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Research Article

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Transfer of Rheumatoid Arthritis into Severe Combined Immunodeficient Mice

The pathogenetic implications of T cell populations oligoclonally expanding in the rheumatoid joints

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Abstract

To investigate the pathogenicity of T cells infiltrating in the rheumatoid joints, mononuclear cells (MNC), predominantly T cells, isolated from either synovial fluid or synovial tissues of the patients with RA were transferred into severe combined immunodeficient (SCID) mice by intraarticular injections. According to our observations in this experimental system, patients with RA could be classified into at least two groups. In one group of patients, the infiltrating MNC induced synovial hyperplasia in the recipient SCID mice (the positive group). Whereas, in the other group no synovial hyperplasia was observed (the negative group). The induction of synovial hyperplasia observed in the positive group was prevented by an anti-human CD3 antibody (OKT3), indicating T cell mediation. Analysis of T cell receptor (TCR) Vβ usage by reverse transcriptase polymerase chain reaction in the infiltrating MNC transferred into SCID mice revealed a marked skew towards the preferential use of certain Vβ genes, which was not seen in the peripheral blood MNC, in only the positive group. The patterns of TCR/Vβ skew were not uniform among the patients. The analysis of the PCR-amplified genes of such skewed TCR/Vβ by single strand conformational polymorphism showed distinct bands, indicating that the T cell populations expanding in rheumatoid joints of the positive group were oligoclonal. Furthermore, the enrichment of the T cell populations expressing such skewed TCR/Vβ by in vitro stimulation of peripheral blood MNC of the patients with the relevant superantigen enabled the induction of synovial hyperplasia in the SCID mice. These results suggest that the pathogenic T cells could be activated locally in rheumatoid joints by certain antigens in some, but not in all patients with RA. (*J. Clin. Invest.* 1995. 96:1746–1758.) Key words: synovial infiltrating T cell • synovial hyperplasia • reverse transcriptase-PCR • T cell receptor/Vβ repertoire • superantigen

Introduction

RA is one of the most common chronic inflammatory diseases characterized by destructive polyarthritis (1). Although the etiology of RA remains unknown, an autoimmune process is thought to be involved in the pathogenesis. Considerable evidence has been accumulated for the critical role of T cells in the initiation and perpetuation of RA (2). (*a*) Activated T cells, specifically the CD4+ phenotype, predominate in the infiltrating mononuclear cells (MNC)[1] of the rheumatoid joints (3–7). (*b*) Susceptibility to RA is associated with particular alleles (DR4) of the MHC class II genes (8–11). (*c*) Partial elimination or inhibition of T cells by immunosuppressive drugs and anti-T cell antibodies can ameliorate the disease (12–16). In the mouse model such as collagen-induced arthritis (CIA), the disease process is T cell mediated, necessitating a particular combination of TCR/Vβ genes and MHC class II genes and the efficacy of anti-TCR/Vβ therapy has been demonstrated (17–21). These observations suggest that a similar scenario may be taking place in human RA. In fact, the TCR/Vβ repertoire in the infiltrating T cells of the patients with RA has been extensively studied using a variety of techniques, such as anti-TCR/Vβ antibodies, RFLP, and PCR (22–30). However, as to the question of whether there is a preferential use of certain Vβ genes in the infiltrating T cells, no clear answer has yet been provided. This may be caused by the pathogenetic heterogeneity of RA because the clinical features of individual patients with RA vary and the diagnosis of RA is made according to the standard criteria (31). Moreover, recent investigations using the mice carrying and expressing the transgenes of inflammatory cytokines (32) and retrovirus (33) suggest non-T cell–mediated mechanism of the pathogenesis of RA. Therefore, the strategy to identify the pathogenic T cells in RA by the analysis of TCR/Vβ repertoire could be applied for some, but not all patients, in whom the pathogenesis of RA is thought to be T cell mediated. However, there have been no particular means how to distinguish between the patients with RA, whose pathogenesis is T cell mediated or not.

Mosier et al. and McCune et al. demonstrated the reconstitution of human immune response in severe combined immunodeficient (SCID) mice by the transfer of either human peripheral blood MNC (PBMNC) or lymphoid tissue (34, 35). Recently, in multiple sclerosis (MS) which is thought to be a T cell-mediated autoimmune disease as well as RA, we have demonstrated the development of MS like pathology, including demyelination localized in the central nervous system, in SCID mice by transferring MNC infiltrating in cerebrospinal fluid of the patients with MS (36). Therefore, it may be possible to investigate the pathogenicity of T cells infiltrating in the rheumatoid joints using such cell transfer experiments in SCID mice.

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1. *Abbreviations used in this paper:* MNC, mononuclear cell; RT-PCR, reverse transcriptase PCR; SCID, severe combined immunodeficient; SEC2, Staphylococcus enterotoxin C2; SF, synovial fluid; SIMNC, synovial tissue infiltrating MNC; SSCP, single strand conformational polymorphism; TCR/Vβ, T cell receptor Vβ.

