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Immunology

The T-cell-receptor repertoire in the synovial fluid of a patient with rheumatoid arthritis is polyclonal

(inverse polymerase chain reaction/autoimmune disease/overrepresentation of β chain variable region gene segments)

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ABSTRACT **We have analyzed the T-cell-receptor repertoire expressed in the synovial fluid of a patient with rheumatoid arthritis by using an inverse polymerase chain reaction. Total RNA was isolated from Ficoll-purified mononuclear cells and converted into circularized double-stranded cDNA. Specific amplification of α- and β-chain variable regions (Vα and Vβ) was achieved with inverted α- and β-chain constant region (Cα and Cβ) primer pairs, and the amplification products were cloned into phage vectors. A total of 78 α and 76 β clones were sequenced, and 67 and 72 productively rearranged α and β genes were identified, respectively. Thirty-one Vα, 33 α-chain joining region (Jα), 29 Vβ, and 12 β-chain joining region (Jβ) gene segments were found in the productively rearranged clones, indicating that the T-cell repertoire expressed in the synovial fluid of this RA patient is highly heterogenous and polyclonal. Comparison of peripheral blood and synovial fluid repertoires showed that the most abundant Vβ sequences, Vβ2.1 and Vβ3.1, were enriched in the inflamed joint by a factor of 2 to 3. It is possible that T cells expressing these Vβ gene segments, which recognize bacterial superantigens, play a role in the disease.**

Human autoimmune diseases are a complex group of about 40 diseases affecting 5–7% of the population (1). Among these rheumatoid arthritis (RA), a systemic autoimmune disease that affects females more often than males, is the most common with a prevalence between 1.6 and 5%. The cause of RA is unknown. Patients suffer from a progressive joint destruction, presumably due to autoimmune attack, and often end up with severe disablement. The pathological changes are divided into four stages (synovitis, loss of articular cartilage, joint erosion, and joint destruction), and the clinical course is characterized by exacerbations and remissions. Current treatment is unspecific and unsatisfactory. An intensive effort is underway worldwide to find more effective treatment modalities (2).

T lymphocytes appear to play an important role in the pathology of RA. (*i*) Activated T cells are found in the inflamed synovium (3). (*ii*) Susceptibility to RA is associated with particular alleles of the HLA complex, primarily subtypes of HLA-DR4 (4). (*iii*) Treatment with immunosuppressive drugs, like cyclosporine, shows clinical efficacy (2). To investigate the repertoire of infiltrating T cells, several laboratories have recently started to analyze the expressed T-cell receptor (TCR) genes in synovial T cells since an oligoclonal repertoire could open up approaches to therapy and prevention (refs. 5–11; for a review on TCR genes, see ref. 12). Expressed TCRs may provide highly selective drug targets, could constitute a starting point for vaccine development, and may also be used to identify relevant autoantigens.

Analysis of animal models of human autoimmune diseases has indeed provided some evidence for TCR oligoclonality. The most striking results have been obtained for experimental allergic encephalomyelitis (EAE) in mice and rats (13). In B10.PL mice, for instance, it has been shown that an oligoclonal receptor repertoire is involved in the induction and maintenance of the disease (14). Antibodies directed against the expressed β-chain variable (Vβ) regions can be used to prevent and treat the disease (15). Furthermore, peptides derived from the corresponding V regions are able to induce resistance against EAE in rats (16, 17).

To analyze the T-cell repertoire in RA, serological (8, 9) and molecular genetic techniques (5–7, 10, 11) have been used in the past. Controversial findings on the heterogeneity of T cells in the synovium have been obtained. The techniques employed so far, however, have severe limitations. Only a few monoclonal antibodies specific for certain human Vβ regions are available. Southern blot analyses are inappropriate to study rearrangements of the TCR α locus due to the large size of the α-chain joining (Jα) chromosomal region.

