Characterisation of T cell clonotypes that accumulated in multiple joints of patients with rheumatoid arthritis

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Accepted for publication
4 May 1999

Abstract

***Objective*—To investigate whether identical T cell clonotypes accumulate in multiple rheumatoid joints, the clonality of T cells that had infiltrated into synovial tissue (ST) samples simultaneously obtained from multiple joints of patients with rheumatoid arthritis (RA) was analysed.**

***Methods*—T cell receptor (TCR) β gene transcripts, amplified by reverse transcription-polymerase chain reaction from ST and peripheral blood lymphocytes of five RA patients, were subjected to single strand conformation polymorphism analysis and DNA sequencing.**

***Results*—Approximately 40% of accumulated T cell clonotypes found in one joint of a patient were found in multiple joints in the same patient. Furthermore, identical amino acid sequences were found in TCR β junctional regions of these clonotypes from different patients with at least one HLA molecule match.**

***Conclusions*—The T cell clonotypes accumulating in multiple rheumatoid joints may be involved in the perpetuation of polyarthritis by reacting to antigens common to these multiple joints.**

(*Ann Rheum Dis* 1999;**58**:546–553)

Rheumatoid arthritis (RA) is a systemic disease characterised by chronic inflammation of synovial tissue in multiple joints. Pathologically, hyperplasia of the cells of the synovial lining and perivascular accumulation of T cells in synovial tissue are observed in affected joints. The onset of RA is statistically associated with particular HLA molecules such as DR4 and DR1.[1 2] This supports the hypothesis that such HLA molecules may affect the formation of the T cell repertoire or that the HLA molecules efficiently present RA associated antigens to T cells, or both. Because of the possible involvement of T cells in the pathogenesis of RA, many studies have focused on T cells, including T cell receptors (TCRs), which are antigen recognising molecules of T cells.[3–8] Previous analyses of the TCR repertoire of RA patients revealed that T cells in RA affected joints expressed skewed BV gene use compared with that in peripheral blood lymphocytes (PBLs).[9–14] The patterns of the bias, however, were found to differ among various cohorts of RA patients examined.

Recently, studies have focused on complementarity determining region (CDR) 3 of TCRs in RA.[15–21] The CDR loops in α and β chains were reported to be important for interaction with the MHC-peptide complex.[22 23] CDR1 and 2 mainly make contact with MHC molecules, while CDR3 mainly makes contact with antigenic peptides. The nucleotide sequence of CDR3 is extremely diverse because of the addition and/or deletion of nucleotides at the V-D and D-J junctions. As the rearrangement of TCR β genes results in allelic exclusion, the nucleotide sequence of CDR3 β is unique for each T cell. Therefore, analysis of CDR3 makes it possible to detect clonal accumulation of T cells. The detection of clonally accumulating T cells is thought to be important in investigating antigen driven immune responses in RA affected joints, as antigenic stimulation gives rise to clonal proliferation of T cells that specifically respond to antigens.

Previously, we analysed TCR β gene clonotypes by reverse transcription-polymerase chain reaction (RT-PCR) and subsequent separation by their single strand conformation polymorphism (RT-PCR/SSCP). By this method, highly expressed TCR β gene clonotypes, which reflect clonal accumulation of T cells, are detected as sharp bands on smear-like backgrounds.[24] Thereby, we reported that healthy PBL showed few bands, suggesting an almost heterogeneous T cell population.[24] In contrast, synovial fluid and synovial tissue (ST) from RA patients showed a large number of bands, which indicated oligoclonal T cell accumulation in RA joints. As RA PBL contained a much smaller number of clonally accumulated T cells compared with that in joints, the clonally accumulated T cells were thought to be joint specific. Furthermore, we also reported that identical T cell clonotypes were accumulated in different sites of the same joint in RA patients.[25] These T cells may recognise ubiquitous antigens in the joints. If such accumulating T cell clonotypes recognised joint related antigens, they would accumulate in multiple affected joints, even if the time of onset of inflammation differed among the joints. Therefore, we compared T cell clonotypes among multiple joints. Specifically, we compared the TCR β gene clonotypes in multiple joints in five RA patients by RT-PCR/SSCP and DNA sequencing to detect commonly accumulating T cells. This showed that

