Multiple Mechanisms Support Oligoclonal T Cell Expansion in Rheumatoid Synovitis

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ABSTRACT

Background: The synovial T cell infiltrate in rheumatoid arthritis (RA) is diverse but contains clonally expanded $CD4^+$ populations. Recent reports have emphasized that RA patients have a tendency to develop $CD4^+$ T cell oligoclonality which also manifests in the peripheral blood. Clonal dominance in the tissue may thus result from antigen specific stimulation in the synovial membrane or may reflect the infiltration of expanded clonotypes present throughout the lymphoid system. We have explored to what extent clonal populations amongst tissue $CD4^+$ T cells display joint specificity as defined by their restriction to the joint, their persistence over time, and their expression of markers indicative for local activation.

Materials and Methods: Matched samples of peripheral blood and synovial fluid or synovial tissue were collected from 14 patients with active RA and $CD4^+$ IL-$2R^+$ and $CD4^+$ IL-$2R^-$ T cells from both compartments were purified. Clonal populations of $CD4^+$ T cells were detected by RT-PCR amplification of T cell receptor (TCR) transcripts with BV and BJ specific primers followed by size fractionation and direct sequencing of dominant size classes of TCR transcripts.

Results: Clonal $CD4^+$ T cells were detected in the synovial fluid and synovial tissue of all patients. All patients carried synovial clonotypes that were undetectable in the blood but were present in independent joints or at several non-adjacent areas of the same joint. These joint restricted $CD4^+$ clonotypes were generally small in size, were preferentially found in the IL-$2R^+$ subpopulation, and persisted over time. A second type of clonogenic T cells in the synovial infiltrate had an unrestricted tissue distribution and was present at similar frequencies amongst activated and nonactivated T cells in the blood and affected joints. Ubiquitous clonotypes isolated from two different patients expressed sequence homologies of the TCR β chain.

Conclusions: Two types of expanded $CD4^+$ clonotypes contribute to the T cell infiltrate in rheumatoid synovitis. Differences in the distribution pattern and in molecular features suggest that distinct mechanisms are supporting the clonal outgrowth of these two groups of clonotypes. Clonally expanded T cells restricted to the joint but present in several independent joints appear to respond to locally residing antigens. Clonogenic cells with an unrestricted distribution pattern and widespread activation in the blood and tissue may react to a different class of antigens which appear to be shared by multiple patients. T cell recognition in RA may be involved at several different levels and may be related to more than one pathomechanism.

INTRODUCTION

Rheumatoid arthritis (RA) is characterized by the accumulation of inflammatory cells in the synovial membrane resulting in irreversible damage of the joint architecture. The finding of a genetic association with MHC class II polymorphism has led to the model that the underlying pathologic event is the recognition of antigen by lesional T cells (1–6). Indirect evidence for a critical role of T cells has been provided by studies on the T cell receptor (TCR) gene segment usage in affected tissues (7,8). Originally, these studies were designed to identify a shared T cell response in the

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rheumatoid joint. Although such a common denominator in the synovial T cell response has not been reported, most investigators have been able to describe the presence of oligoclonal T cell populations in the synovial infiltrate (9–16). In general, the diversity of tissue infiltrating T cells was extensive (17,18). Individual proliferating T cell clones were small and did not lead to a major shift in the TCR BV gene segment repertoire (7,15). Each individual patient expressed a unique set of T cells proliferating in the synovium.

The finding of clonal T cell populations in the synovial inflammation is suggestive for the recognition of locally residing antigens. Specificity for a joint-specific antigen of the synovial T cell clonotypes would be supported by their local enrichment. Their limited size could be explained by the co-occurrence of antigen induced proliferation and antigen induced programmed cell death. The co-existing diversity of the global infiltrate may simply reflect that antigen specific T cells remain a minority in the lesions and only very few antigen specific T cells may be necessary to maintain the immune response. However, this model has been questioned because in situ T cell proliferation has not been observed and T cell activation products are infrequent in the synovium.

Alternative explanations for oligoclonal T cell populations in the synovial compartment include the selective enrichment of certain T cells based upon their ability to transmigrate into the tissue (19–21). Enhanced capability for tissue migration has been associated with a memory phenotype of T cells, and indeed tissue infiltrating T cells in rheumatoid synovitis are almost exclusively composed of memory T cells. Thus, T cell clonotypes detected in the joint could simply reflect the advantage of preactivated T cells to home to the tissue. Oligoclonal T cell proliferation in the synovial tissue could also result from the release of tissue specific growth factors facilitating T cell growth. It has been proposed that resident cells in the joint can release IL-15, which then supports the proliferation and clonal expansion of IL-15 receptor (IL-15R) expressing T cells (22).

Recent reports on the oligoclonality of circulating T cells in RA patients have added an additional level of complexity to the interpretation of dominant T cell populations in the synovial tissue. It is now accepted that $CD8^+$ T cells are not as diverse as previously thought and regularly include expanded clonotypes (23–26). Clonal expansion of $CD4^+$ T cells appears to be infrequent in normal individuals but is frequently encountered in RA patients (27,28).

Clonogenic $CD4^+$ T cells in RA patients express an unusual phenotype: they lack expression of the CD28 molecule (29,30). The recirculation patterns of such $CD4^+$ $CD28^-$ T cell clones are unclear. Particularly, it is unknown whether they preferentially home to sites of inflammation.