The polymerase chain reaction (PCR) appears to be the method of choice for repertoire analysis since it allows the determination of TCR sequences even if only small numbers of T cells are available. Anchored PCR (18–21), V-region-specific primers (22–26), and V-region consensus primers can be used to determine T-cell repertoires. These techniques, however, are not optimal since anchored PCR is difficult to set up and V-region-specific or consensus primers might introduce a bias into the results obtained.

Here we show that the inverse PCR, as described for the amplification of unknown flanking sequences in genomic DNA (27), can be used to amplify and clone Vα and Vβ sequences from RNA of synovial fluid and peripheral T lymphocytes of a patient with RA. The technique is easy to set up and does not require information on V-region sequences. Analysis of 67 and 72 productively rearranged Vα and Vβ sequences, respectively, shows that the TCR repertoire in the synovial fluid of this patient is polyclonal. Comparison of synovial fluid and peripheral blood libraries by hybridization with specific oligonucleotide probes indicates, however, that the two most abundant Vβ gene segments are enriched in the inflamed joint.

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Abbreviations: PCR, polymerase chain reaction; TCR, T-cell receptor; RA, rheumatoid arthritis; V, J, and C, variable, joining, and constant regions, respectively; V*n*, J*n*, or C*n*, *n* chain of the V, J, or C, respectively; EAE, experimental allergic encephalomyelitis.

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MATERIALS AND METHODS

Materials. Synovial exudating and peripheral blood mononuclear cells were obtained from a 33-year-old female patient, HLA-DR4 positive, who was first diagnosed as having RA 17 years previously. When cells were taken, the patient had a generalized flare-up of her rheumatoid disease including the knee from which the fluid was aspirated. The fluid was highly cellular in keeping with its inflammatory nature. The patient shows erosive joint disease, is rheumatoid-factor-positive and anti-nuclear-factor-negative, and has no extraarticular disease. Treatment with hydroxychloroquine, D-penicillamine, and methotrexate was not effective; gold treatment resulted in skin rash. Current treatment is with nonsteroidal antiinflammatory drugs.

PCR. Total RNA was isolated from 6 million Ficoll-purified mononuclear cells obtained from synovial fluid or peripheral blood according to Chomczynski and Sacchi (28). cDNA synthesis and PCR were carried out using methods fully described elsewhere (29). Briefly, oligo(dT)-primed double-stranded cDNA was synthesized from 1 μg of total RNA using Moloney murine leukemia virus-derived reverse transcriptase, RNase H, *Escherichia coli* DNA polymerase I, and *E. coli* DNA ligase, followed by incubation with T4 DNA polymerase for blunt-end formation. The blunt-ended cDNA was circularized with T4 DNA ligase in a volume of 10 μl. The ligated material (1 μl) was used as template for the PCR. α and β chain amplifications were separately performed in 50-μl reaction mixtures [67 mM Tris·HCl, pH 8.8/16.6 mM $(NH_4)_2SO_4$/bovine serum albumin (100 ng/μl)/2 mM $MgCl_2$/200 μM dATP/200 μM dCTP/200 μM dGTP/200 μM dTTP/PCR constant region (C) primers either for α or for β chains (each at 300 nM)/*Taq* polymerase (0.05 unit/μl)]. The PCR C primers used are as follows: Cα forward primer (5'-GGGTCGACGACCTCATGTCTAGCACAGT), Cα inverse primer (5'-GCATGCGGCCGCCCTGCTATGCTGTGTGTCT), Cβ forward primer (5'-GGGTCGACCTGTGCACCTCCTTCCCATT), and Cβ inverse primer (5'-GCATGCGGCCGCATGGCCATGGTCAAGAGA). Each forward and inverse primer contains artificial *Sal* I and *Not* I sites, respectively. After 30 cycles of PCR (denaturation at 95°C for 0.5 min, annealing at 62°C for 0.5 min, and extension at 72°C for 1.0 min), the Klenow fragment of *E. coli* DNA polymerase I was added to ensure full-length DNA synthesis.

