11 McHugh NJ, James IE, Fairburn K, Maddison PJ. Autoantibodies to mitochondrial and centromere antigens in primary biliary cirrhosis and systemic sclerosis. Clin Exp Immunol 1990; 81:244–9.
12 Szostecki C, Guldner HH, Will H. Autoantibodies against 'nuclear dots' in primary biliary cirrhosis. Semin Liver Dis 1997; 17:71–8.
13 Kinoshita H, Omagari K, Whittingham S *et al.* Autoimmune cholangitis and primary biliary cirrhosis – an autoimmune enigma. Liver 1999; 19:122–8.
14 Fida S, Myers MA, Whittingham S, Rowley MJ, Ozaki S, Mackay IR. Autoantibodies to the transcriptional factor SOX13 in primary biliary cirrhosis compared with other diseases. J Autoimmun 2002; 19:251–7.
15 Manns MP, Johnson EF, Griffin KJ, Tan EM, Sullivan KF. Major anitgen of liver kidney microsomal autoantibodies in idiopathic autoimmune hepatitis if cytochrome P450db1. J Clin Invest 1989; 83:1066–72.
16 Miyakawa H, Kako M, Nagai K *et al.* HCV-RNA in type 2 autoimmune hepatitis. Am J Gastroenterol 1991; 86:1688–9.
17 Mulder AH, Horst G, Haagsma EB, Limburg PC, Kleibeuker JH, Kallenberg CG. Prevalence and characterization of neutrophil cytoplasmic antibodies in autoimmune liver diseases. Hepatology 1993; 17:411–7.
18 Miyachi K, Matsushima H, Hankins RW *et al.* A novel antibody directed against a three-dimensional configuration of a 95-kDa protein in patients with autoimmune hepatic diseases. Scand J Immunol 1998; 47:63–8.
19 Koller KJ, Brownstein MJ. Use of a cDNA clone to identify a supposed precursor protein containing valosin. Nature 1987; 325:542–5.
20 Zhang X, Shaw A, Bates PA *et al.* Structure of the AAA ATPase p97. Mol Cell 2000; 6:1473–84.
21 Hetzer M, Meyer HH, Walther TC, Bilbao-Cortes D, Warren G, Mattaj IW. Distinct AAA-ATPase p97 complexes function in discrete steps of nuclear assembly. Nat Cell Biol 2001; 3:1086–91.
22 Scheuer PJ. Primary biliay cirrhosis. Proc R Soc Med 1967; 60:1257–60.
23 Scheuer PJ. Ludwig Symposium on biliary disorders – part II. Pathologic features and evolution of primary biliary cirrhosis and primary sclerosing cholangitis. Mayo Clin Proc 1998; 73:179–83.
24 Smolen JS, Butcher B, Fritzler MJ *et al.* Reference sera for antinuclear antibodies. II. Further definition of antibody specificities in international antinuclear antibody reference sera by immunofluorescence and immunoblotting. Arthritis Rheum 1997; 40:413–8.
25 Miyachi K, Gupta RC, Dickson ER, Tan EM. Precipitating antibodies to mitochondrial antigens in patients with primary biliary cirrhosis. Clin Exp Immunol 1980; 39:599–606.
26 Fritzler MJ, Lung C-C, Hamel JC, Griffith K, Chan EKL. Molecular characterization of golgin-245: a novel Golgi complex protein containing a granin signature. J Biol Chem 1995; 270:31262–8.
27 Griffith KJ, Chan EKL, Hamel JC, Miyachi K, Fritzler MJ. Molecular characterization of a novel 97 kDa Golgi complex autoantigen recognized by autoimmune antibodies from patients with Sjögren's syndrome. Arthritis Rheum 1997; 40:1693–702.
28 Nakagawa T, Hirano Y, Inomata A *et al.* Participation of a fusogenic protein, glyceraldehyde-3-phosphate dehydrogenase, in nuclear membrane assembly. J Biol Chem 2003; 278:20395–404.
29 Sasagawa S, Yamamoto A, Ichimura T, Omata S, Horigome T. *In vitro* nuclear assembly with affinity-purified nuclear envelope precursor vesicle fractions, PV1 and PV2. Eur J Cell Biol 1999; 78:593–600.
30 Smythe C, Newport JW. Systems for the study of nuclear assembly, DNA replication, and nuclear breakdown in *Xenopus laevis* egg extracts. Meth Cell Biol 1991; 35:449–68.
31 Chan EKL, Pollard KM. Detection of antibodies to ribonucleoprotein particles by immunoblotting. In: Rose NR, de Macario EC, Folds JD, Lane HC, Nakamura RM, eds. Manual of clinical laboratory immunology. Washington: American Society for Microbiology, 1997; 928–34.
32 Kondo H, Rabouille C, Newman R *et al.* p47 is a cofactor for p97-mediated membrane fusion. Nature 1997; 388:75–8.
33 Rabouille C, Kondo H, Newman R, Hui N, Freemont P, Warren G. Syntaxin 5 is a common component of the NSF- and p97-mediated reassembly pathways of Golgi cisternae from mitotic Golgi fragments in vitro. Cell 1998; 92:603–10.
34 Stock D, Gibbons C, Arechaga I, Leslie AG, Walker JE. The rotary mechanism of ATP synthase. Curr Opin Struct Biol 2000; 10:672–9.
35 Yasuda R, Noji H, Kinosita K Jr, Yoshida M. F1-ATPase is a highly efficient molecular motor that rotates with discrete 120 degree steps. Cell 1998; 93:1117–24.
36 Mashima J, Nagahama M, Hatsuzawa K *et al.* N-ethylmaleimide-sensitive factor is associated with the nuclear envelope. Biochem Biophys Res Commun 2000; 274:559–64.
37 Dang CV, Tan EM, Traugh JA. Myositis autoantibody reactivity and catalytic function of threonyl-tRNA synthetase. FASEB J 1988; 2:2376–9.
38 Tan EM. Autoantibodies in pathology and cell biology. Cell 1991; 67:841–2.

Immunogenetic Features in 120 Japanese Patients with Idiopathic Inflammatory Myopathy

TAKEFUMI FURUYA, MASAYUKI HAKODA, NAOYUKI TSUCHIYA, SHIGERU KOTAKE, NAOMI ICHIKAWA, YUKI NANKE, AYAKO NAKAJIMA, MEGUMI TAKEUCHI, MAKOTO NISHINARITA, HIROBUMI KONDO, AYA KAWASAKI, SHIO KOBAYASHI, TSUNEYO MIMORI, KATSUSHI TOKUNAGA, and NAOYUKI KAMATANI

ABSTRACT. ***Objective.*** To examine the role of HLA-DRB1 and tumor necrosis factor (TNF) promoter genotypes in the development and the autoantibody profiles of idiopathic inflammatory myopathy (IIM) in Japanese patients.