The ultimate goal of repertoire studies is to identify T cells which react to disease inducing antigens. Such T cells could be used to screen for antigens and thus represent unique tools. Attempts to utilize T cell clonotypes isolated from the tissue to detect arthritogenic antigens will depend upon the ability to choose the relevant T cell. This study was designed to explore whether synovial dominant clonotypes are a homogenous population based upon their distribution pattern, their activation status, and their persistence. Expression of the IL-2R α chain (CD25) was chosen as a marker of T cell activation. Clonal $CD4^+$ T cell populations were identified and the distribution of such clones in the $IL\text{-}2R^-$ and $IL\text{-}2R^+$ subsets of synovial and peripheral T cells were compared. Within the tissue infiltrate, two different types of clonally expanded $CD4^+$ T cells could be distinguished. One type of clonogenic $CD4^+$ T cells was restricted to the joint being compatible with antigen specific stimulation restricted to the synovial microenvironment. It coexisted with a second type of clonally expanded $CD4^+$ T cells which appeared to be in equilibrium between the blood and the joint and possibly recognized antigens not restricted to the joint. The distinction of two types of proliferating $CD4^+$ T cells in the RA synovium raises the possibility that T cells are involved in different aspects of RA pathology.

MATERIALS AND METHODS

Patients and Samples

Fourteen patients fulfilling the 1987 American College of Rheumatology criteria for the diagnosis of RA were studied (31). Synovial tissue was harvested at the time of joint replacement surgery or synovectomy of 10 patients. Tissue from either two distinct joints or from anatomically independent sites within the same joint was available from eight of these patients. All patients had active synovitis. Samples were only included

TABLE 1. Demographics of the study population

Patient	Age (years)	Sex	Disease Duration (months)	Treatment[a]	RF	HLA	Source	% $CD4^+$ T Cells Expressing IL-2R	
								Blood	Synovium
CRB	68	M	8	NSAID	+	n.d.[b]	F[c]	9.2	12.6
DC	68	F	240	NSAID, DMARD	−	DRB1*0401/07	F	4.2	21.8
DMP	53	F	84	NSAID	+	DRB1*01/08	T, F	5.4	18.9
EK1	24	F	120	NSAID, DMARD, Pred	+	DRB1*01/0401	T	6.8	5.4
EK2	54	F	24	NSAID, DMARD, Pred	−	DRB1*01/13	T	1.3	5.7
HW	62	M	132	NSAID, DMARD	−	DRB1*04/13	T	n.d.	n.d.
JAB	56	M	432	NSAID	+	DRB1*0401/0404	T, F	6.9	17.9
JE	54	M	408	NSAID, Pred	−	DRB1*01/01	T	1.7	12.5
JH	44	M	6	NSAID, DMARD	+	DRB1*0401/07	F	6.2	10.2
KB	23	F	2	NSAID	−	DRB1*0401/13	F	n.d.	n.d.
LT	68	M	240	NSAID, Pred	+	DRB1*0401/0404	T	0.6	3.3
RC	61	M	66	NSAID, DMARD, Pred	+	DRB1*03/0404	T	1.4	6.2
RL	68	F	60	NSAID, DMARD	+	DRB1*01/03	T	0.9	7.4
RMB	46	M	240	NSAID	−	DRB1*01/0401	T	n.d.	n.d.

[a]NSAID, nonsteroidal anti-inflammatory drugs; DMARD, disease modifying antirheumatic drugs; Pred, prednisone.
[b]n.d., not done.
[c]F, synovial fluid; T, synovial tissue.

if a minimum of 2×10^6 mononuclear cells could be recovered from the synovial tissue. Paired samples of synovial fluid and peripheral blood were ascertained in six patients. Thirteen of the 14 study patients were characterized for their HLA-DRB1 alleles as described (32) and expressed at least one RA associated allele. Eight of the 14 patients produced rheumatoid factor (RF). Disease duration varied between 2 months and 36 years. Table 1 shows the demographic data of the study population.

Cell Purification

For TCR analysis, synovial tissue was cut into small pieces and digested for 50 min at 37°C with 30 mg collagenase, 10 mg hyaluronidase, and 1 mg deoxyribonuclease (Sigma Chemical, St. Louis, MO) dissolved in 10 ml HEPES buffered RPMI 1640 per 1 g of tissue. The cell suspension was separated by Percoll gradient. Peripheral blood mononuclear cells (PBMC) and synovial fluid mononuclear cells were isolated by Ficoll (Pharmacia LKB, Piscataway, NJ) separation. Subsequently, cells were stained with anti-CD25-PE (IL-2Rα) and anti-CD4-FITC (Becton Dickinson, San Jose, CA) in RPMI 1640 supplemented with 10% FCS (Hyclone, Logan, UT) and $CD4^+$ IL-$2R^+$ and $CD4^+$ IL-$2R^-$ cells were sorted by flow cytometry.

T Cell Receptor Analysis

RNA was obtained by guanidinium chloride phenol chloroform extraction (Trizol, Life Technologies, Grand Island, NY) from $CD4^+$ IL-$2R^+$ and $CD4^+$ IL-$2R^-$ T cells. To avoid a bias due to sample size, a minimum of 5×10^4 IL-$2R^-$ and IL-$2R^+$ cells were analyzed unless samples from distinct joints were available. cDNA was amplified by polymerase chain reaction (PCR) (94°C 1 min, 55°C 2 min, 72°C 2 min, 30 cycles) by using BV specific primers (BV3, 5S1, 8, 14, and 17) and a BC primer. The PCR product was diluted 1:200 and further amplified under the same PCR conditions with the appropriate BV primer and a BJ specific primer (BJ1S1, 1S2, 2S1, 2S3, 2S5, and 2S7). A total of 30 amplification products was screened for each sample. The BV segments studied represent approximately 25% of the total repertoire (33) and included some of the previously implicated BV gene elements (7,8). The set of BJ primers covered approximately 70% of the BV–BJ paring combination frequencies for a given BV gene arrangement (34,35). The primer sequences have been recently described (28,36).