Table 1 Patients and clinical specimens

	RA 1	*RA 2*	*RA 3*	*RA 4*	*RA 5*
HLA phenotypes	A2/A26 B35/B61 Cw9 DR8/DR9 (DRB1*0803/0901) DQ6/DQ9 (DQA1*0103/03) (DQB1*03032/0601) (DPB1*0501)	A24/A33 B54/B70 Cw1/Cw7 DR4 (DRB1*0401/0405) DQ7/DQ4 (DQA1*03) (DQB1*0301/0401) (DPB1*0201/0501)	A24 B51 Cw1 DR4/DR12 (DRB1*0403/1201) DQ7/DQ8 (DQA1*03/05) (DQB1*0301/0302) (DPB1*0301)	A31/A33 B44/B61 Cw10 DR4/DR9 (DRB1*0405/0901) DQ4/DQ9 (DQA1*03) (DQB1*03032/0401) (DPB1*0201/0501)	A26/A33 B54/B7 Cw10 DR1/DR4 (DRB1*0101/0405) DQ4/DQ5 (DQA1*03/0101=0104) (DQB1*0401/0501) (DPB1*0401/0501)
Stage	IV	IV	IV	IV	IV
Disease duration (y)	14	24	18	8	18
ESR (mm 1st h)	41	20	21	110	77
CRP (mg/dl)	2.5	0.4	1.2	11	7.6
RF (U)	270	53	<20	28	46
Examined joints	left ankle right ankle left PIP left elbow	right MP right knee	right MP right elbow	left knee right wrist	left knee right knee

*In each patient, synovial tissue samples were simultaneously obtained from above joints by therapeutic multiple synovectomy.

44% of clonally accumulating T cells in one joint were detected in multiple joints in the same patient. Furthermore, we found several identical amino acid sequences in the TCR β CDR3 of T cells that were accumulated in multiple joints.

Methods

CLINICAL SPECIMENS

The five patients (RA1-RA5) in this study had been diagnosed as having RA based on the RA criteria of the American Rheumatism Association revised in 1987.[26] Patient RA1 in this study is the same as patient RA3 in the study of Ikeda and colleagues.[25] The HLA-C allele of this patient, which was described as Cw1/3 in reference 25, was found to be Cw9/9 upon additional repeated testing. A different PBL sample from this patient was used in this study than that used in reference 25. The PBL sample for this study was taken two months after the therapeutic multiple synovectomy. As for the other patients, PBL samples and ST specimens were simultaneously obtained (table 1). All ST specimens were obtained at the time of therapeutic radical multiple synovectomy by open surgery.

ANALYSIS OF T CELL CLONALITY BY RT-PCR/SSCP

The RT-PCR/SSCP method for analysing T cell clonality has been described previously.[24] In brief, total RNA was extracted from each sample by the acid guanidinium thiocyanate-phenol-chloroform method.[27] Approximately 2–4 μg of RNA was converted to cDNA by use of reverse transcriptase (Superscript, BRL, Gaithersburg, MD) and 100 pmol of a random hexamer oligonucleotide primer (BRL). Next, PCR was performed with 100–200 ng of cDNA, 5 nmol of dNTPs, 1 U of Taq polymerase (Promega Co, Madison, WI), 50 pmol of each of the 20 BV primers and 50 pmol of a common BC primer for 35 cycles in a thermocycler (TaKaRa PCR Thermal Cycler, TaKaRa Co, Otsu, Japan). The sequences of the primers have been described previously.[28] For separation of the amplified DNA fragments based on differences in their single strand conformation, they were diluted, heat denatured and then electrophoresed on non-denaturing 4% polyacrylamide gels containing 10% glycerol. The electrophoresed DNA fragments were transferred to membranes (Immobilon-S, Millipore Intertech, Bedford, MA) and hybridised with a biotinylated internal BC probe (5'-A(AC) A A(GC)GTGTTCCCACCCGAGGTCGC TGTGTT-3'). Finally, the bound BC probe was detected by use of a Plex Luminescence kit (Millipore Intertech), after which the gels were dried and stored until DNA sequencing.

DNA SEQUENCING OF TCR β CDR 3 REGIONS

To determine the nucleotide sequence of the TCR β gene transcripts that had been detected as a distinct band on an SSCP gel, we recovered a small piece of the dried SSCP gel that corresponded to the detected band. The TCR β gene transcripts, extracted from the gel fragment into TE.1 (10 mM TRIS-HCl/0.1 mM EDTA) by repeated boiling and freezing, were amplified by PCR using two primers, a matched BV primer with an EcoRI recognising sequence and a common BC primer with a Hind III recognising sequence. After digestion with EcoRI and Hind III (Wako, Osaka, Japan), the re-amplified TCR β gene transcripts were subcloned into a plasmid vector (pBluescript II, TOYOBO, Osaka, Japan). Finally, approximately 10 clones derived from each band were subjected to di-deoxy DNA sequencing (373A DNA Sequencing System, Perkin Elmer/Applied Biosystems, Foster, CA).