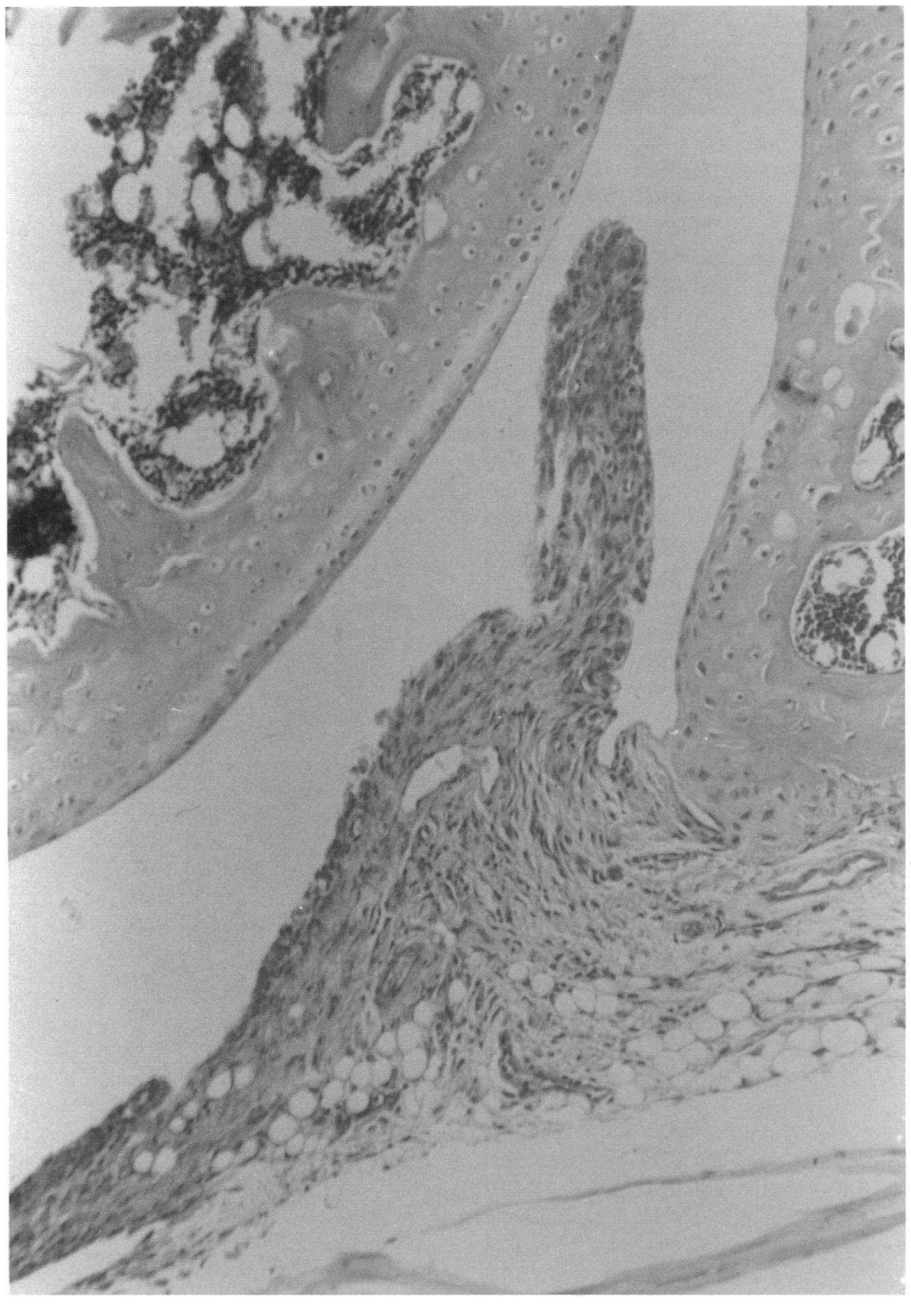

Figure 1. The representative histopathological changes observed in the injected SCID mice. SF or synovial tissue were obtained from the affected joints of the patients with RA. The MNC were prepared from the synovial samples, as described in Methods. 1.0×10^6 MNC were transferred into SCID mice by intraarticular injections at the knee joints of posterior legs. 4 or 8 wk after cell transfer, injected mice were killed with anesthesia. Bilateral knee joints of posterior legs were removed and fixed in buffered formalin. After decalcified and embedded in paraffin, seven serial sections were cut at 3 μm thickness. Sections were stained with hematoxylin and eosin and subjected to histopathological examinations. The histopathological changes characterized with villous changes of synovium with proliferation of synovial cells, angiogenesis, and infiltration of inflammatory cells were observed in the injected joints of the SCID mice.

In this study, we investigated the pathogenicity of T cells infiltrating in the rheumatoid joints by transferring the MNC from synovial fluid (SF) or synovial tissue obtained from the affected joints into SCID mice. In the patients with RA, whose T cells could transfer the RA-like pathology, the TCR repertoire of the T cells infiltrating in the joints was investigated by analysis of TCR/Vβ genes usage using reverse transcriptase PCR (RT-PCR).

Methods

Patients and synovial samples. Human experimentation (use of patients SF and synovial tissues) in this study was approved on May 27, 1992 by the Ethical Committee of Osaka University Medical School. We obtained samples from 41 patients with informed consent according to the guidelines of the Ethical Committee. All patients with RA fulfilled the 1987 standard diagnostic criteria proposed by the American Rheumatism Associations, being followed at either Osaka-Minami National Hospital or the Department of Medicine III, Osaka University Medical School. SF were obtained by intraarticular puncture. Synovial tissues were obtained from the affected joints at the surgical treatments. At the time of sampling all patients had active inflammatory disease.

Isolation of MNC (synovial fluid mononuclear cells [SFMNC], synovial tissue infiltrating mononuclear cell [SI-MNC]) from synovial samples. Single cell suspensions were prepared from synovial samples (SF or synovial tissues) by enzymatic treatment. Briefly, SF was incubated with 5 U/ml of heparin (Novo Nordisk A/S, Bagsvaert, Denmark) and 5 U/ml of hyaluronidase (Mochida Pharmaceutical Co. Ltd., Tokyo, Japan) for 20 min at 37°C. In the case of synovial tissues, they

Table I. Induction of Synovial Hyperplasia in the SCID Mice Injected

Donor	Sex	Disease	HLA-DR	Cell transferred	No. of cells transferred ($\times 10^5$)	Number of injection	Incidence of synovial hyperplasia (Positive mice/total mice)
Patient 1	F	RA	DR2/DR4/DRw53	SIMNC	1.0	×1	3/6
Patient 2	F	RA	DR4/DRw15	SFMNC	10.0	×3	2/5
Patient 3	F	RA	DR1/DR8.1	SFMNC	1.0	×5	2/3
				SFMNC	1.0	×4	2/3
Patient 4	F	RA	DR4/−	SFMNC	10.0	×4	3/4
				SFMNC	10.0	×4	2/2
Patient 5	F	RA	N.T.	SFMNC	3.0	×1	2/5
Patient 6	F	RA	N.T.	SIMNC	3.0	×1	4/5
Patient 7	F	RA	DR4/−	SFMNC	1.0	×1	2/6
Patient 8	M	RA	DRw8.2/DRw15	SFMNC	10.0	×4	1/1
Patient 9	F	RA	DR4/−	SFMNC	10.0	×1	2/4
Patient 10	F	RA	DRw12/DRw15	SFMNC	10.0	×1	1/6
Patient 11	F	RA	DR4/−	SFMNC	1.0	×1	1/1
Patient 12	M	RA	DR1	SFMNC	10.0	×4	0/4
				SFMNC	10.0	×3	0/5
Patient 13	F	RA	N.T.	SFMNC	10.0	×1	0/4
Patient 14	F	RA	N.T.	SFMNC	10.0	×3	0/2
				SFMNC	1.0	×1	0/5
Patient 15	F	RA	DR4	SFMNC	10.0	×1	0/4
Patient 16	F	RA	N.T.	SFMNC	10.0	×4	0/2
Patient 17	M	RA	DR4/DR9	SIMNC	1.0	×1	0/13
Patient 18	M	RA	DR4/−	SFMNC	10.0	×6	0/1
Patient 19	M	RA	DR4/−	SFMNC	10.0	×3	0/2
				SFMNC	10.0	×4	0/4
Patient 20	F	RA	DR4/−	SFMNC	1.0	×2	0/2
				SFMNC	10.0	×4	0/2
				SFMNC	10.0	×1	0/2
Patient 21	F	RA	DR1/DR4	SFMNC	10.0	×1	0/5
Patient 22	F	RA	N.T.	SFMNC	10.0	×1	0/4
Patient 23	F	RA	N.T.	SFMNC	10.0	×1	0/5
Patient 24	M	RA	DR4/−	SFMNC	1.0	×4	0/2
Patient 25	F	RA	N.T.	SFMNC	10.0	×1	0/3
Patient 26	F	RA	N.T.	SFMNC	10.0	×1	0/5
Patient 27	M	RA	N.T.	SFMNC	10.0	×4	0/1
Patient 28	F	RA	N.T.	SFMNC	10.0	×1	0/4
Patient 29	F	RA	N.T.	SFMNC	10.0	×1	0/5
Patient 30	F	RA	DRw8.1/DRw12	SFMNC	10.0	×1	0/4
Patient 31	F	RA	N.T.	SFMNC	10.0	×1	0/5
Patient 32	F	RA	DR4/−	SFMNC	10.0	×1	0/4
Patient 33	F	RA	N.T.	SFMNC	10.0	×1	0/4
Patient 34	F	RA	DR4/DRw8.2	SFMNC	10.0	×1	0/3
Patient 35	F	RA	N.T.	SFMNC	10.0	×1	0/3
Patient 36	M	RA	DR1/DRw15	SFMNC	1.0	×1	0/5
Patient 37	F	RA	N.T.	SFMNC	10.0	×4	0/4
Patient 38	F	RA	DRw8.1/−	SFMNC	10.0	×1	0/8
Patient 39	M	RA	DR4/−	SFMNC	1.0	×3	0/3
Patient 40	F	RA	N.T.	SFMNC	10.0	×4	0/1
Patient 41	F	RA	N.T.	SFMNC	1.0	×2	0/2
T.T.	F	polyarthritis	N.T.	SFMNC	10.0	×1	0/3
				SFMNC	10.0	×1	0/5
S.F.	M	OA	N.T.	SIMNC	1.0	×1	0/3
K.N.	M	OA	N.T.	SFMNC	0.5	×1	0/2
N.N.	F	OA	N.T.	SFMNC	10.0	×1	0/2
S.F.	F	SLE	N.T.	SFMNC	0.1	×1	0/2
S.K.	M	Normal	N.T.	PBMNC	1.0	×1	0/2
M.I.	M	Normal	N.T.	PBMNC	1.0	×1	0/3

Table 1. (Continued)

Donor	Sex	Disease	HLA-DR	Cell transferred	No. of cells transferred ($\times 10^5$)	Number of injection	Incidence of synovial hyperplasia (Positive mice/total mice)
				PBMNC	10.0	×5	0/1
N.F.	M	Normal	N.T.	PBMNC	10.0	×5	0/1
N.A.	M	Normal	N.T.	PBMNC	10.0	×5	0/1
T.M.	M	Normal	N.T.	PBMNC	10.0	×2	0/3
				PBMNC + PHA	10.0	×2	0/3
S.O.	M	Normal	N.T.	PBMNC	10.0	×2	0/3
				PBMNC + PHA	10.0	×2	0/4
A.O.	M	Normal	N.T.	PBMNC	10.0	×2	0/4
				PBMNC + PHA	10.0	×2	0/3

N.T., not tested. To investigate the pathogenicity of the MNC infiltrating in the rheumatoid joints, MNC isolated from synovial samples (SF or synovial tissues) of 41 patients with RA were transferred into SCID mice by intraarticular injections. As a control, the MNC from SF of five patients with non-RA inflammatory arthropathies and either PHA-activated or nonactivated PBMNC from seven healthy volunteers were transferred into the SCID mice in exactly the same way. 4–8 wk after the injections, all mice were killed with anesthesia and the bilateral knee joints of the posterior legs of the mice were removed. Histopathological examinations of each sample were performed in seven serial sections stained with hematoxylin and eosin. The incidence of synovial hyperplasia was presented by the ratio of the positive mice to the total injected mice. The positive mice were judged by the incidence of synovial hyperplasia in at least one out of two injected joints of the individual mouse.

were minced into small pieces and treated with 0.2 mg/ml of DNase (Sigma Chemical Co., St.Louis, MO) and 2 mg/ml of collagenase (Sigma Chemical Co.) for 3 h at 37°C. MNC were isolated from such single cell suspensions by Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation.

