nucleotide sequences that encoded the identical LGTEG junctional amino acids and identical TCR $\beta$-chain, and three different nucleotide sequences that encoded the identical PGTEG-containing TCR $\beta$-chain. These receptors used differing aliphatic leucine (L), proline (P), alanine (A), or aliphatic hydroxyl (serine; S) amino acids at position 94 and either glycine (G) or asparagine (N) at position 98 of the TCR $\beta$-chain sequence, but maintained a core CDR3 sequence of glycine (G), threonine (T), and glutamate (E) as well as CDR3 length. It is important to note that PCR sequence errors did not account for the variation in the CDR3 nucleotide sequence. Thus, in these 28 different cDNA clones, analysis of the invariant TCRB segments (i.e., BV, BJ, and BC) showed only one sequence error in over 10,000 nucleotides sequenced (data not shown). As shown in Figure 2A, the set of related BV14 clones comprised approximately 25% of the BV14 subset in the two joints of patient CS-1. Considering that Vp14$^+$ cells were 3 to 4% of the synovial CD4$^+$ T cell population (Fig. 1), this set of related clones comprised ~1% of the total CD4$^+$ T cells within the knee joints of this patient.

Our analysis of TCRB gene sequences also showed that a relatively large fraction of the TCRs is identical in different joints, but there is little overlap with T cells in the circulating pool (Fig. 2 and Table II). For example, in patient CS-1, 29 of 159 clones (18%) in the right knee joint were also present in the left knee, but none was present in the peripheral blood. The data in Table II are expressed as the fraction of total sequences (rather than clones) that were shared among different joints and peripheral blood lymphocytes. For example, in patient CS-2, 61 of 189 sequences (32%) in the right knee matched sequences in the left knee compared with none in the blood ($p = 5 \times 10^{-11}$, by Fisher’s exact test). Overall, the data showed that the synovial T cell repertoire is not random and is not a passive reflection of the circulating T cells.

Interestingly, immunofluorescence analysis (Fig. 1) failed to accurately predict the presence of large clones identified by sequence analysis. For example, large clones were present in the left knee