Alternatively, for determination of the frequency of individual T cell clonotypes, the first PCR products of TCR β gene transcripts were directly re-amplified with the two primers mentioned above and subcloned into plasmids for DNA sequencing.

Results

DETECTION OF ACCUMULATING T CELL CLONOTYPES IN RA PATIENTS

We first analysed T cell clonality in 20 major BV families in PBL and ST samples from five patients with RA. The TCR β gene transcripts, which were amplified by RT-PCR, were separated by their SSCPs for detection of accumulated TCR clonotypes.

The ST specimens from all the patients showed dozens of distinct bands, which indicated oligoclonal T cell accumulation.

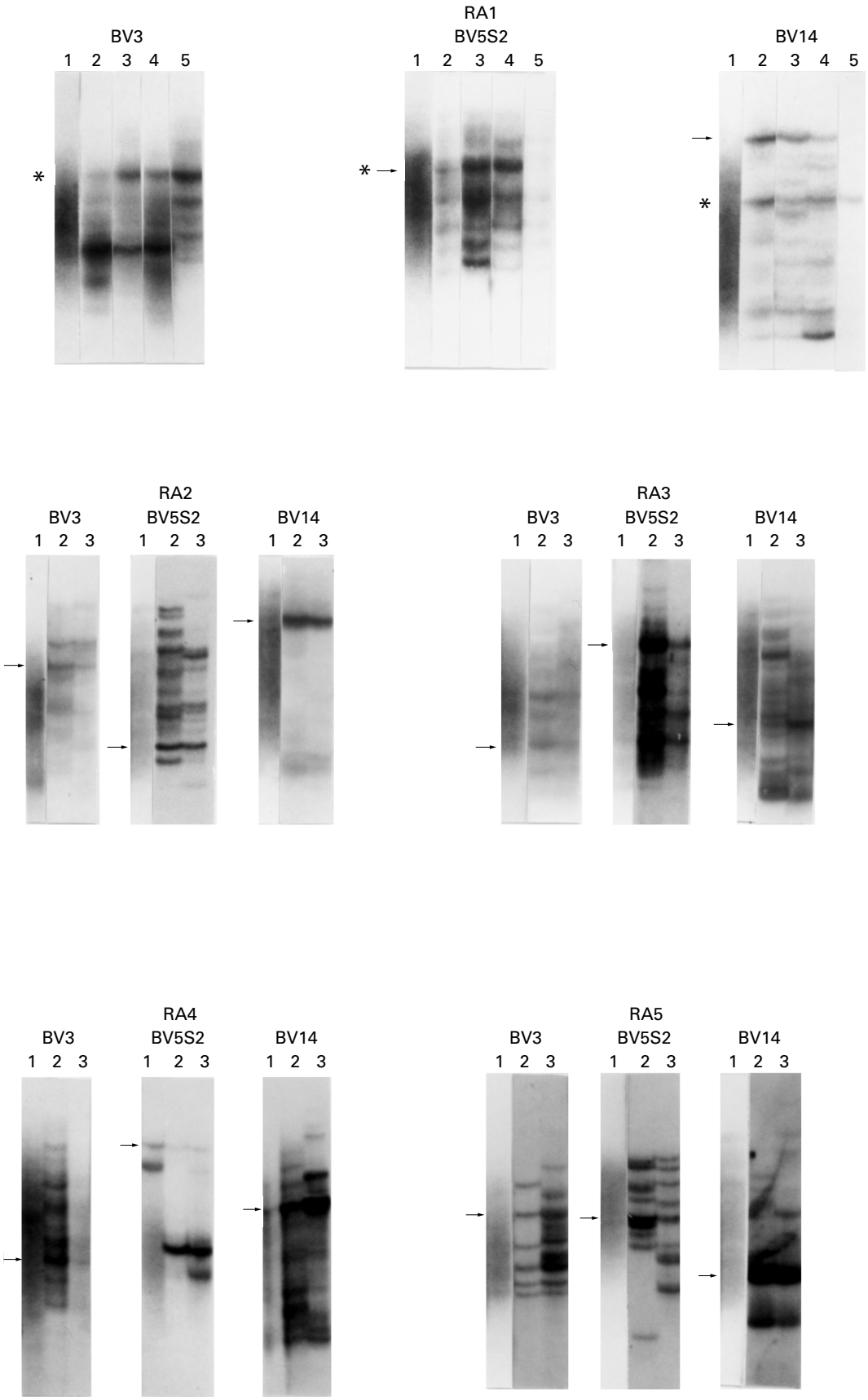

Figure 1 Comparison of accumulated TCR β gene clonotypes in different joints by RT-PCR/SSCP analysis. ST specimens were derived from four different joints of RA1 and two different joints of RA2–RA5, respectively. TCR β gene transcripts, amplified from each specimen by RT-PCR, were separated based on their single strand conformation polymorphism on a single gel to detect TCR β gene bands with identical migrating positions. Some of the distinct TCR β gene bands derived from different specimens were found to migrate to identical positions (indicated by asterisks). This indicates that T cells that possessed TCR β gene transcripts corresponding to these bands accumulated clonally in multiple joints. Results of representative three BV families (BV3, BV5S2 and BV14) are shown. Arrows indicate bands from which TCR β gene transcripts were recovered for determination of nucleotide sequences. The results of RA1 are shown as follows: (lane 1, PBLs; lane 2, ST from the left ankle; lane 3, ST from the right ankle; lane 4, ST from the left PIP; and lane 5, ST from the left elbow). The results of RA2–RA5 are shown as follows: RA2: lane 1, PBL; lane 2, ST from the right MP; lane 3, ST from the right knee. Similarly, RA3: lane 1, PBL; lane 2, ST from the right MP; lane 3, ST from the right elbow. RA4: lane 1, PBL; lane 2, ST from the right wrist; lane 3, ST from the left knee. RA5: lane 1, PBL; lane 2, ST from the right knee; lane 3, ST from the left knee.