Animals. Animal experimentations in this study were performed according to the guidelines of the Animal Experimentation Committee of Osaka University. Inbred C.B-17 scid/scid mice, 6–8 wk old, were supplied by the Central Institute for Experimental Animals. Mice were matched for age and sex in each experiment. Mice were housed in sterilized microbarrier units under germ-free conditions.

Cell transfer into SCID mice by intraarticular injections. $1.0 \times 10^5 - 1.0 \times 10^6$ MNC, suspended in 50 μl of HBSS, were transferred into SCID mice by intraarticular injections with a 28-gauge needle syringe at the knee joints of the bilateral posterior legs.

Histopathological examinations. 4–8 wk after the cell transfer, the SCID mice were killed with anesthesia. Bilateral knee joints of the posterior legs of the mice were removed and fixed in 10% buffered formalin for 3 d, and decalcified in 25% formic acid and 10% sodium citrate for two or three days. After decalcification, the samples were embedded in paraffin and cut serial seven slices at 3 μm thickness. The serial sections were stained with hematoxylin and eosin, subjected to histopathological examinations.

FACS® analysis of MNC (SFMNC and SIMNC) from synovial samples. 1×10^5 MNC were stained with the labeled monoclonal antibodies specific for CD3, CD4, CD8, CD14, CD19, CD25, and HLA-DR (Becton Dickinson Immunocytometery Systems, Mountain View, CA). Cell staining was determined using a FACScan® flow cytometer (Becton Dickinson Immunocytemetory Systems). The data were analyzed using the Consort 30 software program (Becton Dickinson Immunocytemetory Systems).

Analysis of TCR/Vβ genes usage in the infiltrating MNC (SFMNC, SIMNC) and PBMNC. Total RNA were prepared from the MNC using acid guanidinium thiocyanate-phenol-chloroform (AGPC) method as described elsewhere (37), subjected to the synthesis of the first cDNA strand by reverse transcriptase and oligo-dT. The reaction was stopped by heating at 95°C before PCR. The cDNA were amplified by PCR with a relevant Vβ specific oligomer or 5′-Cβ oligomer, and 3′ Cβ oligomer (38). The number of cycles were determined for each cDNA to ensure that the amount of products proportional to the amount of Vβ specific mRNA present in the original sample. The number of PCR cycles used in this experiment was from 33 to 37. Within this difference of PCR cycle, the result could not be altered. PCR was carried out on a DNA thermal cycler (Perkin-Elmer Cetus Corp. Norwalk, CT) according to the following: for the denaturing step, at 94°C for 1 min; for the annealing step, at 60°C for 1 min; for the extension step, at

Table II. The Comparison of Clinical Characteristics of the Patients between the Positive and the Negative Group

	Disease duration (yr) (mean±SD)		C-reactive protein (mg/dl) (mean±SD)		Rheumatoid factor (IU/ml) (mean±SD)		Radiographical stage*			
							I	II	III	IV
Positive Group (n = 11)	6.88±6.45	$P < 0.05$	3.79±3.31	NS	145.18±114.64	NS	1	3	3	4
Negative Group (n = 30)	13.04±8.32		3.14±2.90		182.47±185.06		3	8	11	8

* According to the criteria of Steinbrocker et al (44). To examine the differences for the clinical characteristics between the positive and the negative groups, the disease duration, c-reactive protein, and rheumatoid factor were statistically analyzed with the Wilcoxon-Mann-Whitney *U* test. The mean of the disease duration of the positive group was shorter than that of the negative group ($P < 0.05$). The others had no significant differences between both groups.

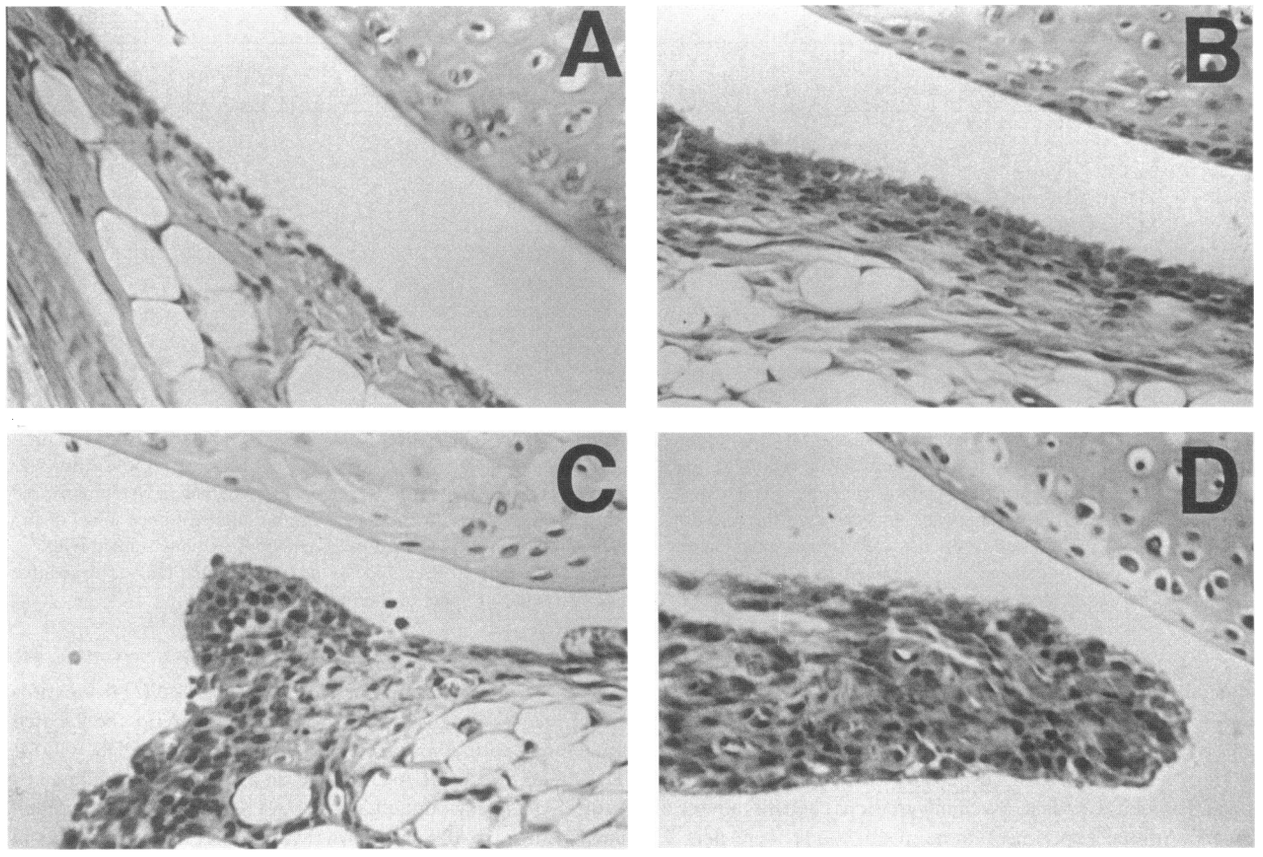

Figure 2. Grading the histopathological changes observed in the injected SCID mice. The histological changes of the synovial tissue were graded as follows. Grade 0: normal *(A)*. Grade 1: thickening of the synovial lining cells with hypertrophy *(B)*. Grade 2: villous changes of synovium *(C)*. Grade 3: villous changes of synovium and proliferation of synovial sublining cells *(D)*.