Amplified products were radioactively labeled and size-fractionated on a denaturing 5% polyacrylamide gel. In this type of analysis, a Gaussian distribution of band intensities is characteristic for a polyclonal population and dominant bands are highly suggestive for clonal expansion (37–39). Dominant bands shared between different RNA samples (e.g., activated and nonactivated T cells, peripheral and synovial T cells, samples from distinct joints, etc.) were excised, eluted, and then reamplified with a T7–BV primer and the BJ primer over 30 cycles under the same PCR conditions as outlined above. The amplified product was directly sequenced by reverse transcriptase mediated dideoxysequencing as described (40). Mixing experiments of T cell clones with known sequences showed that the direct sequencing approach is sensitive enough to detect clonotypes which contribute more than 25% of the total PCR product (27).

RESULTS

Influence of Extra-articular T Cell Activation on the Repertoire of Synovial T Cells

It could be hypothesized that preactivation of T cells in extra-articular lymphoid tissue is per se sufficient to ascertain recruitment to the synovial site of inflammation. If this assumption is correct then $CD4^+$ IL-$2R^+$ T cells dominant in the blood should consistently infiltrate the joint. In the peripheral blood, 4% of the $CD4^+$ T cells expressed IL-2R whereas 11.8% of synovial T cells were preactivated, consistent with either local activation or selective homing (Table 1). Purification of IL-$2R^+$ and IL-$2R^-$ T cells from both the blood and the joint allowed for directly addressing the question whether the population of preactivated T cells in the synovial membrane is foremost a reflection of T cell stimulation outside of the joint with subsequent preferential homing of such activated cells. TCR sequences were amplified from purified subsets with BV and BJ gene segment-specific primers sets and separated by size to compare the different repertoire. Dominant bands of equal size were eluted and directly sequenced to search for shared clonotypes. Although there was a trend for the number of third complementarity determining region (CDR3)

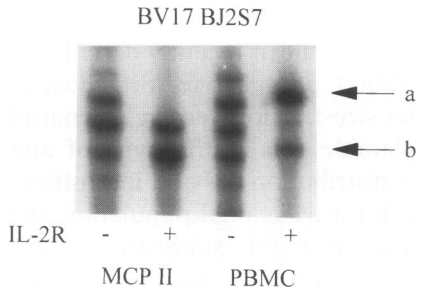

FIG. 1. The repertoire of activated peripheral and synovial T cells is different

$CD4^+$ IL-2R^+ and $CD4^+$ IL-2R^- T cells from peripheral blood and synovial tissue were purified by cell sorting. A sample of 10% of the total repertoire was screened for oligoclonal populations by PCR with BV and BJ specific primers. The amplified product was separated by size fractionation. Dominant bands were eluted, reamplified and directly sequenced. A representative example of the BV17-BJ2S7 amplification product from synovial tissue of metacarpal joint (MCP) II and peripheral blood (Patient RC) is shown. The repertoires of IL-2R^+ $CD4^+$ T cells in peripheral blood and synovial tissue were different. The amplification product from IL-2R^+ $CD4^+$ peripheral cells contained two dominant bands, one of which (a) was not present in the IL-2R^+ synovial T cells. For band b different sequences were found in the peripheral blood and synovial tissue (BV17 CACRDPSYEQYF BJ2S7 in the IL-2R^+ fraction of MCP II;. BV17 CASSSKASSYEQYF BJ2S7 in the IL-2R^+ fraction from PBMC).

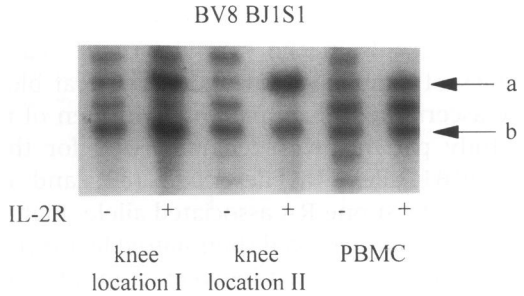

FIG. 2. Identification and characterization of joint-specific clones

$CD4^+$ IL-2R^+ and $CD4^+$ IL-2R^- T cells from two anatomical distinct locations and peripheral blood were obtained and a sample of TCR β chain repertoire was analyzed as described in Fig. 1. The BV8-BJ1S1 amplification products of Patient JE are shown as a representative example. Results of 8 patients are summarized in Table 2. Joint-specific T cell clones were frequently found in the IL-2R^+ fraction of synovial $CD4^+$ lymphocytes (e.g., a = BV8 CASSSLTGMNTEAFF BJ1S1). Only a few clones were found in both the IL-2R^- and IL-2R^+ subsets (e.g., b = BV8 CASSLVEGTEAFF BJ1S1). Both sequences were not detected in the IL-2R^+ and IL-2R^- subsets of PBMC but were present in two nonadjacent tissue segments from the knee.

size classes in the joint to be slightly lower than in the corresponding population in the blood, the comparison of CDR3 size classes emphasized that even $CD4^+$ IL-2R^+ T cells in the synovial tissue were highly diverse (data not shown).