Table 2 Deduced amino acid sequences in the junctional regions of TCR β chains carrying BV3 gene segment derived from (A) right ankle and (B) the left elbow joint of RA1

A

**F (%)*	*BV3*	*ᴺDᴺ*	*BJ*	
8 (24.2)	CASSL	TGR	TGELF	J2S2
2 (6.0)	CAS	TTLPGLVW	F	J2S1
2 (6.0)	CASS	PMKGG	TDTQY	J2S3
2 (6.0)	CASSL	GG	AKNIQY	J2S4
1 (3.0)	CASS	LGLMD	TEAF	J1S1
1 (3.0)	CASS	SPRQGD	TEAF	J1S1
1 (3.0)	CAS	RPGAAD	TEAF	J1S1
1 (3.0)	CASS	LAPNL	YGYT	J1S2
1 (3.0)	CASS	AGTGTL	YGYT	J1S2
1 (3.0)	CASSL	GGAG	NQPQH	J1S5
1 (3.0)	CAS	RTRGS	SYNSPLH	J1S6
1 (3.0)	CAS	TSD	SYNEQF	J2S1
1 (3.0)	CASS	SRLAT	SYNEQF	J2S2
1 (3.0)	CAS	SRQP	TGELF	J2S2
1 (3.0)	CASS	QAGGP	DTQY	J2S3
1 (3.0)	CASSL	GSA	DTQY	J2S3
1 (3.0)	CASS	QSGYIS	DTQY	J2S3
1 (3.0)	CASS	NRGY	TDTQY	J2S3
1 (3.0)	CASSL	FGV	YEQY	J2S7
1 (3.0)	CAS	RPSTSGT	YEQY	J2S7
1 (3.0)	CASS	PDYL	YEQY	J2S7
1 (3.0)	CAS	WQGT	YEQY	J2S7
1 (3.0)	CASS	PPDGS	SYEQY	J2S7

*Frequency of each clonotype. Total of 33 subcloned TCR β gene transcripts were analysed.

B

†F (%)	*BV3*	*ᴺDᴺ*	*BJ*	
13 (44.8)	CASSL	TGR	TGELF	J2S2
3 (10.3)	CAS	TTLPGLVW	F	J2S1
2 (6.9)	CASS	PMKGG	TDTQY	J2S3
2 (6.9)	CAS	SPAGT	GANVLT	J2S6
1 (3.4)	CAS	FTRDN	EAF	J1S1
1 (3.4)	CASS	QAVY	TEAF	J1S1
1 (3.4)	CAS	TLGGSR	QPQH	J1S5
1 (3.4)	CA	SSFSGL	NEQF	J2S1
1 (3.4)	CASSL	GRGN	NEQF	J2S1
1 (3.4)	CASS	NRGY	TDTQY	J2S3
1 (3.4)	CAS	VRQEGL	DTQY	J2S3
1 (3.4)	CASS	SDVTGV	YEQ	J2S7
1 (3.4)	CA	RRPGISGT	YEQ	J2S7

†Total of 29 subcloned TCR β gene transcripts were analysed.

There was no obvious bias of BV gene use in the accumulating T cell clonotypes. In contrast, only a few bands were detected in the analysis of PBL samples. Figure 1 shows the representative results. These results were consistent with our previous findings.[24 25] In addition, we tried to analyse T cell clonality in synovium from patients with osteoarthritis as a control for non-inflammatory joint diseases. However, the number of infiltrated T cells was too small for analysis of clonality (data not shown).