Cloning of PCR Products. PCR products were purified by phenol extraction, precipitated with ethanol, and digested with restriction endonuclease in excess amounts of *Sal* I and *Not* I. Fragments of expected sizes for the cDNAs were enriched by preparative low-melting-point agarose gel electrophoresis. Recovered DNA fragments were ligated to phagescript vector arms (Stratagene) obtained by *Sal* I and *Not* I digestion. Phages were grown on XL-1 Blue *E. coli* cells (Stratagene). A total of 300,000 to 1 million clones were obtained per μg of RNA. Hybridization with upstream Cα and Cβ probes showed that more than 80% of the clones contained TCR sequences. Single plaques were picked and grown up, and recombinant phage DNA was purified for DNA sequence determination.

Sequencing Reactions. Sequencing reactions were done manually using the Sequenase sequencing system (United States Biochemical) or by automated sequencing (Applied Biosystems) with *Taq* polymerase.

Sequence Analysis. Homology searches were performed with GenBank and EMBL libraries (30) on November 8, 1990. Sequence alignments were performed by using a program (C. Broger, personal communication) that is based on published algorithms (31, 32).

Hybridization of cDNA Libraries Generated by Inverse PCR. Recombinant plaques were transferred from YT plates to GeneScreen*Plus* membranes (New England Nuclear) as described (33). Membranes were hybridized with Cα (5'-TCTCTCAGCTGGTACACGGCAGGGTCAGGG)-, Cβ (5'-CCAGGCACACCAGTGTGGCCTTTTGGGTGT)-, Vα23.1 (5'-TACGTCAGCAACTGGAGACCTGCTCCAGGT)-, Vβ2.1 (5'-TTGCTCGTATGTGGCCTTGGAGCCCTCATT)-, and Vβ3.1 (5'-GGTCTTGTCGATACCAGAACATATTTTCA)-specific oligonucleotides as recommended by New England Nuclear. The frequency of V gene usage was calculated from the ratios of the numbers of plaques positive with V- and C-region probes.

RESULTS

A Heterogenous Repertoire of Vα and Jα Segments Is Found. Out of 78 α clones sequenced, 67 (86%) showed productive rearrangements (sequences are available upon request). On average 200 and 400 base pairs of sequence were determined for each clone using manual and automated procedures, respectively. Cα sequences were determined for 17 clones, to check the reliability of the PCR procedure. Five clones showed a total of six nucleotide substitutions as compared to the published Cα sequence (34). From this we conclude that the mutation rate of the PCR in our procedure is about 1 in 350 base pairs. All of the 10 nonproductive clones showed a frame-shift mutation making it unlikely that they were generated by PCR artifacts.

A total of 31 Vα and 33 Jα gene segments were identified in the pool of 67 productively rearranged clones (Fig. 1). No single Vα or Jα gene segment occurred more than 5 times. Vα23.1 and Jα25 were found 5 times each. If we assume that the germ-line repertoire contains 50 Vα and 50 Jα gene segments (36) and that the individual gene segments are randomly represented in the expressed repertoire, then the probability of finding an individual α gene segment 5 times in a total of 67 clones is less than 1% ($P < 0.01$). On the other hand, we know that the peripheral repertoire in the mouse is not random but skewed by positive and negative selection in the thymus and perhaps by preferential rearrangements (40, 41). It is likely that the peripheral repertoire in humans is shaped by similar forces.

It is of interest to note that one of the clones contained a functionally rearranged Vδ1 gene segment. The shared usage of V gene segments between TCR α and δ chains has been observed (42).

A Heterogenous Repertoire of Vβ and Jβ Segments Is Found. Out of 76 β clones analyzed, 72 (95%) contained productive rearrangements (sequences are available upon request). The pool of 72 productively rearranged sequences represents at least 29 Vβ gene segments. Only 12 of the 13 Jβ gene segments of the human β locus were found in the clones analyzed. The Vβ3.1 and Vβ2.1 gene segments were identified 11 and 7 times, respectively, and the Jβ2.5 gene segment was identified 9 times (Fig. 2). The overrepresentations of the Vβ2.1 and Vβ3.1 gene segments are statistically significant ($P << 0.01$) if one assumes that the peripheral repertoire contains a completely random representation of the 70 Vβ gene segments that have been estimated to exist (36). As discussed above, however, this is unlikely to be true. In this regard it is important to note that an overrepresentation of the Vβ2, -3, -6, -8, and -12 gene families, evident from our analysis (Fig. 2), has also been observed by others (22, 36) in peripheral blood lymphocytes.