Surprisingly, we did not find evidence for an accumulation of circulating $CD4^+$ IL-2R^+ T cell clonotypes in the inflammatory lesions. The pattern of CDR3 length analysis of T cell clones in the preactivated $CD4^+$ population in the blood and in the joint were strikingly dissimilar. Direct sequencing of dominant bands which were shared between peripheral and synovial $CD4^+$ IL-2R^+ populations revealed different CDR3 amino acid sequences (Fig. 1). Clonal populations which were exclusively dominant among peripheral blood $CD4^+$ IL-2R^+ cells have likely been activated outside of the synovial compartment. Although such clonal populations were frequent, they did not cross over into the joint in sufficient numbers to reach clonal dominance. This observation suggested that the peripheral and synovial $CD4^+$ IL-2R^+ populations were distinct and that the increased frequencies of IL-2R^+ cells in the synovial tissue were a consequence of local activation.

Synovial T Cell Infiltrate Includes T Cells that Proliferate Specifically in the Joint

Antigen driven T cell stimulation in the synovial lesions should lead to the preferential outgrowth of a few $CD4^+$ T cells. If the antigen resides in the joint, these T cells should have a survival advantage in synovial lesions but should be diluted out in the blood. Provided the antigen is disease relevant, it should be available at different sites of inflammation, in particular it should induce T cell responses in independent joints. We therefore explored whether dominant clonotypes could be identified which, at a given time, proliferated in more than one joint and were enriched in the synovium as compared to the peripheral blood. Biopsy material from eight patients that derived from two distinct joints or from non-adjacent sites of the same joint was available. $CD4^+$ IL-2R^- and $CD4^+$ IL-2R^+ T cells were purified from these different tissue sources by cell sorting. TCR β chain sequences were amplified, the amplification product was size-frac-

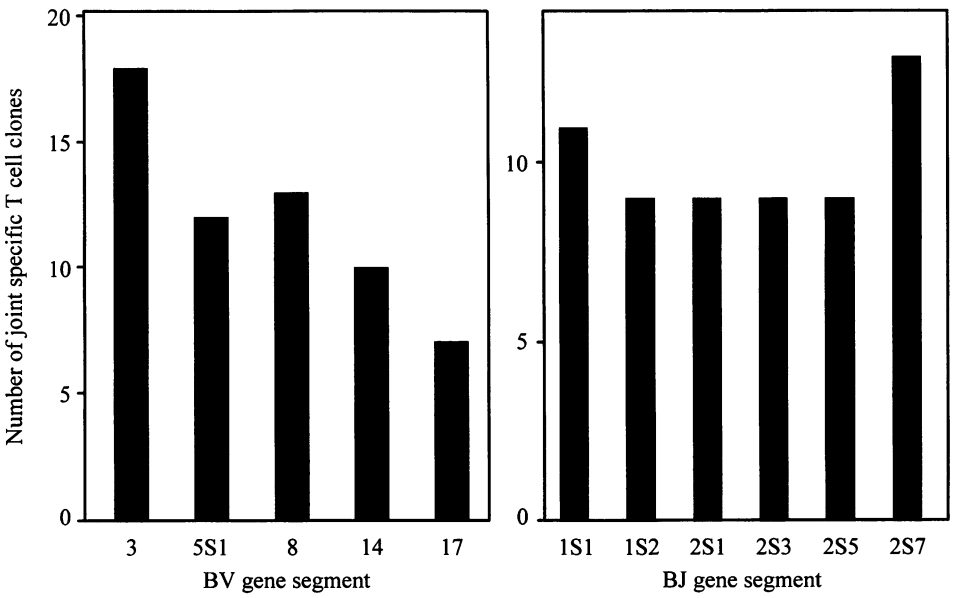

FIG. 3. TCR gene segment usage of joint-specific clones

TCR β chains from T cell clones which were overrepresented at two different sites of the synovial inflammation, but not in the peripheral blood (as shown in Fig. 2) were identified and clonal identity was confirmed by sequencing of eluted bands. TCR BV (left) and TCR BJ (right) gene segment usage is shown. The T cell response was heterogeneous with a trend towards usage of the BV3 segment.

tionated, and dominant bands were identified. Direct sequence analysis of eluted shared bands documented that the expression of a particular TCR specificity at independent sites of the inflammation was not uncommon. In all eight patients TCR sequences were found which had the following features: they had reached clonal dominance, they were transcribed at high frequencies in the joint but not in the blood, and they were expressed in several independent tissue samples (Fig. 2). In the eight patients, 60 joint restricted $CD4^+$ clonotypes were detected. The TCR β chain sequences are listed in Table 2. Within the window of the TCR repertoire tested, the frequency of such clonotypes ranged from 1 to 13 per patient.

Joint-specific $CD4^+$ T cells exhibited several distinguishing features. Many, but not all, of the joint-specific clonotypes displayed preference for the preactivated $CD4^+$ T cell subset. Of the 60 clonotypes that fulfilled the criteria for joint-specificity, 34 were exclusively found among $CD4^+$ IL-$2R^+$ T cells (Table 2; Fig. 2). A set of 18 clonotypes was present among both IL-$2R^-$ and IL-$2R^+$ T cells. Eight of the clonotypes were limited to the $CD4^+$ IL-$2R^-$ population and did not seem to be activated.