Methods. HLA-DRB1 and TNF promoter genotypes were determined, and serum antinuclear autoantibodies were identified in 120 adult Japanese patients with IIM [72 with dermatomyositis (DM), 30 with polymyositis (PM), 18 with myositis overlapping with other collagen vascular diseases], as well as in 265 controls.

Results. Forty-two patients (35%) were positive for myositis-specific autoantibodies (MSA), including 37 (31%) for anti-aminoacyl-tRNA synthetase (ARS) autoantibodies. Allele carrier frequency of HLA-DRB1*0803 was increased in the patients with IIM [p = 0.02, corrected p (pc) NS, 23% vs 14%, odds ratio (OR) = 1.9 (95% confidence interval, CI = 1.1–3.2)], with PM [p = 0.006, pc NS, 33%, OR 3.1 (95% CI 1.3–7.1)], and with anti-ARS autoantibodies [27%, p = 0.04, OR 2.3 (95% CI 1.0–5.1)] compared with controls. DRB1*0405 was increased in patients with anti-ARS autoantibodies compared with controls [41% vs 25%, p = 0.04, pc NS, OR 2.1 (95% CI 1.0–4.3)]. TNF promoter genotype was associated with the presence of interstitial lung disease (ILD). The carriage of a TNF-α haplotype formed by –1031C, –863A, and –857C was increased in the patients with ILD versus those without ILD [33% vs 18%, p = 0.05, pc NS, OR 2.3 (95% CI 0.94–5.5)].

Conclusion. HLA-DRB1 alleles were associated with development of IIM and MSA in a Japanese population. (J Rheumatol 2004;31:1768–74)

Key Indexing Terms:
IDIOPATHIC INFLAMMATORY MYOPATHY HLA-DRB1 AUTOANTIBODIES
JAPANESE TUMOR NECROSIS FACTOR-α PROMOTER

From the Institute of Rheumatology and Department of Neurology, Tokyo Women's Medical University, Tokyo; Department of Clinical Studies, Radiation Effects Research Foundation, Hiroshima; Department of Rheumatology, Taga General Hospital, Ibaraki; Department of Internal Medicine, Kitasato University School of Medicine, Kanagawa; Department of Human Genetics, Graduate School of Medicine, The University of Tokyo; and Department of Rheumatology and Clinical Immunology, Kyoto University Graduate School of Medicine, Kyoto, Japan.

Supported by a grant-in-aid from the Ministry of Education, Culture, Science and Technology of Japan.

T. Furuya, MD, PhD, Instructor; S. Kotake MD, PhD, Assistant Professor; N. Ichikawa, MD, PhD, Instructor; Y. Nanke, MD, PhD, Assistant Professor; A. Nakajima, MD, PhD, Assistant Professor; N. Kamatani, MD, PhD, Director and Professor, Institute of Rheumatology, Tokyo Women's Medical University; M. Hakoda, MD, PhD, Chief, Division of Clinical Laboratories, Department of Clinical Studies, Radiation Effects Research Foundation; M. Takeuchi, MD, PhD, Assistant Professor, Department of Neurology, Tokyo Women's Medical University; M. Nishinarita, MD, PhD, Department of Rheumatology, Taga General Hospital; H. Kondo, MD, PhD, Professor, Department of Internal Medicine, Kitasato University School of Medicine; A. Kawasaki, BSc, Technician; N. Tsuchiya, MD, PhD, Associate Professor; K. Tokunaga, PhD, Professor, Department of Human Genetics, Graduate School of Medicine, The University of Tokyo; S. Kobayashi, AS, Research Assistant; T. Mimori, MD, PhD, Professor, Department of Rheumatology and Clinical Immunology, Kyoto University Graduate School of Medicine.

Address reprint requests to Dr. T. Furuya, Institute of Rheumatology, Tokyo Women's Medical University, 10-22 Kawada-cho, Shinjuku-ku, Tokyo 162-0054, Japan. E-mail: furuyat@ior.twmu.ac.jp

Submitted August 12, 2003; revision accepted March 25, 2004.

We previously reported the association of HLA class I and class II alleles with idiopathic inflammatory myopathy (IIM) by analyzing 84 Japanese patients[1]. Our previous study did not examine myositis-specific autoantibodies [MSA; anti-aminoacyl-tRNA synthetases (ARS), anti-signal recognition particle (SRP)] or myositis associated autoantibodies (MAA; anti-RNP, Ro, La, Ku) except for anti-Jo-1 autoantibody.

MSA appear to define clinical features, prognosis, and response to treatment[2]. In Caucasian patients, IIM in the presence of MSA is reportedly associated with HLA-DRB1*0301[3]. Although HLA-DRB1*0301 is common (around 20%) in the Caucasian population, it is very rare (0.1–0.2%) in Japanese[4]. There have been only a few small studies analyzing HLA alleles of patients with IIM among Japanese[1,5,6]. There have been no reports of an association of HLA alleles with MSA in Japanese.

In our previous study, HLA-DRB1*08 alleles were significantly increased in the Japanese patients with IIM compared with controls[1]. However, the actual mechanism by which HLA regulates susceptibility to myositis remains to be defined. Tumor necrosis factor-α (TNF-α), encoded within the MHC, is a potent proinflammatory cytokine with

a wide range of activities. It plays a critical role in the pathogenesis of inflammatory or autoimmune diseases[7]. A recent report showed blockade of TNF may be beneficial for dermatomyositis (DM) and polymyositis (PM)[8].

Among the single nucleotide polymorphisms (SNP) of the 5'-flanking region of the TNF gene, the –308A allele has been shown to be associated with high promoter activity[9] as well as susceptibility/severity to juvenile[10] and adult[11] IIM. However, the frequency of –308A allele is very low in the Japanese population (less than 3%)[12].

New SNP, which may affect transcriptional activity, were recently reported at position –1031(T/C), –863(C/A), and –857(C/A) in the 5'-flanking region of TNF, and each substitution was found in a substantial proportion of the Japanese population[12]. Four major haplotypes were identified, namely, TCC, TCT, CAC, and CCC, which we tentatively designated TNF-U01, U02, U03, and U04, respectively[13]. The associations of TNF-U03 with Crohn's disease[14] and TNF-U01 with asthma[15] have been reported in the Japanese.

In the present study, we aimed to confirm our previous findings of an association of HLA-DRB1*08 alleles with Japanese IIM patients by analyzing a larger number of patients, and to examine whether the polymorphisms of HLA-DRB1 and TNF promoter contribute to susceptibility and autoantibody production among Japanese with IIM.