Table III. Prevention of the Synovial Hyperplasia in the SCID Mice Injected with the Infiltrating MNC by Anti-human CD3 Antibody

		Grade of synovial hyperplasia				Incidence
	Treatment	0	1	2	3	Positive/ total joints
Patient 5	IgG2a	7	1	1	1	3/10
	OKT3	9	1	0	0	1/10
Patient 6	IgG2a	3	5	2	0	7/10
	OKT3	9	1	0	0	1/10

To examine whether or not T cells mediate the induction of such synovial hyperplasia observed in the SCID mice injected with the infiltrating MNC from the patients of the positive group, the MNC from affected joints were injected into the knee joints of posterior legs in the presence of 100 μg/ml of either OKT3 or isotype-matched control (murine IgG2a). 4 wk after the injection, mice were killed with anesthesia and subjected to histopathological examinations. The histopathological changes were graded as described in Fig. 2. In patient 5, synovial hyperplasia were observed in 3 out of 10 joints in five mice injected with murine IgG2a. On the other hand, synovial hyperplasia as graded 1 was observed in 1 out of 10 joints in five mice of the OKT3-treated group. In patient 6, synovial hyperplasia was observed in 7 out of 10 joints in five mice of the murine IgG2a-treated group and in only 1 out of 10 joints in five mice of the OKT3-treated group. Thus, the incidence and the severity of synovial hyperplasia were markedly decreased in the mice injected with OKT3 compared to the control.

72°C for 1 min. The quantitative analysis of the amplified products with the ^{32}P end-labeled 3′ primers (1×10^6 cpm each) was made by Image analyzer (Fujix BAS 2000; Fujifilm I & I Co. Tokyo, Japan) after separation on 5–15% polyacrylamide gels. The relative expression of TCR/Vβ genes was determined by normalization of the density in the Vβ band to that in the Cβ band as an internal control.

Single strand conformational polymorphism (SSCP) analysis. The cDNA were amplified by PCR with a relevant Vβ specific oligomer and an oligomer for upstream of Cβ using 150 kilobecquerels of α[^{32}P]dCTP (DuPont/NEN Research Product Boston, MA). PCR was carried out on 35 cycles according to the following: for the denaturing step, at 94°C for 45 s; for the annealing step, at 60°C for 45 s; for the extension step, at 72°C for 1 min. The synthesized products, of which size estimated 240–50 bp, were heated at 96°C for 5 min with the dye containing formamide and separated on 8% polyacrylamide gels containing 10% glycerol.

In vitro stimulation of PBMNC with a relevant superantigen Staphylococcus enterotoxin C2 (SEC2). PBMNC isolated from two of the patients of the positive group (patient 3 and patient 5), were cultured with 10 ng/ml of SEC2 (Serva Feinbiochemica GmbH & Co., Heidelberg, Germany), which is known as a superantigen to stimulate T cell populations expressing such skewed TCR/Vβs (Vβ12, Vβ13), in complete medium (10% FCS RPMI-1640) for 4 d. After the harvest, the cells were washed with HBSS solution four times. Before the cell transfer, enrichment of the T cell populations expressing such skewed TCR/Vβ (Vβ12, Vβ13) was confirmed by the analysis of

TCR/Vβ genes usage using RT-PCR. As a control, PBMNC from the same individual were cultured with 2 μg/ml of PHA as a nonspecific stimulation without enrichment of such populations in exactly the same condition. Then, 1.0×10^6 MNC from each cell culture were transferred into SCID mice in exactly the same way described above. 4 wk after the cell transfer, the injected mice were killed with anesthesia and subjected to histopathological examinations.

Reagents. Hyaluronidase was obtained from Mochida Pharmaceutical Co. Ltd). OKT3 was from Janssen-Kyowa Co. Ltd., Tokyo, Japan.

Statistics. Clinical characteristics of patients, such as disease duration, C-reactive protein (CRP) and rheumatoid factor, were statistically analyzed using the Wilcoxon-Mann-Whitney *U* test.

Results

Induction of synovial hyperplasia in the SCID mice. To investigate the pathogenicity of T cells infiltrating in the rheumatoid joints, MNC, predominantly T cells, isolated from synovial samples (SF or synovial tissues) of 41 patients with RA were transferred into SCID mice by intraarticular injections. Although, we examined the incidence of arthritis in the mice by daily observations of clinical signs such as swelling, limitation of range of motion, and disturbance of gait after the transfer, no such clinical signs were observed. Then, 4–8 wk after the injections all mice were killed with anesthesia. The bilateral knee joints of the posterior legs of the mice were removed and subjected to histopathological examinations. Some of the mice injected with the infiltrating MNC developed histopathological changes in the synovium characterized with thickening, villous changes of synovial lining cells, and proliferation of sublining cells as shown in Fig. 1. The proliferating synovial cells were stained by immunohistochemistry with anti-murine MHC class I antigen (H-2K) antibody, indicating that they derive from the mice. No human leukocytes could be detected in the lesions by immunohistochemistry with anti-CD45 antibody (data not shown). However, human DNA was detected in the joints injected with human MNC 4 wk after the cell transfer by PCR using the primer for human specific *Alu* (39) (data not shown), indicating that human MNC could exist in the injected joints for at least 4 wk.

On the other hand, mice injected with either mitogen (PHA) activated or nonactivated PBMNC from seven healthy volunteers did not develop such synovial hyperplasia, indicating the histopathological changes observed in the SCID mice injected with the infiltrating MNC unlikely result from graft vs host reactions (Table I). The traumatic effects of articular injections are also unlikely to the cause of such observations, because multi-injections did not develop such synovial hyperplasia (Table I). Moreover, MNC of SF from the five patients with non-RA inflammatory arthropathies did not induce such synovial histopathological changes either, suggesting that our observations seem to be specific for RA (Table I). Interestingly, in some, but not all patients (11 out of 41 patients) studied, such synovial hyperplasia were observed (Table I). In other words, the patients with RA studied in this experiment could be classified into at least two groups. One is the group of the patients, whose infiltrating MNC induced synovial hyperplasia in the recipient SCID mice (the positive group). The other is the group without induction of synovial hyperplasia (the negative group). To investigate the factors for successful induction of synovial hyperplasia, the following were analyzed, *(a)* disease duration, *(b)* radiographical stages of the disease, *(c)* C-reactive protein, *(d)* rheumatoid factor. As a result, the mean duration of the disease of the patients in the positive group was significantly shorter than that of the negative group. However, the other factors had no significant difference between the two groups (Table II).

Table IV. FACS Analysis of the Infiltrating MNC Injected into the SCID Mice in the Representative Patients of the Positive and Negative Groups

			FACS analysis of MNC (%)					
	Donors	Cells transferred	CD3	CD25/ CD3	DR/ CD3	CD4/ CD8	CD19	CD14
	Patient 1	SIMNC	63	N.D.	N.D.	0.7	9.1	23.9
	Patient 3	SFMNC	92	13	89	0.6	1.0	1.3
	Patient 4	SFMNC	94	9	52	0.8	<1	3.0
(P)	Patient 7	SFMNC	93	4.4	48	3.1	5.0	N.D.
	Patient 8	SFMNC	87	5.8	60	1.1	1.0	5.0
	Patient 9	SFMNC	95	3.8	61	1.1	1.0	0.3
	Patient 10	SFMNC	81	4.9	28	2.1	2.0	0.5
	Patient 11	SFMNC	87	N.D.	N.D.	0.6	1.0	11
	Patient 12	SFMNC	75	13	84	1.9	9.0	<1
	Patient 14	SFMNC	96	6	68	1.4	1.3	5.0
	Patient 16	SFMNC	93	7	76	1.1	2.0	<1
(N)	Patient 17	SFMNC	65	N.D.	N.D.	0.7	18.3	21.0
	Patient 18	SFMNC	88	7	88	0.6	0.8	9.0
	Patient 19	SFMNC	81	8	77	1.5	5.0	2.0
	Patient 20	SFMNC	92	11	77	2.0	1.0	4.0
	Patient 21	SFMNC	91	0	78	1.2	0.1	3.5

(P), the positive group; (N), the negative group; N.D., not done. To examine the differences in the phenotypic characteristics of the infiltrating MNC injected into SCID mice between the two groups, the cells were analyzed by FACS®. 1.0×10^5 MNC were stained with the labeled monoclonal antibodies specific for CD3, CD4, CD8, CD14, CD19, CD25, and HLA-DR. Cell staining was determined using a FACScan® flow cytometer. The data were analyzed using the Consort 30 software program. The FACS® analysis revealed the predominance of T cells in both groups. The phenotypic characteristics, such as the CD4/CD8 ratio and the expression of activation markers (CD25 and HLA-DR), had no significant difference between the two groups.