Molecular analysis identified BV3 as the most frequent gene segment used by joint-specific $CD4^+$ T cells. As given in Fig. 3, 30% of the clonotypes displaying joint restriction had rearranged the BV3 gene segment. BV17 was encountered infrequently among this group of T cell specificities. Based upon frequencies of BV usage in peripheral T cells, BV3, BV8 and BV17 are about equally frequent in polyclonal unbiased populations, suggesting that there was a trend toward nonrandomness in the recruitment of these T cells. The use of BJ gene elements was about equal for the six different BJ gene segments. Among the joint-specific $CD4^+$ T cells, BJ2S7 was most frequently encountered followed by BJ1S1.

Amino acid sequences of the CDR3 region should provide the most insight into antigen specificity. Among the 60 clones, the sequence and the length of the CDR3 region varied widely. The only two TCR β sequences which were isolated from two different patients and exhibited sequence similarities (FVL versus TVL; Table 2) were found in clones with different BV and BJ gene segment usage and different CDR3 length.

To identify parameters that predicted the occurrence of joint-specific $CD4^+$ clonotypes, the clinical characteristics of the patients were correlated with the frequency of joint-restricted clo-

TABLE 2. TCR β chain sequences of joint-specific clonotypes

Patient	BV	N-D-N	BJ	IL-2R$^-$	IL-2R$^+$
DMP	3 CASS	F V L	NEQFF 2S1	+	+
	5S1 CASS	L G G G	STNEQFF 2S1	+	+
	14 CASS	P I S G K	ETQYF 2S5	+	+
EK1	5S1 CASS	L D S G G G V L T	SYEQYF 2S7	−	+
	8 CASS	L V W H S G D	NYGYTF 1S2	−	+
	14 CAS	G L	TEAFF 1S1	+	+
	17 CASS	T A G R	SDTQYF 2S3	−	+
EK2	3 CASS	Q G Q G R P F	NTEAFF 1S1	−	+
	3 CAS	S L R S S R R G S I G G	NEQFF 2S1	−	+
	5S1 CASS	G G Q G T L P N	EQYF 2S7	+	+
	5S1 CASS	L D A R	NEQFF 2S1	−	+
	5S1 CASS	L V	QETQYF 2S5	−	+
	8 CASS	L A V Q G T R	YGYTF 1S2	−	+
	8 CASS	F N A L T G G R	ETQYF 2S5	−	+
	14 CASS	C S V	NTEAFF 1S1	+	+
	14 CAS	S Y S A	NYGYTF 1S2	+	+
	17 CASS	R G G	GYTF 1S2	−	+
JAB	3 CASS	L A G V	STNEQFF 2S1	+	−
JE	3 CAS	S L A G	STNEQFF 2S1	+	+
	3 CASS	L L G R	NTEAFF 1S1	−	+
	3 CAS	S S S G T G A	YEQYF 2S7	−	+
	5S1 CASS	S Y D E T G T G G L P	YEQYF 2S7	−	+
	8 CASS	L V E G	TEAFF 1S1	+	+
	8 CASS	L D L R Q G E W	ETQYF 2S5	+	+
	8 CASS	T N	YEQYF 2S7	+	+
	8 CASS	S L T G M	NTEAFF 1S1	−	+
	8 CASS	M L V R R G	NYGYTF 1S2	−	+
	14 CASS	L R G R T	DTQYF 2S3	−	+
	14 CASS	L T G K	TQYF 2S5	−	+
LT	3 CAS	S L G G N S	NTEAFF 1S1	+	+
	3 CAS	R N P A G S R V G	SYEQYF 2S7	+	+
	3 CASS	L L	DTQYF 2S3	+	−
	3 CASS	L R E	TQYF 2S5	+	−
	3 CAS	C F G G N S	NTEAFF 1S1	+	−
	3 CAS	S A G Q A P F L	YGYTF 1S2	−	+
	3 CASS	N H N P L G V L G	TDTQYF 2S3	−	+
	14 CASS	P H L L G S	YEQYF 2S7	−	+
	17 CASS	A L V P G Q G P V G	NEQFF 2S1	+	+
	17 CASS	H G G L A G I	ETQYF 2S5	+	−
RC	3 CASS	S G T S G	EQFF 2S1	−	+
	3 CASS	L G D R G	TDTQYF 2S3	−	+
	3 CASS	I R D R G A Q Y H	YEQYF 2S7	−	+
	5S1 CASS	S L G R V	NTEAFF 1S1	−	+
	5S1 CASS	P L E G R	SYEQYF 2S7	−	+
	8 CASS	C T H G	TEAFF 1S1	−	+
	14 CASS	S R Q D	STDTQYF 2S3	−	+
	14 CASS	R T G G	YEQYF 2S7	−	+

TABLE 2. *Continued*

Patient	BV	N-D-N	BJ	IL-2R$^-$	IL-2R$^+$
RL	3 CAS	SFRTG	TQYF 2S5	+	+
	5S1 CASS	LDGA	YGYTF 1S2	+	+
	5S1 CASS	PGGGVH	TDTQYF 2S3	+	−
	5S1 CASS	LGR	ETQYF 2S5	−	+
	5S1 CAS	RDRQGIL	SYEQYF 2S7	−	+
	8 CASS	LSGV	YGYTF 1S2	+	+
	8 CASS	LYAGA	EAFF 1S1	+	+
	8 CASS	FRRN	NEQFF 2S1	−	+
	8 CASS	PGQ	SYEQYF 2S7	−	+
	14 CASS	FVCV	NYGYTF 1S2	−	+
	17 CASS	NGE	TDTQYF 2S3	+	−
	17 CASS	TVL	SYEQYF 2S7	+	−
	17 CA	FRARGN	TDTQYF 2S3	−	+

nogenic CD4$^+$ cells. This analysis showed a trend for patients with shorter disease duration (up to 5 years) to express a higher number of clonotypes. On average, these patients had 10 T cell clones whereas patients with a disease duration of longer than 5 years carried approximately 5 joint-specific CD4$^+$ T cell clonotypes. The HLA-DRB1 genotype was not predictive for a low or high number of joint-specific T cells. The number of clonotypes was also independent of antirheumatic therapy and the production of RF.