Accumulation of Vβ2.1 and Vβ3.1 Sequences in the Synovial Joint. To find out whether the most abundant Vα and Vβ sequences are enriched in the synovial fluid of this patient as compared to the peripheral blood, we hybridized cDNA libraries generated by the inverse PCR with specific oligonucleotide probes and counted positive plaques. As shown in Table 1, about 4% of the α clones generated from synovial fluid or peripheral blood mononuclear cells hybridized to a

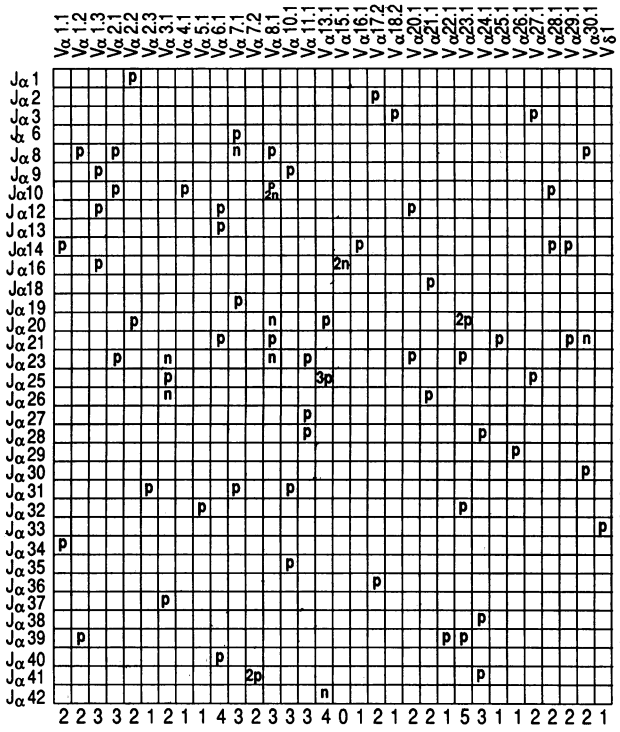

FIG. 1. Combinatorial joins in the α repertoire analyzed. Vα and Jα nomenclature is as described for Vα1 to Vα19 (35); Vα20.1 to Vα24.1 correspond to TCRAI1, HAVT15, HAVT20, HAVT31, and HAVT32, respectively (36, 37); Vα27.1, Vα29.1, and Vα30.1 correspond to Vαw25, Vαw23, and Vαw28 (38); Vα17.2, Vα18.2, Vα25.1, Vα26.1, and Vα28.1 have not been described, to our knowledge; Jα1 to Jα24 correspond to JαA to JαX (36); Jα25 to Jα32 correspond to AA13, AB11, AB22, AC17, AC24, AC9, AD17, and AP511, respectively (35). AP511 has been described to contain an in-frame stop codon but, in our analysis, there is a point mutation that has changed this segment into a functional one. Jα33 to Jα36, Jα38, Jα41, and Jα42 correspond to IGRJa08, IGRJa12, IGRJa03, IGRJa05, IGRJa01G, IGRJa14, and IGRJa04 (38); Jα37, Jα39, and Jα40 have not been described, to our knowledge. The Vδ1 gene segment has been described (39). P and N indicate productive and nonproductive joinings, respectively. Numbers at the bottom and on the right side of the grid are the frequencies with which individual Vα and Jα gene segments were found in the productive clones.