The severity of the synovial hyperplasia varied among the mice injected with the MNC from the individual patients in the positive group. Then, these synovial hyperplasia could be graded from grade 0 to grade 3 as shown in Fig. 2. The synovial hyperplasia graded as 1 is characterized with thickening of the synovial lining cells with hypertrophy. Although such changes are minimal, they were never observed in the mice injected with the MNC from either the patients of the negative group or those with non-RA inflammatory arthropathies. Therefore, even grade 1 synovial hyperplasia is significant.

Prevention of the induction of synovial hyperplasia by murine monoclonal anti-human CD3 antibody (Table III). To examine whether or not T cells mediate the induction of the synovial hyperplasia observed in the SCID mice, we injected MNC obtained from SF of the two patients of the positive group (patient 5 and patient 6) in the presence of either 100 μg/ml of a murine monoclonal anti-human CD3 antibody (OKT3) or equivalent dose of murine IgG2a (PharMingen, San Diego, CA)

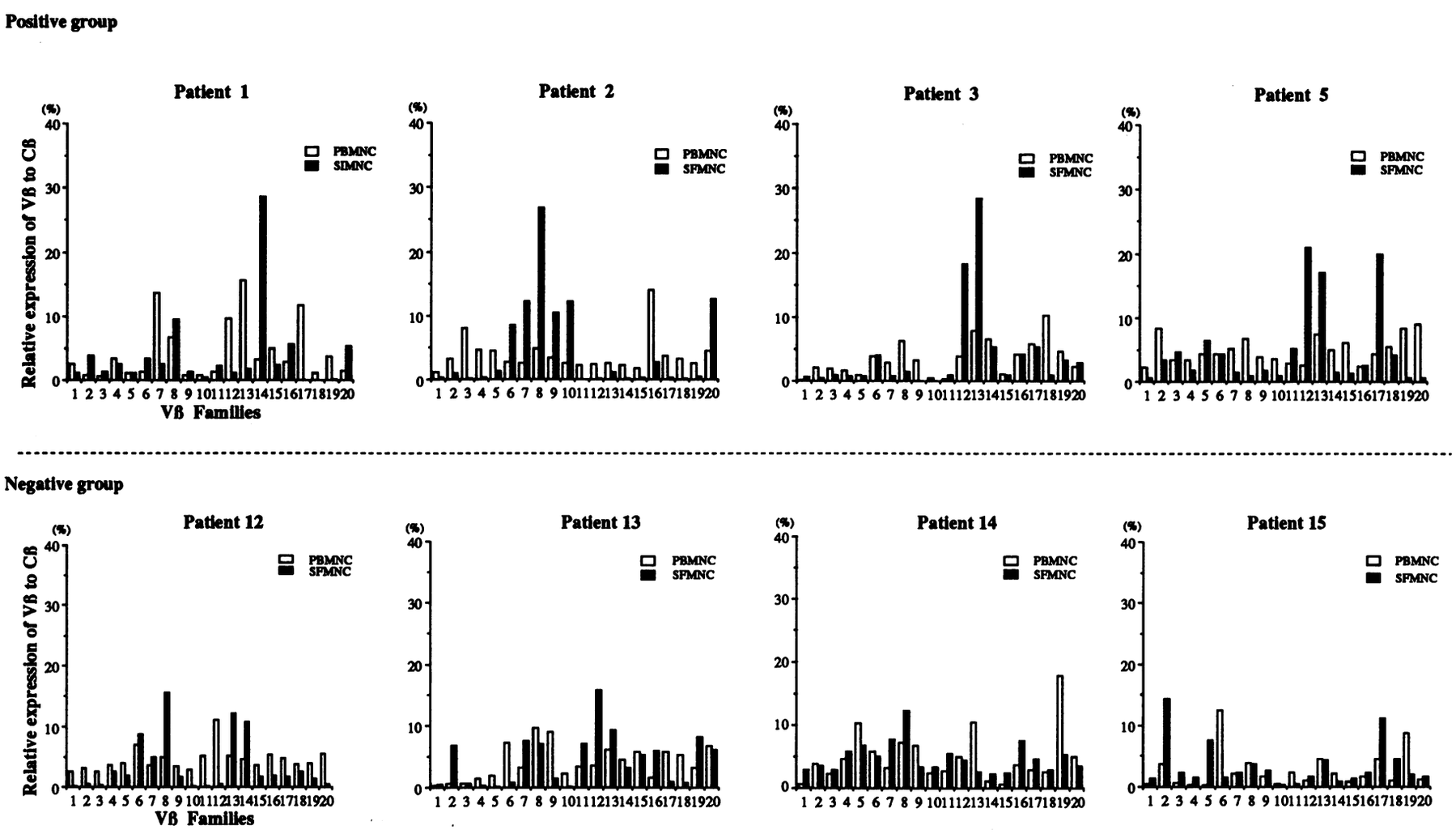

Figure 3. The comparison of TCR/Vβ genes usage of the infiltrating MNC (*SFMNC or SIMNC*) between the positive and the negative groups. TCR/Vβ genes usage of four patients in each group were analyzed by RT-PCR, as described in Methods. The result of TCR/Vβ genes usage of the infiltrating cells (*SIMNC* or *SFMNC*) and the PBMNC of each patient were presented as the relative expression of Vβ to Cβ by closed column and open column, respectively. (*SFMNC* or *SIMNC*, ■; *PBMNC*, □) A marked skew towards the preferential use of certain Vβ genes in the infiltrating MNC (> 20%) (patient 1, Vβ 14; patient 2, Vβ 8; patient 3, Vβ 12 and Vβ 13; patient 5, Vβ 12, Vβ13, and Vβ 13, and Vβ 17), which was not seen in the PBMNC of the individual patients, was observed in the positive group. On the other hand, such skew was not observed in the negative group.

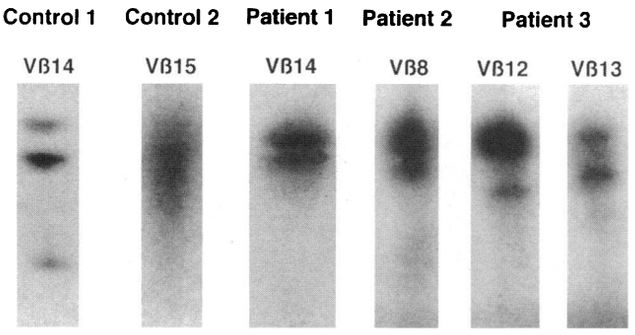

Figure 4. SSCP analysis on the skewed TCR/Vβ genes in the infiltrating MNC (SFMNC or SIMNC) from patient 1, patient 2, and patient 3 of the positive group. To examine whether or not the clonality of T cells could be determined by the SSCP analysis of TCR/Vβ genes, the amplified DNA of either the mixture of three T cell clones (*Vβ 14 positive*) or the polyclonal T cells from peripheral blood were analyzed by SSCP. Briefly, the cDNA of either the T cell clones or the polyclonal T cells were amplified by PCR with a Vβ-specific oligomer (T cell clones, *Vβ14*; polyclonal T cell, *Vβ15*) and an oligomer for upstream of Cβ using 150 kilobecquerels of α-[^{32}P]dCTP. The synthesized products were heated at 96°C for 5 min with the dye containing formamide and separated on 8% polyacrylamide gels containing 10% glycerol. Three distinct bands were observed in the lane of mixture of the T cell clones (*control 1*). On the other hand, no distinct bands were seen in the lane of the polyclonal T cells (*control 2*). This indicated that the clonality of T cells could be determined by the SSCP analysis of TCR/Vβ genes. Then, to examine the clonality of T cell populations expressing a certain skewed TCR/Vβ, the amplified DNA of the skewed TCR/Vβ families (*patient 1, Vβ14; patient 2, Vβ8; patient 3, Vβ12 or Vβ13*) were analyzed by SSCP in exactly the same way described above. A few distinct bands in the smear were seen in the gels. (two bands in Vβ14 of patient 1, one band in Vβ8 of patient 2, two bands in Vβ12 and Vβ13 of patient 3).