MATERIALS AND METHODS

Patients. One hundred twenty Japanese patients with IIM seen from September 1994 to December 2001 were studied: 86 at Institute of Rheumatology; 7 at Department of Neurology, Tokyo Women's Medical University (Tokyo); 19 at Kitasato University School of Medicine (Sagamihara, Kanagawa); 6 at Taga General Hospital (Hitachi, Ibaraki); and 2 at Tokyo Metropolitan Otsuka Hospital (Tokyo). The mean ± standard deviation (SD) age was 47.9 ± 14.8 years. Thirty of the patients were men and 90 were women. Among these patients, 72 and 30 were classified as having dermatomyositis (DM) and polymyositis (PM), respectively, using criteria proposed by Bohan and Peter[16,17]. Among them, 52, 36, and 14 patients were diagnosed as definite, probable, and possible DM/PM, respectively. The mean ± SD age of DM and PM patients was 47.1 ± 14.4 years and 51.8 ± 13.7 years, respectively. Among 72 DM and 30 PM patients, 19 and 9 were men, respectively. Heliotrope and Gottron rash were used to classify the patients as DM. Three patients with DM also had malignancy at the time of diagnosis (uterine carcinoma, malignant lymphoma, and lung cancer).

Eighteen patients had myositis overlapping with other collagen vascular diseases. They fulfilled both sets of Bohan and Peter criteria for myositis[16,17], as well as criteria for primary collagen diseases. Among them, 6, 8, and 4 patients were diagnosed as definite, probable, and possible DM/PM, respectively. Twelve of the patients met the American College of Rheumatology (ACR) preliminary classification criteria for systemic sclerosis[18], 4 met the ACR criteria for systemic lupus erythematosus (SLE)[19], and 2 met the ACR criteria for rheumatoid arthritis[20]. The mean ± SD age of overlap disease patients was 44.4 ± 17.1 years. Among 18 overlap disease patients, 2 were men and 16 were women.

Among the patients, 64 (56%) were diagnosed as having interstitial lung disease (ILD). ILD was defined by the presence of pulmonary fibrosis seen by chest radiography and/or computed tomography. Information regarding ILD was not available for 5 patients including one patient with anti-ARS autoantibody.

After patients gave informed consent, blood samples were obtained.

Controls. The control group consisted of 265 healthy laboratory personnel and students, 148 of the controls were men, 116 were women, and one was unknown. All controls were unrelated Japanese living in the Tokyo area. HLA-DRB1 and TNF promoter genotypes of the control group have been reported[21]. The central part of Japan has been shown to be relatively homogeneous with respect to genetic background[22], permitting the case-control approach to be employed in this study.

Autoantibody studies. We analyzed the plasma samples obtained from September 1994 to December 2001, which had been stored at –20°C until analysis. Autoantibodies were identified by RNA immunoprecipitation technique using HeLa cell extract as an antigen source[23].

Genomic DNA. Genomic DNA from patients and healthy individuals was purified from peripheral blood leukocytes using a standard phenol-chloroform extraction procedure or the QIAamp blood kit (Qiagen, Hilden, Germany).

HLA typing. The HLA-DRB1 genotype was determined using a polymerase chain reaction (PCR) restriction fragment length polymorphism method[24].

TNF promoter genotyping. TNF promoter allele formed by SNP at –1031, –863, and –857 was determined using the sequence-specific oligonucleotide probing followed by melting-curve analysis in a real-time PCR machine (LightCyclerTM, Roche Diagnostics, Mannheim, Germany) based on fluorescence resonance energy transfer technology. The detailed genotyping method is described in a previous report[25].

Statistical analysis. For the comparison of genotypes, allele carrier frequencies (homozygotes and heterozygotes combined) were compared. Statistical significance of the differences between groups was determined by chi-square analysis or Fisher's exact probability test. Corrected p (pc) values were obtained by multiplying the observed p values by the number of alleles examined; namely, 23 for HLA-DRB1, 12 for HLA-DR (serologic specificity), and 4 for TNF. The odds ratio (OR) with 95% confidence interval (95% CI) was calculated. To determine the sex differences in genetics, each allele carrier frequency was compared among patients and controls in each sex.

RESULTS

Autoantibody frequencies in different myositis syndromes. Frequencies of MSA and MAA in different forms of myositis are shown in Table 1. Among 120 patients with IIM, 42 (35%) were positive for MSA, including 37 (31%) for anti-ARS and 5 (4%) for anti-SRP autoantibody. Of the anti-ARS autoantibodies, anti-Jo-1 autoantibody was the most common (15%), and anti-EJ (9%) autoantibody was the second. Anti-SRP autoantibody was found exclusively in patients with PM.

MSA occurred more commonly in patients with PM (63%) than in those with DM [26%, p = 0.0004, OR 4.8 (95% CI 1.9–12.0)] or in patients with overlap disease [22%, p = 0.006, OR 6.0 (95% CI 1.6–23.0)]. Anti-ARS autoantibodies were more common in PM (47%) versus DM [26%, p = 0.046, OR 2.4 (95% CI 1.0–5.9)]. Anti-Jo-1 was present in 27% of patients with PM versus 11% with DM [p = 0.049, OR 2.9 (95% CI 0.98–8.7)]. Six DM patients, 4 PM, and one with overlap disease were positive for anti-EJ autoantibody.

Among 36 patients with anti-ARS autoantibodies, 28 (78%) had complicating ILD, while 36 (46%) of the remaining 79 antibody-negative patients had this pulmonary complication [p = 0.001, OR 4.2 (95% CI 1.7–10.3)].

Table 1. Autoantibody frequencies in Japanese patients with IIM. Values are the number (%) of patients with autoantibodies.

Autoantibody	All IIM, n = 120	DM, n = 72	PM, n = 30	Overlap, n = 18
Myositis-specific				
Anti-aminoacyl-tRNA synthetases	37 (31)	19 (26)	14 (47)*	4 (22)
Anti-Jo-1	18 (15)	8 (11)	8 (27)**	2 (11)
Anti-EJ	11 (9)	6 (8)	4 (13)	1 (6)
Anti-PL-7	4 (3)	2 (3)	2 (7)	0
Anti-PL-12	3 (3)	3 (4)	0	0
Anti-OJ	1 (1)	0	0	1 (6)
Anti-SRP	5 (4)	0	5 (17)	0
Any of above	42 (35)	19 (26)	19 (63)†	4 (22)
Myositis-associated				
Anti-tRNA	1 (1)	0	0	1 (6)
Ro (SSA)	21 (18)	11 (15)	8 (27)	2 (11)
U1-RNP	11 (9)	1 (1)	1 (3)	9 (50)††
U2-RNP	1 (1)	0	0	1 (6)
La (SSB)	1 (1)	1 (1)	0	0
Ku	1 (1)	0	0	1 (6)

IIM: idiopathic inflammatory myopathy; DM: dermatomyositis; PM: polymyositis; Overlap: overlap disease patients. * $p = 0.046$, OR 2.4 (95% CI 1.0–5.9) vs DM; ** $p = 0.049$, OR 2.9 (95% CI 0.98–8.7) vs DM; † $p = 0.0004$, OR 4.8 (95% CI 1.9–12.0) vs DM, $p = 0.006$, OR 6.0 (95% CI 1.6–23.0) vs Overlap; †† $p = 4 \times 10^{-9}$, OR 71 (95% CI 8.0–627.6) vs DM, $p = 0.0001$, OR 29 (95% CI 3.2–261.0) vs PM.

