

Dominant T-cell-receptor β chain variable region $V\beta 14^+$ clones in juvenile rheumatoid arthritis

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ABSTRACT The characteristic histopathology and major histocompatibility complex associations in juvenile rheumatoid arthritis suggest an oligoclonal antigen-specific T-cell population may be critical to pathogenesis. To test this, we analyzed the T-cell repertoire of a polyarticular HLA-DR4⁺ juvenile rheumatoid arthritis patient with an aggressive form of disease that required arthrocentesis of the knee joints and early replacement of both hip joints. A comparison of T-cell-receptor β chain variable region ($V\beta$) gene expression in peripheral blood and synovial fluid performed by semiquantitation of cDNA samples amplified by the PCR revealed overexpression of the T-cell-receptor $V\beta 14$ gene family. To determine the nature of $V\beta 14$ overexpression, we sequenced randomly cloned amplification products derived from two synovial fluid, two synovial tissue, and three peripheral blood samples by using a $V\beta 14/\beta$ chain constant region primer pair. Sequence data showed that the T-cell response in the synovia was oligoclonal. Of four clones found, one was present in all joints examined and persisted over time. This clone accounted for 67% and 74% of all $V\beta 14^+$ clones sequenced in two synovial fluid samples and 75% and 40% in two synovial tissue samples. This clone was also found at a lesser frequency in peripheral blood samples. Further studies provided evidence for the presence of oligoclonally expanded populations of T cells utilizing the $V\beta 14$ T-cell receptor in 6 of 27 patients examined. In contrast to the remaining patients studied, 3 with a late onset polyarticular course who exhibited especially marked clonality were characterized by features typical of adult rheumatoid arthritis (IgM rheumatoid factor-positive and HLA-DR4⁺). These data suggest a role for $V\beta 14^+$ T cells in a group of juvenile rheumatoid arthritis patients.

Juvenile rheumatoid arthritis (JRA) is the most common chronic rheumatic disorder in children with a wide diversity of clinical symptoms found both at onset and during the course of the disease. JRA is classified at disease onset into three major types: pauciarticular (four or fewer joints), polyarticular (five or more joints), and systemic (1). The immunopathology of JRA, like that of other autoimmune diseases, is believed to be an antigen-driven process (2). It has been suggested that recognition of arthritogenic antigen presented in the context of a limited range of major histocompatibility complex molecules by T-cell receptors (TCRs) of disease-promoting T lymphocytes results in the triggering of a pathogenic immune response. The strong association between JRA and certain major histocompatibility complex class II specificities (3–5) and the histopathology of the diseased joint (6–8) support the concept of a role for T cells in the disease.

The nature of the disease-related peptide(s) still remains to be determined. The hypothesis involving a prolonged im-

mune response against autoantigens located in inflamed joints implies a selective or clonal expansion of T cells. Several studies employing restriction fragment length polymorphism analysis of TCR β chain gene rearrangements or the use of semiquantitative PCR and subsequent sequencing have yielded some evidence of oligoclonal expansion of $\alpha\beta$ TCR-bearing cells in the synovial fluid of adult rheumatoid arthritis (RA) patients (9–11). As a result, a limited number of β chain variable region ($V\beta$) genes have been implicated in the pathology of RA. Marked overexpression and clonal expansion of $V\beta 14^+$ T cells have been demonstrated (11). Analysis of $V\beta$ gene expression by interleukin 2 receptor-positive synovial T cells revealed $V\beta 3$, -14, and -17 gene family involvement in RA pathology (10). Oligoclonal expansion of $V\beta 20^+$ T cells in two of six JRA patients was shown through analysis of synovial fluid from interleukin 2 receptor-positive T cells (12). In contrast, several other studies have failed to demonstrate the presence of a dominant clone in the synovial compartment of RA patients (13–15), supporting an alternative concept that activated T cells may be recruited from peripheral blood through nonspecific cytokine-induced inflammatory mechanisms (16).

Initially, we analyzed a JRA patient subtyped as polyarticular, IgM rheumatoid factor-positive (RF⁺), and possessing an HLA-DR4⁺ haplotype. Multiple synovial tissue and fluid samples were available providing a unusual opportunity to analyze the T-cell repertoire in the synovium at various stages of disease. The use of semiquantitative PCR revealed that the $V\beta 14$ transcript was significantly more abundant in synovial fluid than in peripheral blood. Sequence data showed that expansion of a dominant $V\beta 14$ clone was found not only in synovial fluid but also in the synovial tissue. These findings were consistent in all joints examined and persisted over the duration of this study, suggesting a role for the dominant clone in this patient's disease. In addition, we provide evidence that oligoclonal $V\beta 14^+$ T-cell expansion may be characteristic of a proportion of JRA patients.