### Table 1

<table>
<thead>
<tr>
<th>Location</th>
<th>Clone Number</th>
<th>Nucleotide Sequence</th>
<th>Nucleotide Replacement</th>
<th>Amino Acid Replacement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Right Knee</td>
<td>18</td>
<td>TGT GCG ACC AGT</td>
<td>TTA GGG ACT GAC GGC</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td>Right Knee</td>
<td>45</td>
<td>TGT GCC ACC AGG</td>
<td>TTA GGG ACT GAG GGC</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td>Right Knee</td>
<td>48</td>
<td>TGT GCC ACC AGT</td>
<td>TTA GGG ACT GAG GGC</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td>Right Knee</td>
<td>62</td>
<td>TGT GCC ACC AGT</td>
<td>TTA GGG ACT GAG GGC</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td>Right Knee</td>
<td>80</td>
<td>TGT GCC ACC AGT</td>
<td>TTA GGG ACT GAG GGC</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td>Right Knee</td>
<td>85</td>
<td>TGT GCC ACC AGT</td>
<td>TTA GGG ACT GAG GGC</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td>Left Knee</td>
<td>12</td>
<td>TGT GCC ACC AGT</td>
<td>TTA GGG ACT GAG GGC</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CASS LGTEG YGYTFG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right Knee</td>
<td>9</td>
<td>TGT GCC ACC AGT</td>
<td>TTA GGG ACC GAA GGC</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td>Right Knee</td>
<td>61</td>
<td>TGT GCC ACC AGT</td>
<td>TTA GGG ACC GAA GGC</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td>Left Knee</td>
<td>1</td>
<td>TGT GCC ACC AGT</td>
<td>TTA GGG ACC GAA GGC</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CASS LGTEG YGYTFG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right Knee</td>
<td>20</td>
<td>TGT GCC ACC AGT</td>
<td>TTA GGG ACC GAA GGC</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td>Right Knee</td>
<td>70</td>
<td>TGT GCC ACC AGT</td>
<td>TTA GGG ACC GAA GGC</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CASS LGTEG YGYTFG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right Knee</td>
<td>50</td>
<td>TGT GCC ACC AGT</td>
<td>TTA GGG ACA GAG GAT</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td>Right Knee</td>
<td>22</td>
<td>TGT GCC ACC AGT</td>
<td>TTA GGG ACA GAG GAT</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td>Left Knee</td>
<td>52</td>
<td>TGT GCC ACC AGT</td>
<td>TTA GGG ACA GAG GAT</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CASS LGTEN YGYTFG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right Knee</td>
<td>55</td>
<td>TGT GCC ACC AGT</td>
<td>TTA GGG ACA GAA GGC</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td>Right Knee</td>
<td>13</td>
<td>TGT GCC ACC AGC</td>
<td>TTA GGG ACA GAA GGC</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td>Right Knee</td>
<td>65</td>
<td>TGT GCC ACC AGC</td>
<td>TTA GGG ACA GAA GGC</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td>Right Knee</td>
<td>79</td>
<td>TGT GCC ACC AGT</td>
<td>TTA GGG ACA GAA GGC</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td>Left Knee</td>
<td>11</td>
<td>TGT GCC ACC AGT</td>
<td>TTA GGG ACA GAA GGC</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CASS PGTEG YGYTFG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right Knee</td>
<td>47</td>
<td>TGT GCC ACC AGC</td>
<td>TTA GGG ACA GAG GGC</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td>Right Knee</td>
<td>72</td>
<td>TGT GCC ACC AGT</td>
<td>TTA GGG ACA GAG GGC</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CASS SGTEG YGYTFG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right Knee</td>
<td>25</td>
<td>TGT GCC ACC AGT</td>
<td>TTA GGG ACA GAG AAC</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td>Right Knee</td>
<td>53</td>
<td>TGT GCC ACC AGT</td>
<td>TTA GGG ACA GAG AAC</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td>Right Knee</td>
<td>68</td>
<td>TGT GCC ACC AGT</td>
<td>TTA GGG ACA GAG AAC</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td>Right Knee</td>
<td>77</td>
<td>TGT GCC ACC AGT</td>
<td>TTA GGG ACA GAG AAC</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td>Left Knee</td>
<td>20</td>
<td>TGT GCC ACC AGT</td>
<td>TTA GGG ACA GAG AAC</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>TGT GCC ACC AGT</td>
<td>TTA GGG ACA GAG AAC</td>
<td>TAT GGC TAC ACC TTC GGT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CASS AGTEN YGYTFG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table II. Matching of identical TCRB sequences in a different synovial fluid and blood in the same patient

<table>
<thead>
<tr>
<th>Patient</th>
<th>Right to Left Knee</th>
<th>Left to Right Knee</th>
<th>Blood to Either Knee</th>
<th>p valuea</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS-1</td>
<td>58/216 (27%)</td>
<td>58/256 (23%)</td>
<td>0/56 (0%)</td>
<td>(&lt;3 \times 10^{-5})</td>
</tr>
<tr>
<td>CS-2</td>
<td>61/189 (32%)</td>
<td>80/247 (32%)</td>
<td>0/96 (0%)</td>
<td>(&lt;1 \times 10^{-10})</td>
</tr>
<tr>
<td>CS-3</td>
<td>25/102 (25%)</td>
<td>29/121 (24%)</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

*The p values for differences in the matches in blood compared to the matches in the left knee synovial fluid were determined by Fisher’s exact test.

**ND**, not determined.

TCRBV3 and BV17 subsets in patient CS-2, but only the percentage of VB3+ cells was increased in the synovial fluid compared with the blood. Also, the percentage of synovial VB3+ cells was increased in synovial fluid relative to that in blood in patient CS-3, but no large clones were apparent by sequence analysis.