COMPARISON OF ACCUMULATING T CELL CLONOTYPES IN MULTIPLE JOINTS

To test whether identical T cells reside in multiple joints, we first sequenced randomly subcloned BV3 carrying TCR β genes from the right ankle and the left elbow of RA1. Table 2 describes the deduced amino acid sequences of 33 and 29 subcloned TCR β genes. As shown, the right ankle contained four accumulating T cell clonotypes, three of which were also found as accumulating clonotypes in the left elbow (BV3-TGR-BJ2S2, BV3-TTLPGLVW-BJ2S1, and BV3-PMKGG-BJ2S3, table 2A and B). This suggests that a considerable number of accumulating T cell clonotypes would be identical among multiple joints in RA patients.

To confirm and generalise this result, we tried to extend this study to various TCR BV families in multiple joints of all five patients using RT-PCR/SSCP. We amplified TCR β gene transcripts from different joints by PCR, and then loaded the PCR products in lanes adjacent to each other on an SSCP gel to screen for bands that migrated to identical positions. For example, in the case of BV3 in RA1, the amplified TCR β gene transcripts derived from different joints were applied to a gel (lanes 2–5, fig 1). The lanes exhibited bands that migrated to an identical position (indicated by an asterisk), and these bands were defined as "common bands." In our previous studies, common bands had been confirmed to have identical nucleotide sequences.[25 29 30]

In all five patients, we detected a number of common bands, indicating that T cell clonotypes that had TCR β CDR3 sequences corresponding to the common bands had accumulated in the tested multiple joints. We defined such T cell clonotypes as "common clonotypes." Figure 1 shows representative cases of three BV families of RA1–5. In the case of RA1, common clonotypes were detected in ST samples from four different joints (lanes 2–5), but not from PBL (lane 1), as indicated by the asterisks. Similarly, in RA2, RA3 and RA5, the common bands indicated by arrows were obtained from ST samples of two joints (lanes 2 and 3) but not from the PBL samples (lane 1). Thus, these accumulating T cell clonotypes were thought to reside in multiple joints but not in PBLs. In the case of BV3 in RA4, the common bands indicated by an arrow were obtained from the ST samples of the right wrist (lane 2) and left knee (lane 3) but not from the PBL sample (lane 1). On the other hand, with regard to BV5S2 and BV14 in RA4, the bands that migrated to the positions indicated by arrows were obtained not only from the ST samples of the two joints (lanes 2, 3) but also from the PBL sample (lane 1).

The TCR β gene transcripts of the common bands used all of the tested 20 BV families. There appeared no remarkable BV gene use bias in the common clonotypes. We counted the number of SSCP bands detected in two joints from each of the five patients and found that 36–71% of the accumulating T cell clonotypes resided in multiple joints (average 44%, table 3A). Similarly, 45% of accumulating clonotypes were present in all four tested joints in RA1 (table 3B). In addition, in cases RA2 and RA4, who showed some SSCP bands in the PBL results, only a small portion of accumulated T cell clonotypes in joints appeared in PBLs (average 12 of 126, 10%) and the majority appeared only in joints (114 of 126, 90%, data not shown).

COMPARISON OF CDR3 AMINO ACID SEQUENCES OF TCR β CHAINS OF THE T CELL CLONOTYPES ACCUMULATED IN MULTIPLE JOINTS

To characterise the common clonotypes, we determined the DNA sequences of TCR β

Table 3 Number of clonally accumulated T cells detected in multiple joints
A Number of clonally accumulated T cells detected in multiple lesions of five RA patients