Among myositis-associated autoantibodies, anti-Ro (SSA) was most commonly found in myositis patients (18%), while its frequency did not differ significantly among the 3 groups. In contrast, anti-U1 RNP autoantibody frequency was significantly increased in overlap disease patients (50%) compared with DM [$p = 4.4 \times 10^{-9}$, OR 71 (95% CI 8.0–627.6)] or PM [$p = 0.0001$, OR 29 (95% CI 3.2–261.0)]. All patients with anti-U1 RNP autoantibody were women.

HLA-DRB1 alleles in patients with myositis. Among all IIM patients, allele carrier frequency of HLA-DRB1*0803 was significantly increased [$p = 0.02$, pc NS, OR 1.9 (95% CI 1.1–3.2)] as compared with controls (Table 2). When DRB1*0802 and 0803 were combined, the association with all IIM remained significant [$p = 0.02$, pc NS, OR 1.8 (95% CI 1.1–3.0)]. DRB1*1302 was significantly decreased in all IIM patients [$p = 0.01$, pc NS, OR 0.4 (95% CI 0.21–0.85)] compared with control patients (Table 2).

Carrier frequency of DRB1*0101 was significantly increased in patients with overlap disease [$p = 0.002$, pc NS, OR 4.6 (95% CI 1.6–13.3)] compared with control patients (Table 2). Although DRB1*0803 appeared increased in both DM and PM groups compared with controls (Table 2), the difference was significant only in the PM group [$p = 0.006$, pc NS, OR 3.1 (95% CI 1.3–7.1)]. When DRB1*0802 and *0803 alleles were combined, a significant increase was observed in PM compared with controls [$p = 0.02$, pc NS, OR 2.5 (95% CI 1.2–5.6)]. The associations of HLA-DRB1*08 or *0803 with IIM or PM, which we previously reported[1], were confirmed in this study with a larger number of patients.

Carrier frequency of the HLA-DRB1 first hypervariable region sequence (9EYSTS13), shared by DR3, DR5, DR6, and DR8 alleles, reported as candidate epitope for IIM[26], was not different between all IIM patients and controls.

HLA-DRB1 alleles in autoantibody subsets of myositis. Carrier frequency of DRB1*0405 was increased in patients with anti-ARS autoantibodies compared with controls [$p = 0.04$, pc NS, OR 2.1 (95% CI 1.0–4.3)] (Table 2). HLA-DRB1*0802 [$p = 0.047$, pc NS, OR 2.6 (95% CI 0.98–7.2)] and *0803 [$p = 0.04$, pc NS, OR 2.3 (95% CI 1.0–5.1)] were increased in the patients with anti-ARS autoantibody compared with controls (Table 2). The frequency of HLA-DRB1*1502 was significantly decreased in patients with anti-ARS autoantibody compared with controls [$p = 0.03$, pc NS, OR 0.22 (95% CI 0.05–0.96)] and MSA negative patients [$p = 0.007$, pc NS, OR 0.16 (95% CI 0.034–0.70)] (Table 2). The frequency of 9EYSTS13 carriers was not significantly different among MSA negative patients, the patients with anti-ARS autoantibodies, and controls (Table 2).

TNF promoter haplotypes in myositis patients and autoantibody subsets of myositis. Four TNF haplotypes (U01, U02, U03, and U04) were present (Table 3). No significant differences in TNF haplotype distribution were observed between IIM patients and controls, or among patients with PM, DM, or overlap disease.

HLA-DRB1 and TNF promoter genotypes with and without

Table 2. Carrier frequencies of *HLA-DRB1* alleles in Japanese IIM patients and controls. Values are the number (%) of subjects carrying each *HLA-DRB1* allele (homozygotes and heterozygotes combined).

	Clinical Subsets				Antibody Subsets		
HLA-DRB1	All IIM, n = 120	DM, n = 72	PM, n = 30	Overlap, n = 18	MSA (–), n = 78	ARS (+), n = 37	Controls, n = 265
*0101	21 (18)	12 (17)	3 (10)	6 (33)***	15 (19)	6 (16)	26 (10)
*0401	1 (1)	1 (1)	0 •	0	1 (1)	0	6 (2)
*0403	6 (5)	3 (4)	2 (7)	1 (6)	3 (4)	3 (8)	13 (5)
*0405	35 (29)	24 (33)	6 (20)	5 (28)	19 (24)	15 (41)#	65 (25)
*0406	7 (6)	5 (7)	1 (3)	1 (6)	5 (6)	2 (5)	19 (7)
*0407	0	0	0	0	0	0	6 (2)
*0410	4 (3)	2 (3)	2 (7)	0	3 (4)	1 (3)	5 (2)
*0802	11 (9)	8 (11)	2 (7)	1 (6)	5 (6)	6 (16)§	18 (7)
*0803	28 (23)*	14 (19)	10 (33)†	4 (22)	16 (21)	10 (27)§§	37 (14)
*0901	29 (24)	15 (21)	6 (20)	8 (44)	19 (24)	10 (27)	77 (29)
*1101	1 (1)	0	1 (3)	0	0	1 (3)	5 (2)
*1201	5 (4)	5 (7)	0	0	2 (3)	3 (8)	19 (7)
*1202	5 (4)	3 (4)	2 (7)	0	3 (4)	2 (5)	11 (4)
*1302	11 (9)**	8 (11)	3 (10)	0	8 (10)	2 (5)	51 (19)
*1401	8 (7)	7 (10)	1 (3)	0	7 (9)	1 (3)	13 (5)
*1403	5 (4)	4 (6)	1 (3)	0	4 (5)	1 (3)	10 (4)
*1405	4 (3)	2 (3)	1 (3)	0	2 (3)	1 (3)	17 (6)
*1406	2 (2)	2 (3)	0	0	2 (3)	0	7 (3)
*1501	18 (15)	8 (11)	6 (20)	4 (22)	12 (15)	4 (11)	29 (11)
*1502	26 (22)	17 (24)	5 (17)	4 (22)	21 (27)	2 (5)##	54 (20)
*1602	4 (3)	1 (1)	2 (7)	1 (6)	3 (4)	1 (3)	8 (3)
Others	2 (2)	0	0	0	2 (3)	0	13 (5)
9EYSTS13	70 (58)	46 (64)	20 (67)	5 (28)††	43 (55)	24 (65)	161 (61)