Analysis of TCRα sequences coexpressed in T cell clones

Selection by the same conventional Ag should be reflected in both the TCRα and TCRβ gene sequences coexpressed by related T cell clones. Therefore, we cloned synovial CD4+ T cells by limiting dilution, identified those clones that expressed related TCRβ sequences, and sequenced the coexpressed TCRα gene segments. We first focused on the large set of related BV14-LGTEG-BJ1.2 clones in patient CS-1 by cloning synovial fluid CD4+ VB14+ T cells from this patient. Figure 4 shows the sequences of the TCRα genes coexpressed in six clones that have this related CDR3 motif. Two of the six (no. 34 and 337) expressed identical TCRα and TCRβ gene sequences, confirming their derivation from the same precursor clone. The relatedness of the TCRα sequences expressed by the five unique clones was clearly evident (Fig. 4). Thus, each TCRα used AJ22 of about 60 TCRαJ gene segments to choose AJ21 of about 60 TCRβV gene segments. We therefore amplified the synovial fluid CD4+ T cell cDNA with TCRαV1-specific and TCRαC primers. Eleven of fifty-seven total sequences (19%; five clonotypes) were related to the clonal set described above (Fig. 5). Two of the sequences were identical with those expressed by T cell clones characterized above (clones 34/337 and 325). In addition, three unique sequences were found, which maintained the same CDR3 length and invariant CAVR motif. One used TCRαJ44 rather than TCRαJ22, but the amino acid sequences encoded by these AJ gene segments were similar, with mostly conservative substitutions.

To extend the above findings, we generated T cell clones from synovial fluid CD4+ VB3+ T cells of patient CS-2. Two sets of related sequences were found, and the sequences of the coexpressed TCRα and TCRβ genes are summarized in Figure 6. The first set of related clones all expressed TCRβJ2S3 in conjunction with TCRβV3 (Fig. 6, upper). The amino acid, but not nucleotide, sequence of the CDR3 in the first two clones was identical, and the third clone showed two conservative amino acid changes, leucine (L) to serine (S) and serine (S) to threonine (T). The
TCRA sequences also showed conservation of CDR3 length, used TCRAJ10, and used either TCRAV5S1 or 11S1. There was one nonconservative change in clone 103 (glycine (G) to glutamate (E)), but other changes were conservative (leucine (L) to valine (V) or threonine (T) to valine (V)).

The second set of clones recovered from patient CS-2 included 10 isolates expressing four clonotypes (Fig. 6, lower). The TCRB sequences again showed conserved CDR3 length, and whereas all used the TCRBV3 gene segment, they each used a different TCRBJ gene segment. The β-chain junctional region amino acid sequence was identical in two of the clones, and two others showed only single residue changes. The corresponding α-chain sequences were also strikingly similar, and two junctional regions were identical. All clones used TCRAV8S1 and showed conservation of CDR3 length, and three of the four used TCRAJ9.

Discussion

The elucidation of the MHC class II allele association with disease susceptibility (11, 13) and pathologic evidence of synovial CD4⁠⁺ T cell infiltration (4 – 8) have suggested a model in which CD4⁠⁺ T cells play a central role in the pathogenesis of RA. However, the specificity of synovial T cells remains mostly unknown, and because of their heterogeneous TCR repertoire (24), major questions have emerged about whether synovial T cells are selected and accumulate based on Ag recognition in this disease. The present study was designed to eliminate some of the problems of past studies of synovial T cell repertoire and to extend previous findings. For example, we avoided in vitro manipulation of cells before TCR repertoire analysis, studied only the CD4⁠⁺ T cell population, and focused an extensive analysis on a fraction of the repertoire, rather than tried to cover the entire T cell pool. The Vβs and TCRB genes analyzed in the current work cover about 25 to 30% of the repertoire (32 – 34, 39) (Fig. 1) and were chosen based on past studies that suggested their possible importance in this disease (24, 36 – 42). We also addressed aspects of TCR repertoire analysis that would be least subject to PCR amplification variation, including the search for related (rather than identical) clones in synovial fluid, the matching of TCR sequences in the synovial fluid from a separate joint and in blood, and the analysis of coexpressed TCRA sequences for relatedness. The results clearly show that a significant fraction of the synovial fluid CD4⁠⁺ TCR repertoire is distinct from that of the circulating resting pool. Furthermore, the results demonstrate the accumulation in synovial fluid of clonal expansions that persist over time and document sets of related synovial CD4⁠⁺ T cell clones with highly homologous TCRB and TCRA genes.