Vα23.1-specific oligonucleotide, indicating that there is no enrichment of this particular Vα gene segment in the inflamed joint of this patient. In contrast, both the Vβ2.1 and Vβ3.1 gene segments were found to be expressed at increased

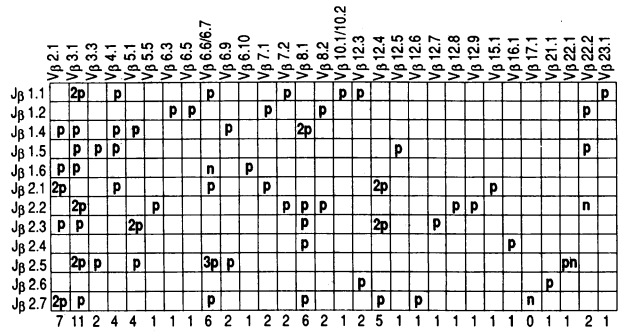

FIG. 2. Combinatorial joins in the β repertoire analyzed. Vβ and Jβ nomenclature is according to Kimura *et al.* (36) except that Vβ12.5, -5.5, -22.1, and -22.2 correspond to 17A2 (23), IGRb07, Vβw21, and Vβw22 (43), respectively. Vβ6.10, -12.6, -12.7, -12.8, -12.9, -21.1, and -23.1 have not been described, to our knowledge. Productive (P) and nonproductive (N) rearrangements and frequencies of individual β gene segments are displayed as in Fig. 1.

Table 1. Frequencies of the most abundant Vα and Vβ gene segments

Source	Vα23.1/Cα	Vβ2.1/Cβ	Vβ3.1/Cβ
Synovial fluid	11/260 (4.2%)	112/850 (13.2%)	121/850 (14.2%)
Peripheral blood	5/121 (4.1%)	33/800 (4.1%)	55/800 (6.9%)

cDNA libraries generated by inverse PCR with total RNA from peripheral blood or synovial fluid mononuclear cells were plated and hybridized with oligonucleotides specific for Cα, Vα23.1, Cβ, Vβ2.1, and Vβ3.1. Numbers of plaques positive for a particular oligonucleotide probe are shown and frequencies of V gene segments expressed are given in percentages.

frequencies in the synovial fluid. A 2-fold increase was identified for Vβ3.1 and a 3-fold increase was identified for Vβ2.1. The frequencies determined for the Vα and Vβ gene segments by hybridization of inverse PCR libraries correlate reasonably well with those determined by sequencing.

Additional Vα, Vβ, and Jα Gene Segments Identified. The repertoire analysis of this patient allowed us to identify five Vα, seven Vβ, and three Jα gene segments that have not been characterized, to our knowledge. Three of the Vα and two of the Vβ gene segments appear to represent distinct families since they are less than 75% identical to members of known V gene families. This assignment, however, remains tentative until these gene segments have been completely sequenced. The other V genes isolated are additional members of defined families. The partial nucleotide and amino acid sequences of the five Vα, seven Vβ, and three Jα gene segments are summarized in Fig. 3.

Combinatorial Joining of V and J Gene Segments. We have analyzed the combinations of V and J gene segments in the α and β repertoires as shown in Figs. 1 and 2. It appears that no Vα/Jα or Vβ/Jβ combination is strikingly overrepresented. In the productively rearranged clones, however, we have picked up the Vα13.1/Jα25 combination three times, the Vα7.2/Jα41 combination twice, and Vα23.1/Jα20 combination twice. The two sequences in each doublet are identical to one another and one of the sequences in the triplet shows a substitution in the N region. A larger number of α chains from the synovial fluid of this patient has to be analyzed to check the relevance of this observation. It is important to note that nonproductive rearrangements, identical to one another with regard to the Vα/Jα join, were also picked up. Furthermore, in the mouse a particular Vα/Jα combination has been found in the periphery at frequencies well above what would be expected based on statistical calculations (44).

One Vβ/Jβ triplet and nine Vβ/Jβ doublets were also identified among the productively rearranged clones (Fig. 2). In contrast to the α chains, all of the Vβ/Jβ pairs show extensive diversity in the junctional regions with the exception of the Vβ2.1/Jβ2.1 pair. This is clearly different from what has been found in EAE where all chains showing the same Vβ/Jβ combination are also highly conserved in the junctional region (14, 45).