MSA: myositis-specific autoantibody; ARS: anti-aminoacyl-tRNA synthetase autoantibody. * p = 0.02, p_c NS, OR 1.9 (95% CI 1.1–3.2) vs controls; ** p = 0.01, p_c NS, OR 0.4 (95% CI 0.21–0.85); *** p = 0.002, p_c NS, OR 4.6 (95% CI 1.6–13.3) vs controls; † p = 0.006, p_c NS, OR 3.1 (95% CI 1.3–7.1) vs controls; †† p = 0.006, OR 0.25 (95% CI 0.086–0.72) vs controls; p = 0.0006, OR 0.22 (95% CI 0.07–0.68) vs DM; p = 0.009, OR 0.19 (95% CI 0.053–0.69) vs PM; # p = 0.04, p_c NS, OR 2.1 (95% CI 1.0–4.3) vs controls; § p = 0.047, p_c NS, OR 2.6 (95% CI 0.98–7.2) vs controls; §§ p = 0.04, p_c NS, OR 2.3 (95% CI 1.0–5.1) vs controls; ## p = 0.03, p_c NS, OR 0.22 (95% CI 0.05–0.96) vs controls; p = 0.007, p_c NS, OR 0.16 (95% CI 0.034–0.70) vs MSA negative patients.

Table 3. Carrier frequency of *TNF* promoter genotypes in Japanese patients with IIM and controls. Values are number (%) of patients carrying each *TNF* allele.

	Clinical Subsets				Antibody Subsets		
TNF	All IIM, n = 120	DM, n = 72	PM, n = 30	Overlap, n = 18	MSA (–), n = 77	ARS (+), n = 37	Controls, n = 265
U01	103 (86)	60 (83)	26 (87)	17 (94)	68 (88)	29 (78)	232 (88)
U02	40 (33)	26 (36)	9 (30)	5 (28)	23 (30)	14 (38)	83 (31)
U03	30 (25)	20 (28)	6 (20)	4 (22)	19 (25)	11 (30)	69 (26)
U04	7 (6)	3 (4)	2 (7)	2 (11)	3 (4)	2 (5)	14 (5)

MSA: myositis-specific autoantibody; ARS: anti-aminoacyl-tRNA synthetase autoantibody.

ILD. No significant association was observed between HLA-DRB1 and the presence or absence of ILD (Table 4). When the TNF promoter was examined, carrier frequency of TNF-U03 was found to be increased in patients with ILD as compared with those without ILD [33% vs 18%, p = 0.05, pc NS, OR 2.3 (95% CI 0.94–5.5)].

Analysis of sex effect. The sex distributions were not significantly different among DM, PM, and overlap disease patients. To examine the sex-specific effects, the frequencies of HLA-DRB1 and TNF alleles were compared between patients and controls in each sex separately (Table 5). When the HLA-DRB1 carrier frequencies were compared, DRB1*0101 was increased in female patients with IIM compared with female controls [p = 0.05, pc NS, OR 2.1 (95% CI 0.99–4.4)]. DRB1*0405 was increased in male IIM patients [p = 0.02, pc NS, OR 2.6 (95% CI 1.1–5.8)] and

Table 4. Carrier frequencies of *HLA-DRB1* and *TNF* promoter genotypes in Japanese IIM patients with and without interstitial lung disease (ILD). Values are the number (%) of patients.

Genotype	ILD (+), n = 64	ILD (–), n = 51	p	OR (95% CI)
*HLA-DRB1*0405*	20 (31)	11 (22)	NS	—
*HLA-DRB1*0803*	14 (22)	13 (25)	NS	—
TNF-U01	55 (86)	44 (86)	NS	—
TNF-U02	19 (30)	17 (33)	NS	—
TNF-U03	21 (33)	9 (18)	0.05	2.3 (0.94–5.5)
TNF-U04	2 (3)	4 (8)	NS	—

NS: not significant.

male IIM patients without MSA [p = 0.05, pc NS, OR 2.5 (95% CI 0.98–6.5)] compared with male controls. DRB1*0803 was increased in both male and female IIM patients compared with individual control subjects. The carrier frequency was significantly increased in male patients with IIM without MSA [p = 0.045, pc NS, OR 2.7 (95% CI 0.99–7.5)] and female IIM patients with anti-ARS autoantibody [p = 0.04, pc NS, OR 2.8 (95% CI 1.0–7.5)] compared with individual controls. The distributions of TNF haplotypes were not significantly different between patients and controls even when male and female patients were analyzed separately (data not shown).

DISCUSSION

The number of patients analyzed in our study is the largest among the Japanese IIM studies examining autoantibodies and HLA-DRB1. The distribution of autoantibody subsets in our series was similar to that in a previous Japanese study examining 91 IIM patients[27]. Anti-EJ was the second most common MSA and seemed to be more common in our series than in those reported in Caucasians[5]. Anti-EJ was associated with PM as well as DM in our series, whereas others found that this autoantibody was associated with DM[28,29]. Ohsone, et al also reported that 4 out of 13 Japanese patients with anti-ARS autoantibodies had anti-EJ, and anti-EJ was the second most common anti-ARS autoantibody. We did not examine anti-Mi-2 autoantibody in this study because this autoantibody has been reported to be very rare (0%) in Japanese IIM patients[5].

In this study, our previous findings of an association of HLA-DRB1*08 and *0803 with Japanese IIM (n = 84)[1] were confirmed by analyzing a larger number of patients (n = 120). We also found significant associations of HLA-DRB1*08 and *0803 with anti-ARS autoantibodies. Since the most common MSA are directed against ARS, these 2 results are consistent. The genetic risk factors for IIM and MSA appear to be different between Japanese (DR8,DRB1*0803) and Caucasians (DR3,DRB1*0301)[3].

DR8 alleles are reportedly increased in African-American IIM patients[5], Hispanic SLE patients[30], and Korean autoimmune thyroiditis patients[31]. Both SLE[30] and autoimmune thyroiditis[32] have been reported to be associated with DR3 in Caucasians. Thus, DR8 alleles might be related to the susceptibility to IIM, SLE, and autoimmune thyroiditis in the ethnic groups in which DR3 alleles are rare. It was suggested that DR8 gene was generated by a gene contraction event in a primordial DR52 haplotype (DR3, DR11, to DR14)[33]. DR3 and DR8 nucleotide sequences have been reported to be similar at introns 4 and 5[34] as well as the 5' end[33]. These shared sequences between DR3 and DR8 may be related to the pathogenesis of IIM.