In the first stage of our analysis of the synovial CD4⁠⁺ T cell repertoire in RA, anti-TCR Vβ specific mAbs were used to accurately quantitate Vβ expression. The results showed a synovial repertoire not so different from that found in blood T cells, which is consistent with the overall heterogeneous TCR expression in synovial fluid. The absence of reciprocal decreases in Vβ percentages in synovial fluid vs blood also suggested that a large expansion within a different Vβ subset is unlikely. For example, if 30% of the synovial T cells expressed a Vβ not analyzed, we would have expected to find, on the average, a 30% decrease in the percentages of T cells expressing Vβs 2, 3.1, 8.1/8.2, 14, and 17.

FIGURE 5. TCRAV1 CDR3 sequences expressed in synovial fluid CD4⁺ lymphocytes from the right knee of patient CS-1. Sequences similar or identical with those shown in Figure 4 are shown. The results also indicate the frequency of each sequence of 57 analyzed and matches with sequences expressed by cloned CD4⁺ T cells from the same fluid (see Fig. 4). These sequences have been submitted to GenBank (accession nos. AF043874 – AF043878).

FIGURE 6. TCRA and TCRB CDR3 sequences expressed in synovial fluid CD4⁺ T cell clones isolated from patient CS-2 by limiting dilution culture. The nucleotide and amino acid sequences from two sets of related clones are shown. These sequences have been submitted to GenBank (accession nos. AF043982 – AF043995).
relative to peripheral blood T cells. Although immunofluorescence staining has the potential advantages of being relatively rapid, easily performed, and accurate for determining the size of a Vβ subset, these data appeared to be somewhat misleading. Most importantly, immunofluorescence analysis of TCR expression did not accurately predict the presence of large clones or sets of related clones in synovial fluid.

We matched the expressed TCRB sequences from one joint with those in a second synovial fluid from the same patient and with those in blood. Remarkably, 20 to 30% of the sequences from the two joints matched. The absence of any matches in blood, concomitantly analyzed, provided a convincing control for the possibility of PCR contamination. Although no matches in blood were found, previous studies, including those from our laboratory, have suggested that synovial CD4+ clones may rarely be present in the circulating pool (36, 49). This may explain how T cell clones travel to different joints, although the original source of the distributed synovial clones is unknown. The extent of TCR matching in different joints is surprising. In studies of experimental autoimmune encephalomyelitis, pathogenic T cells appear to comprise only a small fraction of the total T cell repertoire in involved central nervous system tissue (50–52). Nonspecific influx of T cells into the areas of inflammation has been postulated to account for these findings in experimental autoimmune encephalomyelitis. Based on the extensive amount of inflammation occurring in rheumatoid synovium, a similar nonspecific influx should be occurring, which would result in dilution of the relevant clones.

Within two synovial compartments of an individual patient, the accumulation of related CD4+ T cell was apparent. We found multiple examples of different T cell clones, i.e., with different TCRB nucleotide sequences, encoding the same TCR β-chain amino acid sequence. Sets of related TCRB sequences with the same BV and BJ gene segments as well as highly homologous CDR3s were also found. Furthermore, a number of these related clones could be isolated by limiting dilution culture after sorting for CD4+ Vβ+ synovial T cells. The coexpressed TCR gene sequences were also shown to be related to each other. We believe that these results provide the most convincing evidence that a subset of synovial CD4+ T cells have accumulated in synovium based on the recognition of a common Ag. The apparent importance of the TCRB CDR3 as well as the selection for particular coexpressed TCR sequences indicates that the selecting Ag is a conventional protein (peptide) Ag/MHC complex rather than a superantigen (43, 53). Of course, the identity of this stimulating Ag (or Ags) is currently unknown as is its role in the disease process. The TCRs expressed by these in vivo expanded synovial clones do not appear to be unusual compared with other TCR, and it is currently not possible to predict a particular type of Ag based on TCR structure.