MATERIALS AND METHODS

Patients. Paired synovial fluid and peripheral blood samples were collected during clinic visits from 27 patients with a diagnosis of JRA (17). At the time of onset, 12 patients had polyarticular disease, 10 had pauciarticular disease, and 5 had systemic disease, with 7 of the pauciarticular or systemic patients progressing to a polyarticular course. Twenty-five of 27 patients have been HLA-typed and 4 of 18 were IgM RF⁺.

Abbreviations: RA, rheumatoid arthritis; JRA, juvenile RA; TCR, T-cell receptor; V, D, J, and C, variable, diversity, joining, and constant regions, respectively; $V\beta$, C α , etc., β chain variable region, α chain constant region, etc.; RF, rheumatoid factor.

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Of particular interest was A.H., an African-American male who developed seropositive polyarticular JRA at age 15.5 years. The patient had rapidly progressive disease that resulted in the destruction of both hips, leading to a left total hip arthroplasty and 8 months later a right total hip arthroplasty.

PCR. Synovial cells from therapeutic arthrocenteses, peripheral blood lymphocytes, and synovial tissue residual from joint replacement surgery served as sources of RNA (5×10^6 cells, when available). Total RNA was prepared (18) and the first-strand cDNA was synthesized as described (19).

The cDNAs were subjected to enzymatic gene amplification using specific TCR primers as described (20, 21). The PCR was performed in 50- μ l reaction mixtures containing roughly equivalent amounts of cDNA, 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.02% gelatin, 5 pmol of each primer, all four dNTPs (each at 0.2 mM), and 2 units of *Taq* polymerase (Perkin-Elmer/Cetus) and subjected to 25–30 cycles of amplification (95°C, 1 min; 56°C, 1 min; 72°C, 1 min). These reaction conditions allow for analysis of PCR products in the linear range of amplification as demonstrated by a dose–response relationship between initial amounts of cDNA and quantity of the final products. Each specific V β primer was used in conjunction with a common β chain constant region (C β) primer (5'-ACACCAGTGTGGCCTTT-TGGGTG). As an internal control, coamplification of the α chain constant region (C α) gene segment was included using the primer pair (5'-ATCATAAATTCGGGTAGGATCC and 5'-GAACCTGACCCTGCCGTGTACC).

Quantification was accomplished by Southern blot transfer of the PCR products and hybridization using a mixture of three ³²P-labeled oligonucleotides specific for C β 1, C β 2, and C α (5'-CTCGGGTGGGAACACGTTTTC, 5'-CTCGGG-TGGGAACACCTTGTTTC, and 5'-TTAGAGTCTCTCAG-CTGGTA, respectively). Washing of the blots was followed by analysis of bound radioactivity by autoradiography or PhosphorImager analysis (Molecular Dynamics). The level of expression of each V β gene family was analyzed relative to levels of total C α expression to normalize results and allow for comparisons between samples.

Cloning and Sequencing of PCR Products. Asymmetrical sticky-end cloning was facilitated by the use of a V β 14 family-specific primer (5'-GGGGTCGACGTCTCTC-GAAAAGAGAAGAGGAAT) containing a *Sal* I restriction site and the use of a C β primer (5'-GCTCTACCCAG-GCCTCGGC) that allowed for amplification of a *Bgl* II restriction site within C β . First-round PCR was done as described above and 10 μ l of this product was used as template for 30 additional cycles of amplification.

After the removal of the PCR primers, the PCR products were digested with *Sal* I and *Bgl* II. The appropriate frag-

ments were purified from agarose gels using GeneClean II (Bio 101) and used for cloning into *Sal* I/*Bam*HI-digested M13mp18. The number of clones sequenced (Sequenase; United States Biochemical) provided a sample size large enough to ensure a >95% probability that at least two identical sequences were identified when the expanded population contains 20% of a given V β family.

In instances in which overexpression was not seen by Southern blot analysis, a simplified analysis of the population of PCR products was done (22). This approach was based on the fact that rearranged β genes vary at their recombination junctions in the number of nucleotides incorporated, and therefore their amplification products will show length heterogeneity. Thus PCR products, synthesized using the ³²P-end-labeled C β primer, were analyzed directly in 6% denaturing polyacrylamide gels for the presence of a dominant band indicative of clonal expansion.