DISCUSSION

Inverse PCR for the Analysis of the 5′ and 3′ Ends of cDNA. The inverse PCR was originally developed for the analysis of unknown flanking sequences in genomic DNA (27). Here we have shown that it can also be used for the analysis of unknown Vα and Vβ regions in TCR cDNAs. Likewise, it should be possible to use the technology to study TCR Vγ and Vδ regions and, by extension, any unknown 5′ and 3′ sequences of cDNAs.

We believe that the technology we have developed is more powerful and easier to use than any of the tailing or linker

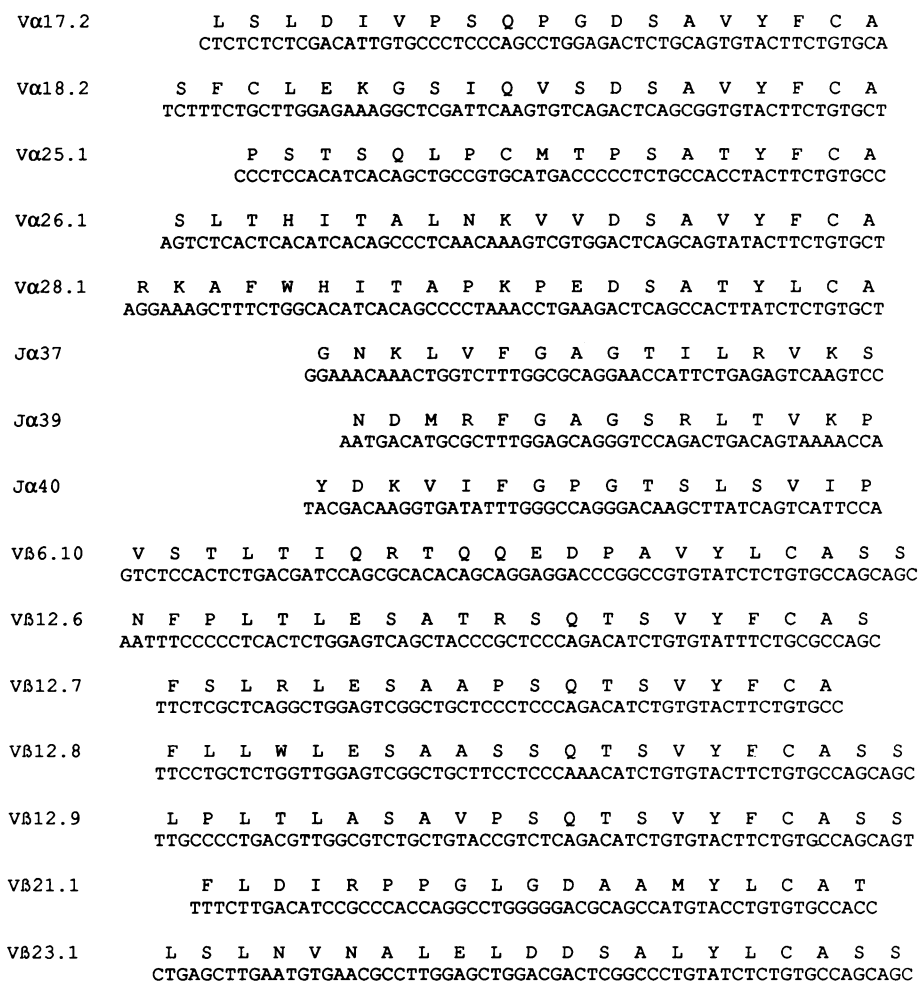

FIG. 3. Nucleotide and amino acid sequences of Vα, Jα, and Vβ gene segments that to our knowledge have not been described. Only the C-terminal sequences of the V gene segments were determined. Vα17.2 and -18.2, Vβ6.10, -12.6, -12.7, -12.8, and -12.9 are additional members of gene families previously described. The other V gene segments constitute distinct families. For nomenclature see legends to Figs. 1 and 2.