Arnett, *et al*[5] reported that DR2 alleles (DRB1*15 alleles) were decreased in Caucasian and African-American patients with PM. Also, we found that DRB1*1502 was significantly decreased in Japanese patients with anti-ARS autoantibodies compared with controls. Thus, Japanese patients with IIM may share some common features of DRB1 alleles with other ethnic groups.

We found an association between DRB1*0405 and anti-ARS autoantibodies in Japanese IIM patients. And the frequency of HLA-DRB1*0405 seemed to be increased in DM patients compared with controls; however, the difference was not statistically significant. Horiki, et al reported a significant association between Japanese PM patients with ILD and DRB1*0405[6]; however, our current study was not able to confirm this. HLA-DRB1*0405 was associated with anti-ARS autoantibodies in our present study. Since 28 (78%) patients with anti-ARS autoantibodies had complicating ILD, it may be possible that the association reported

Table 5. Carrier frequencies of *HLA-DRB1* alleles in Japanese IIM patients and controls by sex. Values are the number (%) of patients.

HLA-DRB1	Male				Female			
	All IIM, n = 30	MSA (–), n = 21	ARS (+), n = 8	Controls, n = 148	All IIM, n = 90	MSA (–), n = 57	ARS (+), n = 29	Controls, n = 116
*0101	1 (3)	0	1 (13)	12 (8)	20 (33)*	15 (26)	5 (17)	14 (12)
*0405	13 (43)**	9 (43)***	4 (50)	34 (23)	22 (24)	9 (16)	11 (38)	31 (27)
*0803	9 (30)	7 (33)†	2 (25)	23 (16)	19 (21)	9 (16)	8 (28)††	14 (12)

MSA: myositis-specific autoantibody; ARS: anti-aminoacyl-tRNA synthetase autoantibody. * p = 0.05, p_c NS, OR 2.1 (95% CI 0.99–4.4); ** p = 0.02, p_c NS, OR 2.6 (95% CI 1.1–5.8); *** p = 0.05, p_c NS, OR 2.5 (95% CI 0.98–6.5); † p = 0.045, p_c NS, OR 2.7 (95% CI 0.99–7.5); †† p = 0.04, p_c NS, OR 2.8 (95% CI 1.0–7.5).

by Horiki, *et al* was primarily due to the association with anti-ARS autoantibodies. In Japanese, DRB1*0405 allele is associated with rheumatoid arthritis[35], Crohn's disease[14], arthritis, and rheumatoid factor in systemic sclerosis[36]. These results suggest a similar genetic background among these autoimmune diseases in Japanese.

Since the HLA-DQA1*0501 or *0401 allele was reported to be commonly increased in Caucasian and African-American patients with IIM, while different DRB1 alleles were associated with the disease in each ethnic population, Arnett, *et al* suggested that susceptibility to IIM may be localized in the DQA1 locus[5]. However, we[1] and others[5,6] did not observe that the susceptibility to IIM was primarily associated with the DQA1 locus in the Japanese. We also examined distribution of MSA in 83 IIM patients for whom DQA1 alleles were analyzed in our previous study and found no significant association of MSA with DQA1 alleles (data not shown).

We found an association of TNF-U03 with ILD in this study. Previous studies concerning the promoter activity of TNF-U03 are conflicting, i.e., increased[12], unchanged[37], or decreased[38]. TNF is considered to have a disease-promoting effect for ILD[39,40] and some reports suggest that blockade of TNF may be beneficial for ILD[41]. TNF-U03 was recently reported to show a higher binding to the transcriptional factor OCT-1, compared with the common TNF-U01 allele[42].

Our results suggest that the association of HLA-DRB1*0101 and HLA-DRB1*0405 with IIM may be influenced by sex. Although previous IIM studies have not showed any sex differences in genetics, we[43] and others[44,45] have reported significant sex influences on modifier loci in other autoimmune diseases. Our data suggest that the association of some loci with IIM may be influenced by sex.

We confirmed in this study our previous findings of immunogenetic features of Japanese patients with IIM. Further studies of non-HLA as well as HLA genes are needed for determining the genetic contribution of the susceptibility and the pathogenesis of IIM.

ACNOWLEDGMENT

We thank Dr. R.L. Wilder for critical review of the manuscript.