	TCR BV families															
Patients joints	*1*	*2*	*3*	*4*	*5S1*	*5S2*	*6*	*7*	*8*	*9*	*11*	*12*	*13S1*	*13S2*	*14*	*15*
RA1																
PBL	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
left ankle	4/6	1/3	2/4	4/5	-/0	4/5	-/7	-/8	5/5	-/10	-/14	-/4	-/3	0/3	5/5	-/6
right ankle	4/6	1/7	2/2	4/8	-/6	4/5	-/10	-/6	5/8	-/13	-/10	-/3	-/3	0/6	5/11	-/4
RA2																
PBL	2/5	S	S	S	S	S	1/3	S	1/2	2/2	2/2	0/1	1/3	0/6	S	S
right MP	2/14	4/9	4/4	3/9	2/11	3/11	1/9	3/7	4/8	6/8	2/4	S	3/7	1/3	2/2	1/2
right elbow	2/10	4/9	4/5	3/7	2/8	3/12	1/6	3/7	4/6	6/6	2/3	S	3/7	1/6	2/3	1/6
RA3																
PBL	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
right MP	7/11	S	2/4	3/6	1/3	4/9	0/3	S	4/9	2/3	2/7	S	1/7	1/7	3/8	1/11
right elbow	7/13	0/4	2/2	3/5	1/5	4/5	0/9	0/4	4/8	2/5	2/3	0/2	1/4	1/4	3/6	1/8
RA4																
PBL	3/5	S	S	S	S	1/2	S	S	1/2	S	3/5	S	0/3	0/4	1/1	S
left knee	5/10	4/4	1/2	0/2	0/1	2/3	1/4	5/8	5/14	4/13	7/11	5/11	0/9	S	3/8	4/13
right wrist	5/10	4/6	1/9	0/2	0/3	2/2	1/3	5/6	5/8	4/14	7/11	5/10	S	S	3/9	4/15
RA5																
PBL	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
left knee	1/3	6/15	5/6	0/3	4/4	3/8	5/8	4/6	2/7	3/6	0/3	0/7	2/7	0/5	2/4	0/3
right knee	1/9	6/6	5/8	S	4/10	3/8	5/12	4/10	2/4	3/6	0/2	0/7	2/6	S	2/5	S

T cell clonality in two joints of each patient was analysed by SSCP. Numbers of SSCP bands that were detected in each joint (total clonotypes) and that were detected in both joints (common clonotypes) are shown as common clonotypes/total clonotypes. Analysis of BV10 and BV19 was not included as these two genes were recently reported to be transcribed pseudogenes.[40] S, smear-like results (no band).

B Number of clonally accumulated T cells detected in multiple joints of RA1

	TCR BV families											*Total number in compared 11 BV families (average)*	*Average in one BV family (%)*
Patients	*1*	*2*	*3*	*4*	*5S2*	*8*	*13S2*	*14*	*17*	*18*	*20*		
Left ankle	4/6	3/3	1/4	3/5	5/5	4/5	0/3	1/5	0/4	5/11	2/5	28/56	
Right ankle	4/6	3/7	1/2	3/8	5/5	4/8	0/6	1/11	0/4	5/10	2/4	28/71	
Left PIP	4/6	3/7	1/2	3/6	5/6	4/8	0/2	1/8	0/4	5/12	2/4	28/65	2.5/5.5
Left elbow	4/4	3/3	1/4	3/5	5/6	4/6	0/4	1/1	S	5/12	2/4	28/49 (28/60)	(45)

T cell clonality in four joints of RA1 was analysed by SSCP. Comparison of the other BV families was not available because of the limited amounts of the samples. Number of SSCP bands that were detected in all four joints were counted as common clonotypes. Results are shown as common clonotypes/total clonotypes. S, smear-like results (no band).

CDR3 of the clonotypes, concentrating on three BV families, BV3, BV5S2 and BV14, as all five patients showed common bands in these three BV families. BV3 of RA1 was analysed by DNA sequencing alone, as described above (table 2). In the other cases, the DNA fragments recovered from the common bands on SSCP gels (indicated by arrows in fig 1) were subjected to DNA sequencing. We recovered DNA fragments of each common band from two lanes in which samples from different joints were loaded, and subcloned them into a plasmid vector for DNA sequencing. When we obtained at least two subcloned genes that carried an identical sequence from each of two joints, we defined the sequence as that of TCR β CDR3 of a common clonotype.

As table 4 shows, we obtained 23 amino acid sequences for TCR β CDR3 of common clonotypes from the sequence results of RA1-BV3 (table 2) and the 14 common bands (indicated by arrows in fig 1). In this analysis, we sequenced 5 to 12 subcloned genes per band and found that 20 to 80% of the obtained sequences showed identity. For example, in the case of the bands in BV14 of RA5 (fig 1, indicated by an asterisk), eight of 10 subcloned genes derived from the right knee band and seven of nine subcloned genes derived from the left knee band possessed the same sequence, BV14-SGQGH (table 4). In some cases, two to three TCR β sequences were obtained from a single band as commonly accumulating clonotypes in multiple joints (that is, BV3 of RA2, table 4). The mechanism for this is discussed later.

We found five pairs of common clonotypes with TCR β chains that carried identical stretches of four or three amino acids, LPGL, SGQG, TSG, GTE, and SGT, in the CDR3 regions (table 4). Interestingly, the paired clonotypes were derived from different patients who possessed several identical HLA class II molecules (tables 1 and 4).