as an isotype matched control. 4 wk after the injection, the mice were killed with anesthesia and subjected to histopathological examination. The histopathological changes are graded as described above (Fig. 2). In patient 5, synovial hyperplasia was observed in 3 out of 10 joints (one for grade 1, one for grade 2, and one for grade 3) in five mice injected with control murine IgG2a. On the other hand, in five mice injected with OKT3 synovial hyperplasia graded as 1 was observed in only 1 out of 10 joints. In patient 6, synovial hyperplasia was observed in 7 out of 10 joints (5 for grade 1 and 2 for grade 2) in five mice treated with the control antibody. However, synovial hyperplasia graded as 1 was observed in only 1 out of 10 joints in five mice treated with OKT3. Thus, the incidence and the severity of synovial hyperplasia were markedly decreased in the mice injected with OKT3 compared to the control, indicating T cell mediation in the induction of synovial hyperplasia observed in the SCID mice.

FACS® analysis of the infiltrating MNC from the patients of the positive and negative groups. The FACS® analysis of the infiltrating MNC injected into the SCID mice was performed using the labeled monoclonal antibodies specific for CD3, CD4, CD8, CD14, CD19, CD25, and HLA-DR in the representative patients (8 patients for each group). As shown in Table IV, the FACS® analysis revealed the predominance of T cells in both groups. The phenotypic characteristics, such as the CD4/CD8 ratio and the expression of activation markers (CD25 and HLA-DR), had no significant difference between the two groups.

Skewed usage of TCR/Vβ genes in the infiltrating MNC (SFMNC or SIMNC) in the patients of the positive group. TCR/Vβ genes usage of the infiltrating MNC and PBMNC in the representative patients of both the positive and the negative groups (4 patients for each group) was analyzed by RT-PCR. As shown in Fig. 3, a marked skew (> 20% of relative expression of Vβ to Cβ) towards the preferential use of certain Vβ genes in the infiltrating MNC was observed in the patients of the positive group, whereas no skew was seen in the PBMNC obtained simultaneously from individual patients. In the case of patients of the negative group, no skew was observed in either PBMNC or infiltrating MNC. Moreover, the patterns of TCR/Vβ genes usage skew were not uniform among the patients (patient 1, Vβ14, patient 2, Vβ8, patient 3, Vβ12 and Vβ13; patient 5, Vβ12, Vβ13, and Vβ17). These results indicate that the T cell populations expressing a certain TCR/Vβ are expanding in the rheumatoid joints of patients of the positive group.

SSCP analysis on the skewed TCR/Vβ genes in patient 1, patient 2, and patient 3. To examine whether or not the clonality of T cells could be determined by the SSCP analysis of TCR/Vβ genes as reported elsewhere (40), the amplified DNA of either the mixture of three T cell clones (Vβ14 positive) or the polyclonal T cells from peripheral blood were analyzed by SSCP. The three distinct bands were observed in the lane of mixture of T cell clones, whereas no distinct bands were seen in the lane of polyclonal T cells (Fig. 4). This indicates that the clonality of T cells could be determined by the SSCP analysis of TCR/Vβ genes. Then, to examine the clonality of T cell populations expressing a certain skewed TCR/Vβ in the rheumatoid joints of patients of the positive group, the PCR-amplified genes of the skewed TCR/Vβ families from three patients of the positive group (patient 1, patient 2, and patient 3) were analyzed by SSCP. As shown in Fig. 4, a few distinct bands in the smear were seen in the gels (two bands in Vβ14 of patient 1, one band in Vβ8 in patient 2, two bands of Vβ12 and Vβ13 of patient 3), indicating that the T cell populations expanding in the rheumatoid joints of patients of the positive group were oligoclonal.

Induction of synovial hyperplasia in the SCID mice by transfer of T cell populations stimulated with a relevant superantigen. To examine the pathogenicity of the T cell populations expressing certain skewed TCR/Vβ in the rheumatoid joints, the PBMNC from the patients of the positive group were transferred into SCID mice after in vitro stimulation with a relevant superantigen. PBMNC isolated from patient 3, whose SFMNC showed a preexisting TCR/Vβ skew (Vβ12 and Vβ13), were cultured with SEC2. SEC2 is known as a superantigen that can stimulate T cell populations with TCR/Vβ12 or Vβ13 (41). After the in vitro SEC2 stimulation, the PBMNC showed a TCR/Vβ skew (Vβ12 and Vβ13) similar to that of SFMNC. Then, those stimulated PBMNC were transferred into SCID mice. As the result of experiment 1 shown in Fig. 5, those PBMNC stimulated with SEC2 induced synovial hyperplasia mimicking that induced by the infiltrating MNC (SFMNC). On the other hand, PBMNC stimulated with PHA, which could not make such TCR/Vβs skew, did not induce such histopathological changes. This result suggests that the selective stimulation of T cell populations expressing such skewed TCR/Vβs induce synovial hyperplasia. Accidentally, in experiment 1, we observed a different TCR/Vβ skew (Vβ4, Vβ5) in the PBMNC before in vitro stimulation, which had not been seen in the PBMNC (the upper panel of Fig. 5) obtained at different time points simultaneously with SFMNC. These data of TCR/Vβ

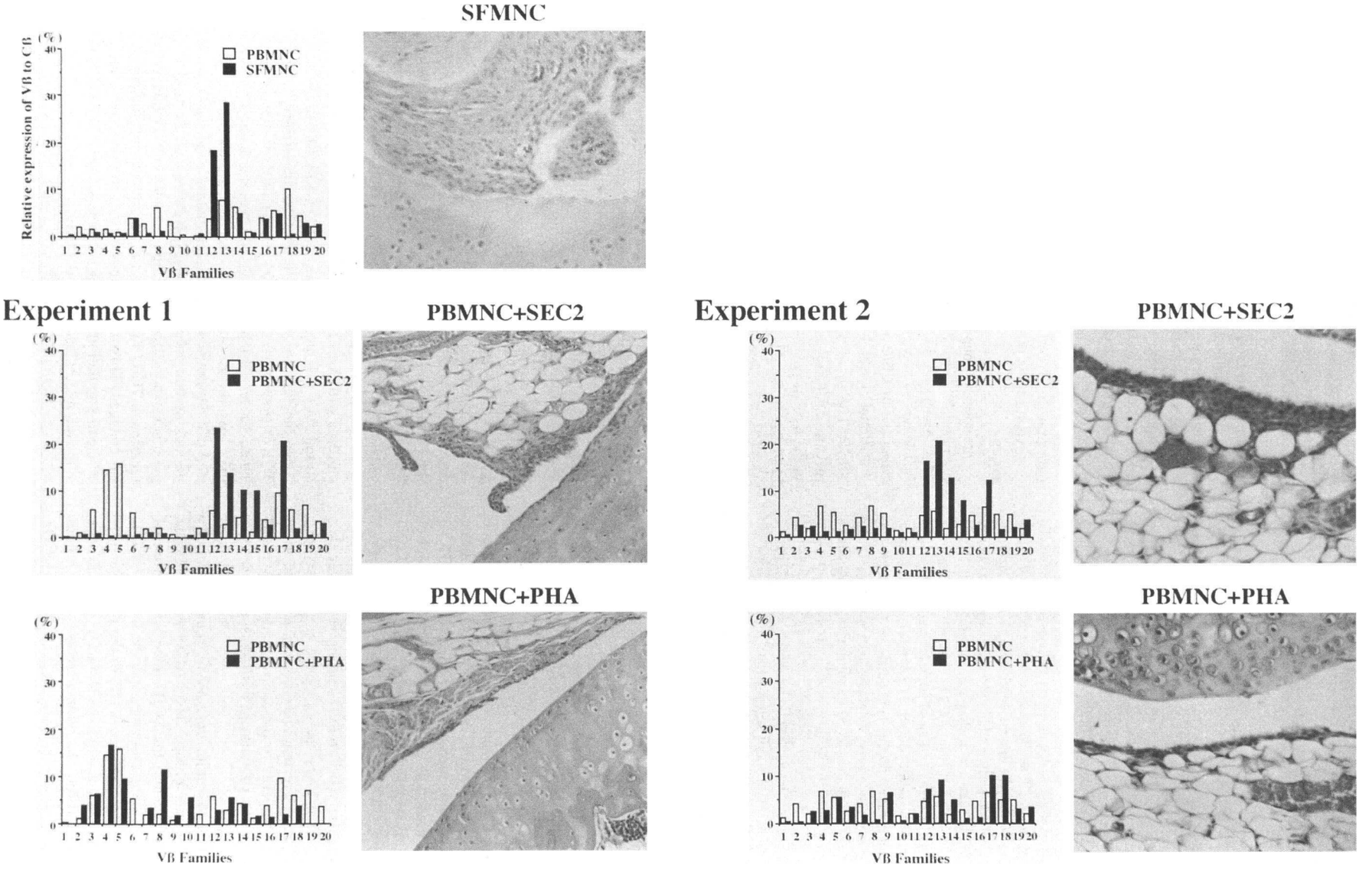
SFMNC
Relative expression of Vß to Cß (%)
PBMNC
SFMNC
Vß Families
Experiment 1
PBMNC+SEC2
PBMNC
PBMNC+SEC2
Vß Families
PBMNC+PHA
PBMNC
PBMNC+PHA
Vß Families
Experiment 2
PBMNC+SEC2
PBMNC
PBMNC+SEC2
Vß Families
PBMNC+PHA
PBMNC
PBMNC+PHA
Vß Families