REFERENCES

1. Furuya T, Hakoda M, Higami K, et al. Association of HLA class I and class II alleles with myositis in Japanese patients. J Rheumatol 1998;25:1109-14.
2. Love LA, Leff RL, Fraser DD, et al. A new approach to the classification of idiopathic inflammatory myopathy: myositis-specific autoantibodies define useful homogeneous patient groups. Medicine Baltimore 1991;70:360-74.
3. Shamim EA, Rider LG, Miller FW. Update on the genetics of the idiopathic inflammatory myopathies. Curr Opin Rheumatol 2000;12:482-91.
4. Imanishi T, Akaza T, Kimura A, Tokunaga K, Gojobori T. Allele and haplotype frequencies for HLA and complement loci in various ethnic groups. In: Sasazuki T, editor. HLA 1991: Proceedings of the Eleventh International Histocompatibility Workshop and Conference. Oxford: Oxford University Press; 1992.
5. Arnett FC, Targoff IN, Mimori T, Goldstein R, Warner NB, Reveille JD. Interrelationship of major histocompatibility complex class II alleles and autoantibodies in four ethnic groups with various forms of myositis. Arthritis Rheum 1996;39:1507-18.
6. Horiki T, Ichikawa Y, Moriuchi J, et al. HLA class II haplotypes associated with pulmonary interstitial lesions of polymyositis/dermatomyositis in Japanese patients. Tissue Antigens 2002;59:25-30.
7. Eigler A, Sinha B, Hartmann G, Endres S. Taming TNF: strategies to restrain this proinflammatory cytokine. Immunol Today 1997;18:487-92.
8. Hengstman GJ, van den Hoogen FH, Barrera P, et al. Successful treatment of dermatomyositis and polymyositis with anti-tumor-necrosis-factor-alpha: preliminary observations. Eur Neurol 2003;50:10-5.
9. Wilson AG, Symons JA, McDowell TL, McDevitt HO, Duff GW. Effects of a polymorphism in the human tumor necrosis factor alpha promoter on transcriptional activation. Proc Natl Acad Sci USA 1997;94:3195-9.
10. Pachman LM, Fedczyna TO, Lechman TS, Lutz J. Juvenile dermatomyositis: the association of the TNF alpha-308A allele and disease chronicity. Curr Rheumatol Rep 2001;3:379-86.
11. Werth VP, Callen JP, Ang G, Sullivan KE. Associations of tumor necrosis factor alpha and HLA polymorphisms with adult dermatomyositis: implications for a unique pathogenesis. J Invest Dermatol 2002;119:617-20.
12. Higuchi T, Seki N, Kamizono S, et al. Polymorphism of the 5'-flanking region of the human tumor necrosis factor (TNF)-alpha gene in Japanese. Tissue Antigens 1998;51:605-12.
13. Matsushita M, Tsuchiya N, Nakayama T, et al. Allele typing of human TNFA 5'-flanking region using polymerase chain reaction-preferential homoduplex formation assay (PCR-PHFA): linkage disequilibrium with HLA class I and class II genes in Japanese. Tissue Antigens 1999;54:478-84.
14. Kawasaki A, Tsuchiya N, Hagiwara K, Takazoe M, Tokunaga K. Independent contribution of HLA-DRB1 and TNF alpha promoter polymorphisms to the susceptibility to Crohn's disease. Genes Immun 2000;1:351-7.
15. Noguchi E, Yokouchi Y, Shibasaki M, et al. Association between TNFA polymorphism and the development of asthma in the Japanese population. Am J Respir Crit Care Med 2002;166:43-6.
16. Bohan A, Peter JB. Polymyositis and dermatomyositis (second of two parts). N Engl J Med 1975;292:403-7.
17. Bohan A, Peter JB. Polymyositis and dermatomyositis (first of two parts). N Engl J Med 1975;292:344-7.
18. Preliminary criteria for the classification of systemic sclerosis (scleroderma). Subcommittee for scleroderma criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee. Arthritis Rheum 1980;23:581-90.
19. Tan EM, Cohen AS, Fries JF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982;25:1271-7.
20. Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315-24.
21. Shibue T, Tsuchiya N, Komata T, et al. Tumor necrosis factor alpha 5'-flanking region, tumor necrosis factor receptor II, and HLA-DRB1 polymorphisms in Japanese patients with rheumatoid arthritis. Arthritis Rheum 2000;43:753-7.
22. Tokunaga K, Imanishi T, Takahashi K, Juji T. On the origin and dispersal of East Asian populations as viewed from HLA haplotypes. In: Szathmary EJ, editor. Prehistoric Mongoloid

dispersals. Oxford: Oxford University Press; 1996:187-97.
23. Hirakata M, Suwa A, Nagai S, et al. Anti-KS: identification of autoantibodies to asparaginyl-transfer RNA synthetase associated with interstitial lung disease. J Immunol 1999;162:2315-20.
24. Uryu N, Maeda M, Ota M, Tsuji K, Inoko H. A simple and rapid method for HLA-DRB and -DQB typing by digestion of PCR-amplified DNA with allele specific restriction endonucleases. Tissue Antigens 1990;35:20-31.
25. Tsuchiya N, Kawasaki A, Tsao BP, Komata T, Grossman JM, Tokunaga K. Analysis of the association of HLA-DRB1, TNF alpha promoter and TNFR2 (TNFRSF1B) polymorphisms with SLE using transmission disequilibrium test. Genes Immun 2001;2:317-22.
26. Goldstein R, Duvic M, Targoff IN, et al. HLA-D region genes associated with autoantibody responses to histidyl-transfer RNA synthetase (Jo-1) and other translation-related factors in myositis. Arthritis Rheum 1990;33:1240-8.
27. Hirakata M, Mimori T, Akizuki M, Craft J, Hardin JA, Homma M. Autoantibodies to small nuclear and cytoplasmic ribonucleoproteins in Japanese patients with inflammatory muscle disease. Arthritis Rheum 1992;35:449-56.
28. Hirakata M, Suwa A, Takeda Y, et al. Autoantibodies to glycyl-transfer RNA synthetase in myositis. Association with dermatomyositis and immunologic heterogeneity. Arthritis Rheum 1996;39:146-51.
29. Targoff IN. Autoantibodies to aminoacyl-transfer RNA synthetases for isoleucine and glycine. Two additional synthetases are antigenic in myositis. J Immunol 1990;144:1737-43.
30. Reveille JD, Moulds JM, Ahn C, et al. Systemic lupus erythematosus in three ethnic groups: I. The effects of HLA class II, C4, and CR1 alleles, socioeconomic factors, and ethnicity at disease onset. LUMINA Study Group. Lupus in minority populations, nature versus nurture. Arthritis Rheum 1998;41:1161-72.
31. Cho BY, Chung JH, Shong YK, et al. A strong association between thyrotropin receptor-blocking antibody-positive atrophic autoimmune thyroiditis and HLA-DR8 and HLA-DQB1*0302 in Koreans. J Clin Endocrinol Metab 1993;77:611-5.
32. Tandon N, Zhang L, Weetman AP. HLA associations with Hashimoto's thyroiditis. Clin Endocrinol Oxford 1991;34:383-6.
33. Andersson G, Lindblom B, Andersson L, Gorski J, Mach B, Rask L. The single DR beta gene of the DRw8 haplotype is closely related to the DR beta 3III gene encoding DRw52. Immunogenetics 1988;28:1-5.
34. Svensson AC, Setterblad N, Sigurdardottir S, Rask L, Andersson G. Primate DRB genes from the DR3 and DR8 haplotypes contain ERV9 LTR elements at identical positions. Immunogenetics 1995;41:74-82.
35. Higami K, Hakoda M, Matsuda Y, Ueda H, Kashiwazaki S. Lack of association of HLA-DRB1 genotype with radiologic progression in Japanese patients with early rheumatoid arthritis. Arthritis Rheum 1997;40:2241-7.
36. Kuwana M, Inoko H, Kameda H, et al. Association of human leukocyte antigen class II genes with autoantibody profiles, but not with disease susceptibility in Japanese patients with systemic sclerosis. Intern Med 1999;38:336-44.
37. Uglialoro AM, Turbay D, Pesavento PA, et al. Identification of three new single nucleotide polymorphisms in the human tumor necrosis factor-alpha gene promoter. Tissue Antigens 1998; 52:359-67.
38. Skoog T, van't Hooft FM, Kallin B, et al. A common functional polymorphism (C→A substitution at position -863) in the promoter region of the tumour necrosis factor-alpha (TNF-alpha) gene associated with reduced circulating levels of TNF-alpha. Hum Mol Genet 1999;8:1443-9.
39. Miyazaki Y, Araki K, Vesin C, et al. Expression of a tumor necrosis factor-alpha transgene in murine lung causes lymphocytic and fibrosing alveolitis. A mouse model of progressive pulmonary fibrosis. J Clin Invest 1995;96:250-9.
40. Piguet PF, Collart MA, Grau GE, Sappino AP, Vassalli P. Requirement of tumour necrosis factor for development of silica-induced pulmonary fibrosis. Nature 1990;344:245-7.
41. Vassallo R, Matteson E, Thomas CF Jr. Clinical response of rheumatoid arthritis-associated pulmonary fibrosis to tumor necrosis factor-alpha inhibition. Chest 2002;122:1093-6.
42. Hohjoh H, Tokunaga K. Allele-specific binding of the ubiquitous transcription factor OCT-1 to the functional single nucleotide polymorphism (SNP) sites in the tumor necrosis factor-alpha gene (TNFA) promoter. Genes Immun 2001;2:105-9.
43. Furuya T, Salstrom JL, McCall-Vining S, et al. Genetic dissection of a rat model for rheumatoid arthritis: significant gender influences on autosomal modifier loci. Hum Mol Genet 2000;9:2241-50.
44. del Rincon I, Battafarano DF, Arroyo RA, Murphy FT, Escalante A. Heterogeneity between men and women in the influence of the HLA-DRB1 shared epitope on the clinical expression of rheumatoid arthritis. Arthritis Rheum 2002;46:1480-8.
45. Meyer JM, Han J, Moxley G. Tumor necrosis factor markers show sex-influenced association with rheumatoid arthritis. Arthritis Rheum 2001;44:286-95.