A number of previous studies of RA patients have presented evidence for expanded CD4+ clones in synovial tissue or synovial fluid based on finding repeated sequences (6, 24–26, 36–38, 54) or discrete bands after separation of TCRB fragments by single-stranded conformational polymorphism (55). Also consistent with our findings, Alam et al. (54) studied two RA patients and found examples of the same clone in two different synovial tissues. These authors also presented evidence for common CDR3 motifs among the different synovial clones found. However, related clones were allowed to have significantly different CDR3 amino acid substitutions and length, and they also frequently differed in BV and BJ usage. The effect of such differences on Ag recognition is likely to be profound (43, 44), and therefore their conclusions regarding a common antigenic stimulus seem limited. Another study (25) concluded relatedness based on the presence of two hydrophobic amino acid residues within the CDR3. Again, other components of the TCR important for recognition, such as CDR3 sequence and length, and BV and BJ gene segment usage, were allowed to vary, raising serious questions about the relatedness of such clones. None of the above studies analyzed coexpressed TCRB sequences for relatedness. Li et al. (26) focused on two potentially related TCRBV17 sequences in synovial tissue that persisted in synovial fluid after synovectomy. These clones varied in CDR3 sequence, CDR3 length, and BJ usage. More importantly, Vβ17+ clones derived from this joint were considered to be related to the original synovial sequences, yet the TCRs being compared varied in CDR3 sequence, length, and/or BJ usage and the two clones showed major differences in the coexpressed TCR sequences. Finally, no previous studies of synovial T cell repertoire have documented the same TCR β-chain to be encoded by different T cell clones, which we found in all three patients studied.

We found one example of an expanded CD4+ Vβ17+ clone in peripheral blood, which did not match any of the synovial clones in this patient. Dominant clonotypes in peripheral blood CD4+ T cells have been described previously, and a preference for T cells expressing BV3, BV14, and BV17 was noted (49). It may be of interest that the CDR3 region of one of our synovial BV3 clones matched a previously reported blood clone in an RA patient (49).

The specificity of the related synovial T cell clones in these RA patients remains unknown. Preliminary studies indicate that these sets of expanded clones from CS-1 and CS-2 do not respond to autologous EBV-transformed B lymphoblastoid cells (M. T. Falta, C. C. Striebich, J. Bill, and B. L. Kotzin, unpublished observations). Thus, it is unlikely that they are directed to EBV trans-activator proteins as recently shown for a subset of CD8+ synovial T cells in RA (56, 57). Our TCR analysis also suggests that many different Ags are involved in the stimulation and selection of different clonal sets, especially in patients with advanced disease. It is emphasized that our TCR analysis and studies of other investigators have not been able to document related T cell clones among different patients. Furthermore, in a comparison of our TCRB sequences to those previously reported to be expressed in RA synovium, no matches were found. This is particularly troublesome considering the association of RA with HLA-DR4 and the hypothesis that DR4 functions as the presenting element for unknown arthritogenic peptide(s). All patients in the current study expressed HLA-DR4, and one patient carried two DR4 alleles. This hypothesis, however, may not explain recent studies that suggest a gene dosage effect in which the presence of two HLA-DR4 alleles confers greater risk of disease and increased severity of disease compared with those in individuals with one copy of the gene (12, 14, 58, 59), and other models for the role of DR4 in Ag presentation and T cell selection have been proposed (7, 60). Thus, the heterogeneity of expanded synovial clones in different RA patients may be explained by responses to multiple Ags, different antigenic determinants, and different presenting class II MHC molecules.

Acknowledgments

We thank Carl Colburn and Daphne Norsworthy for technical assistance with the TCR sequence analysis and thank Drs. John Kappler and Philippa Marrack for helpful discussions.

References

SYNOVIAL T CELLS IN RHEUMATOID ARTHRITIS