RESULTS

V β 14 Is Overexpressed in Diseased Joints. These studies were initiated to investigate the TCR repertoire in the diseased joints and peripheral blood of a JRA patient (A.H.) who had a very aggressive form of the disease. Semiquantitative PCR was used to provide a measure of the level of expression of each V β gene family using a set of 20 V β family-specific primers (20, 21). The autoradiographs shown in Fig. 1 illustrate the level of expression of the V β 2, -6, -12, and -14 families in the synovial fluid relative to the level in the peripheral blood using C α as a control. When the V β /C α ratio in the synovial fluid was at least twice that in peripheral blood, the family was designated as "overexpressed." The level of V β 14 overexpression was \approx 6-fold higher in the synovial fluid than in the peripheral blood (Fig. 1), whereas the other three families illustrated had similar profiles in both the blood and synovial fluid, as did the additional 16 V β families also analyzed (data not shown). Therefore, only a single V β gene family was overexpressed in this particular patient, suggesting the presence of disease-related T cells.

V β 14 Overexpression Is Due to the Presence of a Dominant Clone. To determine the nature of the V β 14 overexpression, we sequenced random-cloned PCR products that were derived from synovial fluid, synovial tissue, or peripheral blood of A.H. by using the TCR V β 14/C β primer pair. The fragment sequenced included the 3' end of V β 14, the ND β N region (where D is diversity region), the complete β chain joining region (J β), and the 5' end of C β . The predicted amino acid sequences from the 3' end of V through the 5' end of J are shown in Fig. 2 since repeated ND β N-J β motifs are indicative of clonal expansion. These results include two

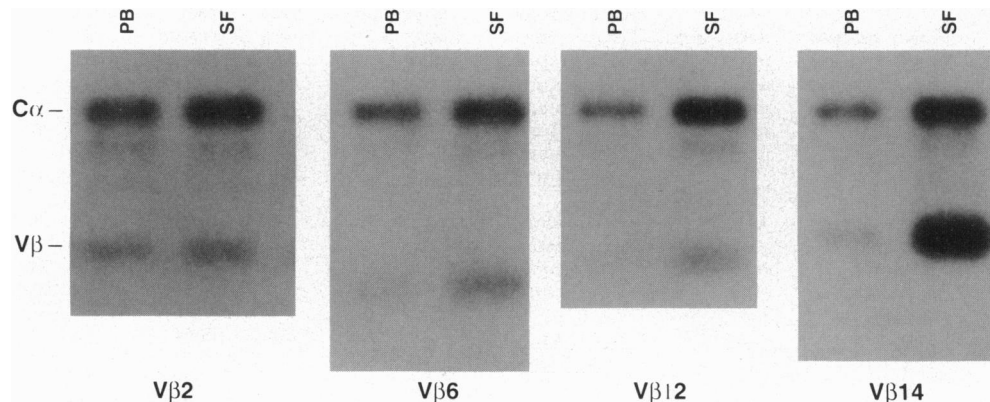


FIG. 1. Southern blot analysis of PCR amplification products derived from TCR transcripts in lymphocytes isolated from the peripheral blood (PB) and synovial fluid (SF). The autoradiograph shows coamplification of the C α gene and various V β families detected by ³²P-labeled C α and C β probes.

amplification, reflecting the reported infidelity of the enzyme (23).

When a peripheral blood sample (PB-1) was examined, we found the same sequence, although to a lesser degree (5 of 21 sequences, Fig. 2B). Two additional expanded clones (CASS-LSSGGH-NEQFF and CASS-PTGTGRS-EQFF) were found exclusively in the peripheral blood.

Dominant Clones Persist Over Time. When samples taken later in the course of disease (SF-2 and PB-2) were examined for clonal expansion, striking similarities were found with SF-1 and PB-1, described above. As seen in Fig. 2A, the same dominant clone was present in the synovial fluid (14 of 20 clones, 70%). The pattern in PB-2 (Fig. 2B) was also similar to that previously described—i.e., all 3 expanded clones were present in roughly the same ratios. Additionally, a third blood sample (PB-3) showed the presence of CASS-LSSGGH-NEQ and CASS-PTGTGRS-TDTQY to the same extent as previously observed, whereas the CAS-GSPDAGW-TDTQY clone was found in only 1 of 21 clones. These results indicate that the expanded T-cell clones persisted over nearly a 2-year period in A.H., although the dominant clone appeared to be declining in parallel with clinical improvement.