techniques that have been described for the analysis of unknown 5′ and 3′ cDNA sequences (18–21). The inverse PCR, therefore, is a more potent tool for the study of TCR repertoires in human diseases such as multiple sclerosis (24, 25), Graves disease (46, 47), primary biliary cirrhosis (48), allograft rejection (23, 49), sarcoidosis (50), malignant disease (26), Crohn disease (51), and RA (5–11). This technology can also be applied to the study of animal models of autoimmune diseases such as EAE, collagen-induced arthritis, etc.

The TCR Repertoire Expressed in the Synovial Fluid of the Patient Studied Is Polyclonal. Using the technology developed, we have shown that the repertoire of productively rearranged TCR α and β genes in the synovial fluid of this patient is highly heterogenous. Thirty-one Vα, 33 Jα, 29 Vβ, and 12 Jβ gene segments were found to be present in the 67 and 72 productively rearranged α and β genes, respectively. To analyze whether the most abundant Vα and Vβ gene segments identified in the productively rearranged genes were enriched in the synovial fluid as compared to the peripheral blood repertoire, we hybridized cDNA libraries generated by the inverse PCR with specific oligonucleotides. No increase of Vα23.1 sequences was found but Vβ2.1 and Vβ3.1 sequences were enriched in the synovial fluid repertoire by a factor of 3 and 2, respectively. As it is evident from the data shown in Fig. 2, these Vβ gene segments are joined to a large number of different Jβ gene segments. Furthermore, since particular Vα gene segments were not found at similar frequencies, it appears that both Vβ2.1 and Vβ3.1 sequences are paired with at least a small number of different Vα chains. We therefore conclude that the repertoire of synovial T cells in this patient is polyclonal.

Why do we find an enrichment of Vβ2.1 and Vβ3.1 sequences in the synovial fluid repertoire? It is interesting to note that T cells expressing these two Vβ sequences can be stimulated by certain bacterial toxins, such as toxic shock syndrome toxin and staphylococcal enterotoxin B of *Staphylococcus aureus*, respectively (22, 52). Perhaps previous bacterial infections in this patient led to stimulation and expansion of T cells expressing these two Vβ gene segments. Some of these T cells might have expressed TCRs recognizing self antigens and, after superantigen stimulation and expansion, they might have initiated the autoimmune disease (52).

Future Analyses of T-Cell Repertoires in RA. It is important to analyze more patients with RA to find out whether our finding of increased frequencies of particular Vβ gene segments in the inflamed joint is of general significance. It could well be that other patients with RA show different Vα or Vβ sequences with increased frequencies, depending on their HLA type and histories of previous infections. It is also important to analyze earlier stages of the disease. We have analyzed a patient with stage III disease. It is possible that in an advanced state of the disease, after extensive tissue destruction, multiple epitopes on self-antigens are being recognized by T cells. This could account for a highly heterogenous T-cell repertoire. In earlier stages of the disease, the pathogenic T cells might be enriched to an even higher degree. A further enrichment of these cells might be

found in synovial membranes as compared to synovial fluid and peripheral blood.

Our technology is sensitive enough to work with less than 1×10^6 mononuclear cells and, therefore, should allow the analysis of subpopulations of T cells from individual lesions without any *in vitro* T-cell expansion. It should be possible, for instance, to analyze activated cells by sorting for high-level p55 interleukin 2 receptor expression or to analyze $CD4^+$ and $CD8^+$ T cells separately.

Note. We have now analyzed an additional 10 RA patients with an anti-Vβ2 monoclonal antibody and found that in all patients Vβ2-positive T cells are highly enriched in the synovium as compared to the peripheral blood (D. Vidovic, Y. Uematsu, M. Follo, M. Muentz, H. van den Boom, W. Siebert, M. Steinmetz, and A.-G. Schmidt, unpublished data).

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