Joint-Specific CD4$^+$ T Cells Persist over Time

The frequent restriction of the joint-specific T cells to the IL-2R$^+$ subpopulation raised the question whether these T cell specificities were only transiently activated and proliferated briefly in the joint. To address this question, consecutive samples were examined for the persistent expression of such T cells. From two patients, serial specimens were collected. In patient KB synovial fluid was obtained from the knee joint on two occasions in a 3-month interval. Three synovial fluid samples were available from patient JH. In Table 3, TCR β chain sequences from four different CD4$^+$ T cell clones are shown. All four T cell specificities were restricted to the joint, were undetected in the blood, and were found at different time points. The T cell clonotype isolated from patient KB was present in two consecutive samples. Three distinct clonotypes identified in patient JH could be demonstrated over a time period of 15 months. Interestingly, joint-specific T cell clonotypes which persisted over time were not restricted to the CD4$^+$ IL-2R$^+$ T cell subset but extended into the IL-2R$^-$ subpopulation. Possibly, the clonotypes persisting in the joint had reached a larger clonal size due to chronic stimulation and not all cells were activated at the time of analysis.

A Second Type of Expanded CD4$^+$ T Cell Exists in the Synovial Lesions

Tracing clonal dominant populations of CD4$^+$ T cells led to the identification of a second type of clonogenic CD4$^+$ T cells. Characteristically, this second clonotype was found in both compartments, blood and synovium, and in both CD4$^+$ subsets, IL-2R$^+$ and IL-2R$^-$. Figure 4 shows an example of such a clonally expanded CD4$^+$ population. A band (a) with identical CDR3 length distorted the Gaussian distribution of CDR3 size classes in all T cell subsets. Clonal identity was confirmed by direct sequencing. Fourteen such clonotypes were isolated from eight patients while six patients did not have this type of ubiquitously distributed clonotype within the repertoire sample tested. Sequences of the TCR β chains are presented in Table 4. Between 0 and 4 of these clonogenic CD4$^+$ populations were en-

TABLE 3. Joint-specific T cell clones persist over time

Patient	TCR β Chain Sequence			Peripheral Blood CD4+ T Cells	
	BV	N-D-N	BJ	IL-2R+	IL-2R−
JH	3 CASS	F S G G L A P	YGYTF 1S2	−	−
	8 CASS	P G P R T S P	YEQYF 2S7	−	−
	17 CASS	F P A G R P	STDTQYF 2S3	−	−
KB	14 CASS	P T T G T G G	NYGYTF 1S2	−	−

[a]n.d., not done.

countered in each patient for the BV–BJ combinations studied.

Although 4 of the 5 analyzed BV gene elements were represented among the 14 TCR sequences, there was a bias toward the preferential usage of BV3. Eight of the 14 clonotypes shown in Table 4 had rearranged a BV3 gene suggesting preferential recruitment of $BV3^+$ T cells. The group of TCR sequences was too small to draw conclusions regarding the BJ gene expression which appeared to be diverse. However, comparison of the CDR3 region revealed a sequence homology between T cell clones derived from two distinct patients (Table 4, bold letters). Both clones used a BV3 gene segment. The TCR sequence found in patient RMB included a FAL stretch in conjunction with a BJ2S1 gene. Patient JAB carried a T cell clone with a FSL sequence combined with a BJ1S1 element.

Analysis of the clinical parameters of the patients studied showed a correlation of the occurrence of broadly distributed $CD4^+$ clonotypes with the seronegative variant of RA. All 6 seronegative patients carried at least one clonotype present in the joint and in the blood. Conversely, only two out of eight RF^+ patients had clonogenic $CD4^+$ cells of that category. The difference in frequency was statistically significant at the $p < 0.005$ level. Disease duration was not a predictor for these clonogenic $CD4^+$ populations. Ubiquitously distributed clonotypes were already detectable in patients with very early disease. Also, the HLA-DRB1 genotype did not predict whether a patient had widely distributed clonal $CD4^+$ cells nor did antirheumatic therapy appear to influence the presence of such T cells.

DISCUSSION

Detailed examination of the synovial T cell infiltrate in this study revealed a series of observations, all of which strongly support the notion of an antigen dependent T cell response in the joint. (*1*) Selected T cell specificities proliferate in situ and reach clonal dominance. (*2*) 85% of these clonotypes express IL-2R, indicative of recent triggering of the TCR. (*3*) T cells carrying identical TCR β chains have gained dominance in nonadjacent tissue fragments and in independent joints. Local enrichment, preactivation, and presence at different sites of the disease process have all been assumed to characterize the dynamics of disease-relevant and antigen-specific T cells. Short of demonstrating the antigen, these findings are highly suggestive for specific T cell responses occurring in the rheumatoid synovium.