Synovial Tissue Lymphocytes Include the Dominant Clone. Sequence data from synovial tissue samples (ST-1 and ST-2, Fig. 2C) revealed the presence of the dominant clone that accounted for 81% of the clones in ST-1 and 37% in ST-2. At the same time a new clone was identified in the synovial tissue. It is worthy to note that a single clone with exactly the same sequence was seen in the SF-2 sample (Fig. 2A). No sequences were found in common with peripheral blood except the dominant GSPDAGW clone, which rules out the possibility that peripheral blood contamination of the tissue was responsible for the results obtained.

TCR V β 14⁺ T-Cell Oligoclonality Is Characteristic of a Proportion of JRA Patients. To assess the frequency of V β 14⁺ lymphocyte expansion in the JRA population, we quantitated V β 14 expression in 26 additional patients. When semiquantitative PCR was used to measure the level of V β 14 transcripts in synovial fluid compared to peripheral blood in these patients, we found that 4 of 27 (including A.H.) had 2-fold or higher levels of V β 14 (Table 1). In addition, two other patients (S.G. and J.F.) with 1.4- and 1.3-fold overexpression were included in subsequent sequence analyses. The remaining 20 samples had approximately equivalent levels of V β 14

expression in the synovial fluid and blood and are not shown. To determine whether the V β 14 overexpression was due to clonal expansion as was shown for A.H., the PCR products from these five additional patients were cloned and sequenced. Sequences that occurred more than one time are shown in Table 1. We found a strikingly high level of expansion in patient A.M. (43% of sequences were identical), who, like A.H., has an aggressive form of the disease. Of the remaining patients, J.F., S.G., and J.G. exhibited clonal expansion in the synovial fluid to a lesser extent, while all TCR sequences for C.D. were unique. Multiple expanded clones were seen for patients A.M., J.G., and J.F. In instances in which expansion was observed in the synovial fluid, the corresponding blood sample was also analyzed. All sequences found in all blood samples analyzed were unique and distinct from those found in the synovial fluids (except A.H., as described above), emphasizing the importance of expanded clones in diseased joints.

Synovial tissue was also available for patient A.M.; although unlike A.H., this sample was obtained many years after the destruction of the hip joint. In this case, the expanded clones identified in the synovial fluid drawn from the knee were not present in the hip tissue.

In instances in which overexpression was not seen, a simplified analysis of the population of PCR products was done. The approach was based on the fact that rearranged TCR β genes vary at their recombination junctions in the number of nucleotides incorporated as well as in J β sequences, and therefore, their amplification products will show length heterogeneity. Productively rearranged genes will differ from each other by intervals of 3 nucleotides. When PCR products (synthesized using ³²P-end-labeled C β primer) were resolved by denaturing polyacrylamide gel electrophoresis multiple copies of the same sequence resulted in a dominant band. Using this approach, we found one additional instance of clonal expansion (N.M.). Cloning and sequencing of the PCR product revealed 11 of 33 sequences were identical (Table 1). Lower levels of expansion may be present in other patients that were not detectable by this methodology.

Thus, 6 of the 27 patients tested demonstrated V β 14 clonal expansion. All 6 of these patients had a polyarticular course of disease, 3 were HLA-DR4⁺, and three were IgM RF⁺ (Table 1). These findings are in contrast to the remainder of the patient population; only 2 of 19 patients HLA-typed were DR4⁺ and 1 of 12 patients tested was IgM RF⁺. These patterns suggest that the 6 patients exhibiting V β 14 oligoclonality have some features typical of adult RA (polyarticular course, DR4⁺, and IgM RF⁺).

DISCUSSION

Current hypotheses of the cause of chronic inflammatory arthropathies including JRA propose that synovial inflammation results from selective or clonal expansion of T cells specific for an unidentified autoantigen located in joints. This "T-cell-centric" hypothesis has been recently challenged (24) because a number of studies have failed to demonstrate evidence for T-cell oligoclonality (15, 16). In this study we provide strong evidence for synovial clonal expansion. This evidence comes from the sequencing of TCRs obtained from the site of disease from a group of JRA patients. In our study, semiquantitative PCR provided for a focused sequencing effort. Correlations have been made between semiquantitative PCR and expression using available V β family-specific monoclonal antibodies (20, 21, 25).