Discussion

To analyse whether identical T cells were present in multiple rheumatoid joints, we first compared nucleotide sequences of TCR β gene transcripts between two joints of RA1 (table 2). Three of four accumulating TCR β clonotypes in one joint were also detected as major TCR clonotypes in the other joint. This encouraged us to determine whether this phenomenon can

Table 3(A) continued

16	*17*	*18*	*20*	*Total number in 22 BV families (average†)*	*Total number in compared BV families (average†)*	*Average in one BV family† (%)*
S	S	S	S	all S	all S	
-/6	3/4	6/11	3/5	114	37/43*	3.4/4.7
-/3	3/4	6/10	3/4	129	37/61*	(71)
				(122)	(37/52)*	
S	0/1	S	1/1	27	9/27	
5/7	0/1	S	1/7	123	47/123	2.4/6.2
5/7	0/1	0/10	1/6	125	47/125	(38)
				(124)	(47/124)	
S	S	S	S	all S	all S	
4/7	3/4	1/7	1/9	115	40/115	2.0/5.6
4/6	3/4	1/4	1/5	106	40/106	(36)
				(111)	(40/111)	
S	S	S	S	22	9/22	
1/8	1/2	1/3	0/6	132	49/132	2.5/6.4
1/9	1/2	1/4	S	123	49/123	(38)
				(128)	(49/128)	
S	S	S	S	all S	all S	
0/2	S	2/3	1/2	102	38/102	1.9/5.0
S	0/1	2/1	1/2	97	38/97	(38)
				(100)	(38/100)	
				(117)‡		(44)§

†Averages were calculated only from the results of joints. -, In the case of RA1, clonotypes in BV5S1, 6, 7, 9, 11, 12, 13S1, 15 and 16 were not compared between the joints because of the limited amounts of the samples, and thus were omitted from the analysis(*). ‡Average number of the total clonotypes in one joint in the five patients. §Average frequency of the common clonotypes in the five patients.

Table 4 Deduced amino acid sequences of the TCR β CDR3 regions of T cell clonotypes that accumulated in multiple joints

	BV3	*NDN*				*BJ*	
	95	*95*	*98*	*100*	*103*		
RA1	CASSL	TGR				TGELF	J2S2
	CAS	TT**LPGL**VW				F	J2S1
	CASS	PMKGG				TDTQY	J2S3
RA2	CAS	TSGTES				YNEQF	J2S1
	CAS	SSVAGIGGL				YGYT	J1S2
RA3	CA*S*	*GT*GGGE				ETQY	J2S5
RA4	CA	IT**SGQG**D				EQY	J2S7
	CASS	PGTEA				NYGYT	J1S2
	CAS	SLGQI				NQPQH	J1S5
RA5	CAS	RPGQPY				SNQPQH	J1S5
	BV5S2	*NDN*				*BJ*	
	95	*96*	*98*	*100*	*103*		
RA1	CASSL	SVFD				EQF	J2S1
RA2	CASS	DPGGVQF				YEQY	J2S7
RA3	CASS	WSCGRL				TDTQY	J2S3
RA4	CASS	L**LPGL**AGKAS				NEQF	J2S1
RA5	CASS	FGQP				NEQF	J2S1
	CASSL	RGI				KNIQY	J2S4
	BV14	*NDN*				*BJ*	
	95	*95*	*98*	*100*			
RA1	CASS	FGL				NTEAF	J1S1
	CASS	SARFH				NEQF	J2S1
RA2	CA	CAGTL				YNEQF	J2S1
RA3	CASS	FGGPG				TGELF	J2S2
	CASS	FRRG				ETQY	J2S5
RA4	CASSL	RRQ				DTQY	J2S3
RA5	CASS	**SGQG**H				YEQY	J2S7

Deduced amino acid sequences of the TCR β CDR3 regions of T cell clonotypes that accumulated in multiple joints in each of the five RA patients. Results of the tested three BV families (BV3, BV5S2, BV14) are shown. All the clonotypes shown here were detected at least in two joints. The identical amino acid sequences detected from different patients are shown as follows: LPGL and SGQG, bold; TSG, underlined; GTE, double underlined; and SGT, italic.

be generalised in RA patients. Recently, Alam *et al* reported comparison of T cell clonotypes of four or five BV families in multiple joints of two RA patients by nucleotide sequencing.[31 32] However, it is difficult to extend this study by DNA sequencing alone to various BV families with a greater number of patients, because such a large number of samples must be sequenced. To avoid this difficulty, we screened accumulated T cell clonotypes using the SSCP method before nucleotide sequencing. Thereby, we were able to analyse 20 BV families in five RA patients. SSCP analysis can detect accumulated TCR β gene clonotypes whose frequency is 1/1600–6400 and higher.[24 33]

In the study by Alam *et al*, the frequency of T cell clonotypes that accumulated in multiple joints differed between the two tested patients. Thus, the frequency of the identical accumulated T cell clonotypes remained to be determined. Our extended study including 20 BV families from the five RA patients revealed that 44% of the dominant T cells were identical between two joints on average (table 3A). The analysis of four joints in RA1 provided a similar frequency of common clonotypes (45%). This frequency of 44% was much lower than that of the clonotypes that resided in different sites of the same joints (60–100%, Ikeda *et al*[25]). Thus, these clonotypes accumulating in multiple joints may be selected because of the ability of their TCR to recognise joint derived peptides, presented by HLA molecules.