The designation of joint-specific clones was based on the definition that sequencing of a dominant band in the size-fractionated PCR product from synovial T cells yielded an unequivocal TCR sequence while the corresponding bands derived from peripheral blood were polyclonal. The finding that joint-specific clones could be identified in different joints indeed suggests that they are present in the circulating T cell population. The direct sequencing approach of dominant bands only allows for the conclusion that the frequencies in the peripheral blood and synovial tissue are markedly different. To determine the actual frequencies of TCR β chain sequences in the peripheral blood compartment, we have established a liquid hybridization assay using CDR3 specific probes in a limiting dilution

TABLE 3. *Continued*

Synovial Fluid CD4+ T Cells					
First Timepoint		Second Timepoint		Third Timepoint	
IL-2R$^+$	IL-2R$^-$	IL-2R$^+$	IL-2R$^-$	IL-2R$^+$	IL-2R$^-$
+	+	+	+	−	+
+	+	+	+	+	+
+	+	+	+	+	+
−	+	−	+	n.d.[a]	n.d.

system. So far, these experiments have shown that joint-specific clones are detectable in the circulation, albeit at very low frequencies of 1 in 10^5 to 1 in 10^7 CD4$^+$ T cells (U. Wagner, J. J. Goronzy, C. M. Weyand, unpublished observations). In contrast, the approach chosen in the present study required a minimal frequency of 1:2000 CD4$^+$ T cells. Thus clonotypes categorized as joint-specific had a significant size and were clearly enriched in the tissue as compared to the blood.

Joint-specific CD4$^+$ T cell clones as defined by tissue-specific accumulation and expression at different nonadjacent regions of the inflammation were not rare but were readily found in all patients. An arbitrarily selected window of 10% of the TCR repertoire was sufficient to reveal an average of 7.5 such CD4$^+$ clonotypes in each patient. It can be concluded that joint-specific CD4$^+$ T cells in RA are a frequent event.

Antigen recognition in the affected tissue appears to involve a wide spectrum of T cells. In each BV family analyzed joint-specific CD4$^+$ clonotypes were found. However, the size of each of the clonotypes remained small. Provided that the CD4$^+$ clonotypes reported here indeed contact antigen residing in the joint and respond with expansion, they still remain a minority among a highly diverse T cell population. The fact that the detection of such clonotypes required PCR for BV–BJ combinations demonstrates that the individual clonotype did not exceed a frequency of about 1% within the CD4$^+$ T cell pool. Most of the T cell clones were only apparent if the analysis was focused on purified CD4$^+$ IL-2R$^+$ T cells. Based on these findings, it cannot be expected that clonal proliferation results in the skewing of the TCR BV repertoire in the synovial tissue of RA patients. This may explain why the comparisons between BV gene segment frequencies in peripheral blood and synovial T cells have yielded conflicting data (7,8,11,13,40,41). Our results are in line with the frequencies found in local antigen-specific responses and in other chronic inflammatory diseases (6,42–45).

Surprisingly, preactivation of circulating T cells as judged by the expression of the IL-2R was

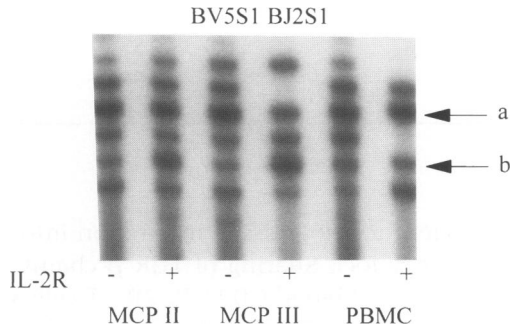

FIG. 4. Identification and characterization of CD4$^+$ T cell clones expanded in the peripheral blood and the synovial tissue

The CD4$^+$ IL-2R$^+$ and CD4$^+$ IL-2R$^-$ subsets were analyzed as outlined in Fig. 1. The BV5S1-BJ2S1 amplification products from peripheral blood and synovial compartments of metacarpal joints (MCP) of patient EK2 are shown. All compartments contained a dominant band of equal size (a) which represented the identical clonotype as determined by direct sequencing (BV5S1 CASSLDARNEQFF BJ2S1). Sequences of ubiquitous clones from 8 patients out of a cohort of 14 patients are shown in Table 4. These clones are generally found in the IL-2R$^+$ as well as the IL-2R$^-$ subset, suggesting continuous stimulation. In addition to this ubiquitous T cell clone, a T cell clone restricted to the IL-2R$^+$ subset (b) of synovial CD4$^+$ T cells was identified (BV5S1 CASSLDARNEQFF BJ2S1).