Initially our efforts focused on one patient (A.H.) with aggressive disease and demonstrated overexpression of a single TCR gene family (V β 14) in the synovial compartment. Specifically, the sequence data showed that the T cells found

Table 1. V β 14 gene expression and clonal expansion in JRA synovial fluids

Patient	SF/PB ratio	Sequences found more than once	Frequency	HLA-DR4	IgM RF
A.M.	7.1:1	CASSLMAEYNSPL (J β 1.6)	9/21	-	+
		CASSAQKGASSYNSP (J β 1.6)	2/21		
A.H.	6.6:1	CASGSPDAGWDTQY (J β 2.3)	16/24	+	+
J.G.	6.5:1	CASSLNNSYNEQ (J β 2.1)	3/29	+	-
		CASSQLGAYYNEFF (J β 2.1)	2/29		
		CASSLFASGANV (J β 2.6)	2/29		
		CASSLTGGNTEAF (J β 1.1)	2/29		
C.D.	3.9:1		0/23	-	-
S.G.	1.4:1	CASSLQGANYEQYF (J β 2.7)	2/19	-	-
J.F.	1.3:1	CASSLTQTTEAFF (J β 1.1)	6/52	-	-
		CASSYAGFGQPQHF (J β 1.5)	8/52		
		CASSFGQGTDTQY (J β 2.3)	2/52		
		CASSPLNGYEYQYF (J β 2.7)	2/52		
		CASSRGLNSNQPF (J β 1.5)	11/33	+	+
N.M.		CASSKFIQGPYEQYF (J β 2.7)	3/33		

Expression of V β 14 is given as a ratio between peripheral blood (PB) and synovial fluid (SF), which were measured relative to levels of C α . Partial amino acid sequence was translated from the 3' end of V β 14, the N+D region, and the 5' end of the J region. Patient N.M. was also included in sequence analysis (see Results).

in peripheral blood and in diseased synovia were oligoclonal. Of the four independent clones found, one was present at high frequency in all joints examined and persisted over time, however in decreasing proportions. In addition, this clone was also found at much reduced levels in the peripheral blood. This report is significant in that it demonstrates the persistence of a T-cell clone throughout the course of disease in both the synovial lining and the synovial fluid of four joints, suggesting its involvement in the pathogenic process.

Next the prevalence of V β 14⁺ T-cell clonal expansion was investigated in a panel of JRA patients. V β 14 oligoclonality was demonstrated in synovial fluids of 6 of 27 patients. Several of these patients with V β 14 oligoclonality including the 3 patients with marked clonal expansion (A.M., A.H., and N.M.) exhibited features typical of adult RA. These results combined with the reports of V β 14 oligoclonality in adult RA (10, 11) are suggestive of a role for V β 14 in this form of disease.

Expansion could result from proliferation of T cells in the synovial compartment or from specific recruitment of previously expanded peripheral blood T lymphocytes by the synovium (26). In this study, the dominant clone of the synovial compartment was present in the peripheral blood for one patient (A.H.). We also found two expanded clones that were present only in the periphery. This fact suggests a mechanism of selective recruitment of peripheral T cells capable of recognizing a synovial autoantigen and eventually associated with joint destruction. The appearance of a second expanded clone in the synovial tissue of A.H. (CASS-IQGANYGTY) may reflect the recognition of newly exposed epitopes as a consequence of the joint destruction as the disease progresses. With the exception of A.H., clonally expanded V β 14 T lymphocytes were identified only in the synovial fluids of patients analyzed in this study. These samples were generally taken at a later stage of disease than in A.H., which could explain these differences. The stage of disease could also explain the dominant clone's absence in the tissue sample in A.M., since this tissue was obtained many years after the joint had been destroyed.

As more patients with all types of JRA are studied for other V β families, common sequence motifs shared by the majority of expanded clones may be identified. It may also be anticipated that other V β families are likely to be involved, given the marked differences in HLA associations between JRA types.

The fact that clonally expanded T lymphocytes utilizing V β 14⁺ gene products were found in a significant proportion (>20%) of JRA patients raises a question about the possibility of therapeutic intervention. The limited heterogeneity of V β usage in experimental allergic encephalomyelitis (27), an animal model of the cell-mediated autoimmunity, has allowed successful treatment of the disease utilizing immunotherapeutic approaches directed against critical V β gene products (27, 28).

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