Figure 5. Induction of synovial hyperplasia in the SCID mice by transfer of T cell populations stimulated with a relevant superantigen. To examine the pathogenicity of the T cell populations expressing a certain TCR/Vβ skew, which were expanding in the rheumatoid joints of the positive group, the PBMNC from the patients of the positive group were transferred into SCID mice after in vitro stimulation with a relevant superantigen. Briefly, PBMNC isolated from one of the patients of the positive group (patient 3), whose SFMNC showed a preexisting Vβ12 and Vβ13 skew, were cultured with 10 ng/ml of SEC2, which is known as a superantigen to stimulate T cell populations expressing such skewed TCR/Vβs (*Vβ12, Vβ13*) in complete medium (10% FCS RPMI-1640) for 4 d. Before cell transfer, enrichment of T cell populations expressing TCR/Vβ (*Vβ12, Vβ13*) was confirmed by analysis of TCR/Vβ genes usage using RT-PCR. As a control, PBMNC from the same individual were cultured with 2 μg/ml of PHA for nonspecific stimulation without enrichment of such populations in exactly the same condition. Then, 1.0×10^6 MNC from each cell culture were transferred into SCID mice in exactly the same way described above. 4 wk after cell transfer, the injected mice were killed with anesthesia. The bilateral knee joints of posterior legs of the mice were removed and subjected to histopathological examinations. PBMNC stimulated with SEC2 induced synovial hyperplasia in the SCID mice. On the contrary, PBMNC stimulated by PHA did not induce. This result suggests that the selective stimulation of T cell populations expressing such skewed TCR/Vβs induce synovial hyperplasia. Accidentally, in experiment 1, we observed a different TCR/Vβ skew (*Vβ4, Vβ5*) before in vitro stimulation, which had not been seen in the PBMNC (*upper panel*) obtained at different time points simultaneously with SFMNC. These data of TCR/Vβ genes usage analysis were reproducible (data not shown). Then, to examine the influence of a preexisting TCR/Vβ skew in PBMNC on this experiment, we reexamined the PBMNC of patient 3 obtained at another time point (*experiment 2*). The repertoire of TCR/Vβ genes usage in PBMNC was similar to that of the upper panel. And these PBMNC also induced synovial hyperplasia in the SCID mice after in vitro SEC2 stimulation. Thus, the transient change of the TCR/Vβ repertoire in PBMNC could not alter the result in this experiment.

Table V. The Reproducibility and Specificity of Induction of Synovial Hyperplasia by the Relevant Superantigen Stimulation of PBMNC from the Patients of the Positive Group

	Skewed TCR/Vβ families in rheumatoid joints	Incidence of hyperplasia (positive/total joints)		
		PBMC + SEC2	PBMC + PHA	PBMC
Patient 3	Vβ12, Vβ13	2/6	0/6	N.D.
		5/10	0/10	0/10
Patient 5	Vβ12, Vβ13, Vβ17	4/10	0/10	N.D.
Patient 1	Vβ14	0/6	N.D.	N.D.
Normal 1		0/10	N.D.	N.D.
Normal 2		0/10	N.D.	N.D.

To examine the pathogenicity of the T cell populations with expressing certain TCR/Vβ skew, which were expanding in the rheumatoid joints of the positive group, the PBMC of the patients of the positive group were transferred into SCID mice after in vitro stimulation with a relevant superantigen. Briefly, PBMC isolated from two of the patients of the positive group (patient 3 and patient 5), whose SFMNC showed a preexisting Vβ12 and Vβ13 skew, were cultured with 10 ng/ml of SEC2, which is known as a superantigen to stimulate T cell populations expressing such skewed TCR/Vβs (Vβ12, Vβ13) in complete medium (10% FCS RPMI-1640) for 4 d. Before the cell transfer, enrichment of T cell populations expressing TCR/Vβ (Vβ12, Vβ13) was confirmed by the analysis of TCR/Vβ genes usage using RT-PCR. As a control, PBMC from the same individual were cultured with 2 μg/ml of PHA as a nonspecific stimulation without enrichment of such populations in exactly the same condition. Then, 1.0×10^6 MNC from each cell culture were transferred into SCID mice in exactly the same way described above. 4 wk after cell transfer, the injected mice were killed with anesthesia. The bilateral knee joints of posterior legs of the mice were removed and subjected to histopathological examinations. PBMC from patient 3 and patient 5, whose SFMNC showed a preexisting Vβ12 and Vβ13 skew, stimulated with SEC2-induced synovial hyperplasia in the SCID mice, although PBMC stimulated with PHA did not induce such histopathological changes. Moreover, PBMC from either the patient 1 whose SFMNC showed a preexisting different skew (Vβ14) or two healthy volunteers stimulated with SEC2 did not induce.

genes usage analysis were reproducible (data not shown). Then, to examine the influence of a preexisting TCR/Vβ skew in PBMNC on this experiment, we reexamined in PBMNC of patient 3 obtained at another time point. As the result of experiment 2 shown in Fig. 5, the PBMNC showed a similar TCR/Vβ repertoire to that of the upper panel. And these PBMNC also induced synovial hyperplasia in the SCID mice after in vitro SEC2 stimulation. Thus, the transient change of the TCR/Vβ repertoire in PBMNC could not alter the result in this experiment. Next, to investigate the reproducibility and specificity of this observation, the same experiments were done in another patient (patient 5) of the positive group with similar TCR/Vβ skew, one patient of the positive (patient 1) group with a different TCR/Vβ skew (Vβ14) and two healthy volunteers. As the results shown in Table V, PBMNC from patient 5, whose SFMNC showed a preexisting Vβ12 and Vβ13 skew, induced synovial hyperplasia in the SCID mice after SEC2 stimulation. On the contrary, SEC2 stimulation of PBMNC from either patient 1, whose SFMNC showed a preexisting different TCR/Vβ skew (Vβ14) or two healthy volunteers did not induce synovial hyperplasia. These results suggest that the T cell populations oligoclonally expanding in the rheumatoid joints of the

patients could be responsible for the induction of synovial hyperplasia.