As 90% of the accumulating T cell clonotypes in joints were not detected in PBLs, it can be supposed that the T cell clonotypes would have accumulated selectively in joints. If antigens of the clonally accumulating T cells were quite unrelated to joint components, and some of the T cell clonotypes happened to be translocated from one joint to other joints, ratios of the common clonotypes to the total accumulating T cell clonotypes would increase according to the duration of the RA. However, the ratios were similar among the five patients despite differences in the durations of disease among these patients. Thus, it is more probable that the T cell clonotypes accumulating in multiple joints were activated by antigens related to joint components and the remaining accumulating T cell clonotypes had less affinity to joint antigens. In this case, T cell clonotypes that recognise joint related antigens in one joint would have been predominantly translocated to other joints in which identical antigens were presented. It is also possible that T cells activated by exogenous antigens cross reactively recognise joint antigens and come to reside in multiple joints.

The sequencing study of the common bands showed that those bands contained identical TCR β sequences of clonally accumulated T cells at ratios of 20–80%. As an SSCP band of an accumulated T cell clonotype lays over a smear-like background consisting of extremely heterogeneous unaccumulated T cell clonotypes, not only one TCR β sequence of an accumulated clonotype but also various other sequences were always detected from a single band. Furthermore, the reasons for our

obtaining two or three dominant sequences from one SSCP band were considered to be as follows: (1) we only visualised antisense strands of the PCR products in the SSCP method, thus sense strands of other dominant clonotypes might migrate nearby to a visible band; (2) although there seemed to be a single band, actually two bands may have been present; and, in addition, (3) the SSCP method cannot separate all genetically diverse TCR β genes.

The interaction between TCRs and MHC-peptide complexes has been investigated by crystallography.[23 34 35] Garcia *et al* reported that, in both α and β chains, CDR1 and CDR2 straddled the central region of the peptide.[35] Garboczi *et al* reported that CDR3 and, to lesser extent, CDR1 of the both chains contacted the peptide.[23] From these results, the 95th–98th, 100th, 101st and 103rd amino acid sequences in the CDR3 of the TCR β chains were considered to directly interact with the peptide.[23 35] We detected five pairs of clonotypes that had identical amino acid sequences in their TCR β CDR3 regions out of the 23 common clonotypes (table 4). The conserved amino acid sequences were loaded within the six amino acid from the 95th to the 100th, indicating that these conserved amino acids may be deeply involved in the antigen recognition. Of note is that these clonotypes having identical 3–4 amino acid sequences in their TCR β CDR3 were derived from different patients who possessed identical HLA molecules. Among those, DRB1*0405, which is the most frequently observed HLA allele in Japanese RA patients,[36 37] was seen in three of the five pairs. The TCRs that carried such identical sequences may recognise similar or identical antigens in the context of the shared HLA molecules. Furthermore, one of our conserved amino acid sequences, TSG, was also observed in the study by Alam *et al*.[32] This TSG sequence was found in HLA-DR4+ patients in both studies. This suggests that an identical antigen may be commonly presented by the DR4 molecule in those RA patients.

Informatively, several studies reported that TCRs recognising a single MHC-peptide complex were highly diverse in their CDR3 sequences.[38 39] Thus, common clonotypes with different CDR3 sequences can respond to the same HLA-peptide complex. In this regard, the oligoclonal T cell accumulation in multiple rheumatoid joints could be caused by highly restricted antigens.

In summary, we detected the T cell clonotypes that accumulated in RA patients and determined their conserved CDR3 amino acid sequences. These clonotypes may recognise restricted antigens that are ubiquitous in joints and are involved in the pathogenesis of RA.

We are indebted to Professor Katsushi Tokunaga in Tokyo University, and Mr Hidenori Tanaka and Mr Kouichi Kashiwase in Japanese Red Cross Central Blood Centre for analysis of HLA haplotypes of the patients. We thank to Professor Takayuki Sumida of Tsukuba University and Dr Tomoko Hasunuma of St Marianna University for their support to our experiments. We also thank Ms Hiroko Sasakawa and Ms Yuka Onoki in St Marianna University for their technical assistant.
Funding: this work was supported in part by grants from the Ministry of Health and Welfare and the Ministry of Education of Japan.

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