TABLE 4. TCR β chain sequences of clonotypes shared between peripheral blood and synovial tissue

Patient	BV	N-D-N	BJ
JAB	3 CASS	**F S L**	NTEAFF 1S1
RMB	3 CAS	**F A L**	SYNEQFF 2S1
KB	3 CASS	R G Q G R D	EQFF 2S1
CRB	3 CASS	L G L A Q	NEQFF 2S1
DC	3 CAS	R F L M G	TDTQYF 2S3
RMB	3 CASS	F L S G	TDTQYF 2S3
RMB	3 CASS	L G G T S P K G L	ETQYF 2S5
RMB	3 CASS	A R Q G V	SYEQYF 2S7
EK2	5S1 CASS	L G R G A	SYNEQFF 2S1
JAB	5S1 CASS	P G L G G	TDTQYF 2S3
KB	14 CAS	R E G Q G Y P	EAFF 1S1
HW	14 CASS	L G I R	ETQYF 2S5
JE	14 CASS	P R R R A P	SYEQYF 2S7
KB	17 CASS	V I P G L A G A E	TDTQYF 2S3

not sufficient to allow tissue infiltration into the inflammatory foci. Sharing of TCR β chains between tissue and blood $CD4^+$ IL-$2R^+$ T cells was a rare event. Most $CD4^+$ T cell clones which were found in the IL-$2R^+$ fraction in the circulation did not cross over into the inflamed synovium. For the vast majority of $CD4^+$ IL-$2R^+$ T cells, the synovium remained an independent unit. These results suggest that there is no free equilibrium between peripheral blood and synovial tissue for $CD4^+$ IL-$2R^+$ T cells. Mechanisms beyond preactivation might be critical in controlling the recruitment of T cells to the synovial membrane.

Joint-specific $CD4^+$ T cells were not the only type of clonogenic cells skewing the TCR repertoire in the synovium. A second type of $CD4^+$ clone with ubiquitous distribution contributed to the synovial infiltrate. Based on CDR3 length analysis, these clonotypes reach a similar clonal size in the different compartments. Clonogenic $CD4^+$ cells present in the joint and blood must have expanded significantly more than joint-specific clones since they have spread throughout a much larger compartment with an equilibrium between the circulation and the joint. In the synovial tissue, ubiquitous clones were generally found in the $CD4^+$ IL-$2R^+$ as well as IL-$2R^-$ T cells, while only a subset of joint-specific clones were found in the IL-$2R^-$ population. Ubiquitous T cell clones, therefore, represent a major fraction of the clonal populations in the synovial tissue. Compared to normal preactivated IL-$2R^+$ $CD4^+$ T cells, these clones differ in their homing pattern and might express adhesion molecules which facilitate their migration to the synovial compartment.

The ubiquitous clonotypes are most likely identical to the clonally expanded $CD4^+$ T cells recently described in the blood of RA patients (27,28). These $CD4^+$ T cell clones were autoreactive and expressed an usual phenotype in that they lacked the expression of CD28 (29). Systematic analysis of $CD4^+$ $CD28^-$ T cells in a cohort of RA patients has led to identification of TCR β chains with almost identical sequences expressed in distinct patients (30). As shown in Table 4, we have again identified clonotypes from two patients with sequence homologies of their TCR β chains. The best explanation is a limited spectrum of antigens driving the clonal expansion of $CD4^+$ $CD28^-$ T cells. A very similar scenario exists for $CD8^+$ T cells. RA patients carry clonally expanded $CD8^+$ populations in the peripheral blood (23,24). These cells lack the expression of CD28 and have a limited TCR β chain diversity with a preferential usage of AV12 and BV3 and sharing of junctional sequences (23,46).

The presence of ubiquitous clonotypes is not a universal feature of all RA patients. All of the patients with seronegative disease and only two of eight patients with seropositive disease carried such clones within the samples tested. This finding is consistent with data from a cohort study where patients with extra-articular disease and

most patients with seronegative RA had increased frequencies of $CD4^+$ $CD28^-$ T cells (47). In contrast, patients with seropositive disease, but without extra-articular manifestations, were less likely to carry these cells. None of the patients with RF+ disease examined in the present study had extra-articular manifestations.

It is curious that the synovial T cell infiltrate contains two distinct variants of dominant $CD4^+$ clones, joint-specific and ubiquitously distributed. The question arises whether a relationship exists between the two types of clonogenic T cells. In terms of molecular features, it was interesting that both types of clonotypes described here displayed a preference for BV3. It could be argued that joint-specific $CD4^+$ clones are precursors of the widely distributed clonotypes. However, ubiquitous clones are already found in patients with early disease and they do not increase in number with disease duration (27,47). In patients who were monitored over time, no change in the distribution pattern could be documented. $CD4^+$ clones restricted to the joint remained joint-specific (Table 3). Also, among the widespread T cell clones, TCR β chains with sequence homologies were readily identified when different patients were compared (30; Table 4). This was not the case for joint-specific T cell clonotypes. The TCRs used by that type of proliferating synovial T cell were highly diverse.

Understanding the differences in the distribution pattern of clonogenic $CD4^+$ cells may come from characterizing the distribution of the relevant antigens. We have evidence that $CD4^+$ $CD28^-$ expanded clonotypes recognize autoantigens which are expressed on PBMC (29). The broad distribution of these clonogenic cells may reflect the tissue expression of the antigen. A recent report by Kouskoff et al. (48) has stressed that T cells specific for ubiquitous self-antigens can cause synovitis without major systemic manifestations. In that model RA could be understood as an example of an autoimmune disease in which self-antigens, although ubiquitously expressed, are preferentially recognized in the joint. Further support for this model comes from the finding that synovial $CD8^+$ T cells recognize Epstein-Barr virus encoded transactivators as expressed on autologous lymphoblastoid cell lines, again suggesting that antigens recognized in RA may not have a joint-specific tissue distribution (49,50). Conversely, joint-specific clonotypes are likely to react to an antigen accessible in the synovial microenvironment. The spatial restriction in the expression of these clonotypes is highly suggestive for a locally residing antigen. The contribution of the two T cell types to RA might be different, and T cells might be involved in distinct dimensions of the disease process.

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