Discussion

Considerable evidence has been accumulated for the critical role of T cells in the initiation and perpetuation of RA. Moreover, in the murine model such as collagen-induced arthritis (CIA), the disease process is T cell mediated, necessitating a particular combination of TCR/Vβ genes and MHC class II genes and the efficacy of anti-TCR/Vβ therapy has been demonstrated. These observations suggest that a similar scenario may be taking place in human RA. In fact, to detect the pathogenic T cells, analysis of TCR/Vβ repertoire on the T cells infiltrating in the rheumatoid joints has been extensively studied using a variety of techniques, such as anti-TCR/Vβ antibodies, RFLP, and PCR. However, with regard to a preferential use of certain TCR/Vβ genes in the infiltrating T cells no clear answer has yet been provided. This might be caused by the heterogeneity of the pathogenesis of RA. Because the clinical features of individual patients vary and the diagnosis is made only by the standard criteria. Moreover, recent investigations using the mice carrying and expressing the transgenes of inflammatory cytokines and retrovirus suggest non-T cell–mediated mechanism of the pathogenesis of RA. Therefore, the analysis of TCR/Vβ repertoire could be applied for some but not all patients, in whom the pathogenesis is thought to be T cell mediated.

In this study, we demonstrated the induction of synovial hyperplasia, which mimicked the histopathological changes observed in patients with RA at the early stages of the disease, in the SCID mice transferred with the infiltrating MNC of the rheumatoid joints by intraarticular injections. The mice injected with either MNC of SF from the patients with other inflammatory arthropathies or mitogen (PHA)–activated PBMNC from healthy volunteers did not develop such synovial hyperplasia, indicating that the synovial histopathological changes observed in the mice injected with the infiltrating MNC of rheumatoid joints were not due to graft vs. host reactions. The traumatic effects of articular injections were also unlikely to cause these observations, since multiple injections did not develop such synovial histopathological changes. Taken together, our results indicated that the MNC infiltrating in the rheumatoid joints could induce synovial hyperplasia in SCID mice. Immunohistochemistry in the lesions of the SCID mice revealed that the proliferating synovial cells derive from the mice. No human cells (mainly T cells) could be detected by immunohistochemistry with monoclonal antibodies to CD45 and CD3, although clusters of mononuclear cells were observed in some of the synovial hyperplastic lesions of the positive mice. One of the considerable explanations is that the antigenicity of the surface molecules such CD45 and CD3 might be affected during the procedures of the fixation and the decalcification of the samples for histopathological examinations. Therefore, we tried to detect human DNA in the total DNA extracted from the injected joints using PCR with primers for human specific *Alu*. We could detect human DNA at 4 wk after the cell transfer. This suggests the presence of human MNC in the injected joints for at least 4 wk after the injection. On the other hand, Sacks et al. reported that the intraarticular injections of single cell suspensions from synovial membrane of patients with RA did not induce formation of inflammatory focus or persistence of human cells except for a few hours (42). Although without definitive reasons for the difference between our result and Sacks', there were some considerable explanations. *(a)* It seems likely that they failed to detect synovial changes due to their histopathological examinations in only three slices of each sample. Because the severity of the synovial histopathological changes varied and they were sometimes minimal. On the other hand, we injected multiple mice with SFMNC or SIMNC from the same individual and examined at least seven serial slices of each sample. *(b)* They did the transfer experiments in only five patients. This number of the patients seems not to be sufficient to select the positive patients. Because the synovial hyperplasia was induced in the SCID mice only by cell samples from a limited population of patients with RA. *(c)* They searched for synovial histopathological changes during only a couple of days after the cell transfer. Our observations seem to be time dependent. We have not observed such synovial hyperplasia during the early days.

The predominance of T cells in the MNC injected into SCID mice suggested that the induction of synovial hyperplasia observed in the SCID mice might be T cell mediated. Indeed, the prevention of the induction of synovial hyperplasia in this experimental system by an anti–human CD3 antibody (OKT3) strongly suggested the T cell mediation.

Interestingly, in some, but not all patients with RA studied, the induction of such synovial hyperplasia was observed. According to the observations in this experimental system, patients with RA could be classified into at least two groups. One is the group of the patients, whose T cells infiltrating in the rheumatoid joints could induce synovial hyperplasia (the positive group). On the other hand, the other is the group did not display the induction of synovial hyperplasia (the negative group). Although false negative cannot be excluded completely because of the limitations of the technique such as histopathological examinations and injections, the possibility of false positive is unlikely because of the following reasons. *(a)* As mentioned before, the synovial hyperplasia observed in this experimental system could not be induced by nonspecific effects, such as graft vs host reactions and traumatic effects of articular injections. And the MNC of SF from patients with non-RA inflammatory arthropathies did not induce such synovial hyperplasia, either. Thus, such observations seem to be specific for RA. *(b)* The repeated experiments in some patients of the positive group (patient 3 and patient 4) showed the reproducibility of induction of synovial hyperplasia in this experimental system. In contrast, the repeated injections in the negative group could not induce the synovial hyperplasia. According to the comparison of clinical characteristics of patients between the positive group and the negative group, only the disease duration could be correlated with the induction of synovial hyperplasia. The mean disease duration of the positive patients is significantly shorter than that of the negative patients. This suggests that "pathogenic" T cells are present in synovial fluid or tissue of the affected joints at the early stages of the disease and they might contribute to the initiation of the disease.

Therefore, we concluded that the strategy to detect the pathogenic T cells in RA by the analysis of TCR/Vβ repertoire could be applied only for the positive group. The analysis of the usage of TCR/Vβ genes by RT-PCR in the infiltrating MNC transferred into SCID mice revealed a marked skew towards the preferential use of certain Vβ genes, which was not seen in the PBMNC, in only the positive group. The patterns of skewed TCR/Vβs were not uniform among the patients. This may reflect the presence of plural causative antigens in RA. For instance, in multiple sclerosis, a T cell–mediated autoimmune disease as well as RA, plural autoantigens, such as MBP (my-

elin basic protein) and PLP (proteolipid protein), are thought to be pathogenetic. If this is true, the patients of the positive group could be divided into subgroups according to the causative antigens. Indeed, the skew of TCR/Vβs (Vβ12, Vβ13) in patient 3 was also seen in patient 5. In this study, there were no significant correlations between the skewed TCR/Vβs and MHC class II antigens(HLA-DR). The analysis of PCR-amplified genes of such skewed TCR/Vβ by SSCP indicated that the T cell populations expanding in the rheumatoid joints of patients of the positive group were oligoclonal. Furthermore, the expansion of the T cell populations expressing such skewed TCR/Vβs by in vitro stimulation of PBMNC with the relevant superantigen in the patients of the positive group enabled the induction of synovial hyperplasia, although PBMNC stimulated with PHA, which could not make such TCR/Vβ skew, did not induce the synovial hyperplasia. Accidentally, we observed a transient change of TCR/Vβ repertoire in the PBMNC of patient 3 before the superantigen stimulation. However, the transient change of the TCR/Vβ repertoire in PBMNC could not alter the result in this experiment. Because the relevant superantigen stimulation of the PBMNC from the positive patient could induce synovial hypereplasia in mice, whether the PBMNC showed a transient change of TCR/Vβ repertoire or not. The cause of this transient change of TCR/Vβ repertoire is not clear. However, one of the plausible explanations for such a transient change of the TCR/Vβ repertoire is the influence of infections. Because, Smith et al. (43) reported that the distinct population of T cells with a certain TCR/Vβ were selectively expanding during viral infections and such population returned to normal at recovery. Therefore, the patient might have a certain infection when PBMNC showed a preexisting different TCR/Vβ skew. Moreover, PBMNC from either one of the patients of the positive group whose SFMNC showed a preexisting different skew or two healthy volunteers could not induce synovial hyperplasia after the same superantigen stimulation. Therefore, the T cell populations oligoclonally expanding in the rheumatoid joints of the patients could be responsible for the induction of synovial hyperplasia. These results might support the pathological role of superantigens and "two step hypotheses" in the stimulation of the pathogenic T cells in RA as the following (41): a superantigen stimulates some populations of T cells expressing certain TCR/Vβs, in which autoreactive pathogenic T cells are included. The activated autoreactive T cells migrate into the target organ (joints) and are restimulated by specific local autoantigens. Thus, the pathogenic autoreactive T cells expressing certain TCR/Vβs could be expanding oligoclonally in the rheumatoid joints.

In summary, the present observations in this SCID mice experimental system provide direct evidence of T cell mediation in some, but not all, patients with RA and suggest that the pathogenic T cells could be activated locally in the rheumatoid joints by certain antigens. Although the pathology developed in the SCID mouse in this experimental system is not mimicking that of advanced RA, the SCID mice reconstituted RA pathology could be a useful tool to clarify the pathogenesis of RA, especially the mechanism of the initiation of the disease.

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