ARTHRITIS & RHEUMATISM
Vol. 50, No. 2, February 2004, pp 660–668
DOI 10.1002/art.20023

Ameliorative Effects of Follistatin-Related Protein/TSC-36/FSTL1 on Joint Inflammation in a Mouse Model of Arthritis

Daisuke Kawabata,[1] Masao Tanaka,[1] Takao Fujii,[1] Hisanori Umehara,[1] Yoshimasa Fujita,[1] Hajime Yoshifuji,[1] Tsuneyo Mimori,[1] and Shoichi Ozaki[2]

Objective. **To clarify the in vivo function of follistatin-related protein (FRP)/TSC-36/FSTL1 in rheumatoid arthritis (RA), we investigated the roles of FRP in a mouse model of arthritis.**

Methods. **Arthritis was induced in BALB/c mice by injecting anti–type II collagen monoclonal antibody and lipopolysaccharide. Mice were treated with daily intraperitoneal injections of 20 μg of recombinant FRP. Development of arthritis was assessed by the clinical score and footpad swelling. Histologic examination of affected paws was performed on day 21 after the onset of arthritis. The gene expression profiles of affected paws in FRP-treated and untreated mice were compared using commercially available complementary DNA (cDNA) arrays. The difference in gene expression was confirmed by real-time quantitative reverse transcription–polymerase chain reaction.**

Results. **Treatment with recombinant FRP showed significant amelioration of the arthritis severity. Histologic analyses confirmed this finding and revealed the alleviation of cellular infiltration into the synovium as well as cartilage damage. The significant decrease in the amount of urinary deoxypyridinoline also indicated the ameliorative effect of FRP on joint destruction. Moreover, cDNA array analysis of the gene expression profile in FRP-treated arthritic lesions revealed a reduced expression of the c-*fos*, *ets*-2, *IL6*, *MMP3*, and *MMP9* genes, some of which are thought to be associated with synovial inflammation and joint destruction.**

Conclusion. **These findings from in vivo experiments suggest that FRP could be one of the key molecules in the treatment of inflammatory joint diseases such as RA.**

Rheumatoid arthritis (RA) is a chronic systemic disease characterized by destructive polyarthritis, and its etiology remains to be elucidated. In the pathogenesis of RA, an antigen-driven immune response involving synovial T cells and B cells is thought to play a central role in persistent joint inflammation (1). Therefore, several self antigens, such as type II collagen (CII), heat-shock protein 60, calpastatin, human cartilage glycoprotein 39, and glucose-6-phosphate isomerase, have been reported to be candidate target antigens of pathogenic autoantibodies in RA (2–6).

In an effort to find such antigens, we have cloned novel target proteins of autoantibodies from RA synovium with the use of RA synovial fluid IgG probes by an expression cloning method; one of these clones was follistatin-related protein (FRP) (7). FRP was originally isolated as the product of TSC-36, one of the genes found to be up-regulated by transforming growth factor β1 (TGFβ1) in the mouse osteoblastic cell line MC3T3-E1 (8). Rat and human homologs have been cloned from glioma cell lines (9).

FRP is a secreted glycoprotein, and it is expressed in all organs, except for peripheral blood leukocytes (7). FRP shares a characteristic structural module (the FS domain) with follistatin, an inhibitor of activin

Supported by grants-in-aid (11557038 and 13557041) from the Ministry of Education, Science, and Culture of Japan, and by grants from the Fujiwara Memorial Foundation and the Kowa Life Science Foundation (A42 of the 15th).

[1]Daisuke Kawabata, MD, Masao Tanaka, MD, PhD, Takao Fujii, MD, PhD, Hisanori Umehara, MD, PhD, Yoshimasa Fujita, MD, PhD, Hajime Yoshifuji, MD, Tsuneyo Mimori, MD, PhD: Kyoto University Graduate School of Medicine, Kyoto, Japan; [2]Shoichi Ozaki, MD, PhD: St. Marianna University School of Medicine, Kawasaki, Japan.

Address correspondence and reprint requests to Masao Tanaka, MD, PhD, Department of Rheumatology and Clinical Immunology, Kyoto University Graduate School of Medicine, 54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: masatana@kuhp.kyoto-u.ac.jp.

Submitted for publication February 4, 2003; accepted in revised form October 1, 2003.

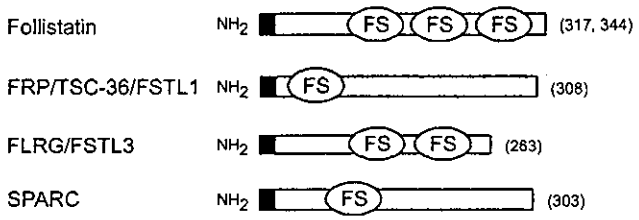

Figure 1. Schematic representation of follistatin and related FS domain–bearing proteins. Follistatin and follistatin-related gene protein (FLRG)/follistatin-like 3 (FSTL3) have more than 2 FS domains in the COOH terminus. Follistatin-related protein (FRP)/TSC-36/FSTL1 contains a single follistatin domain in the NH_2 terminus. Numbers in parentheses represent amino acid residues in the human proteins. Signal peptides are shown in the solid portions of the bars.

(10) (Figure 1). In addition to follistatin and FRP, other FS domain–bearing proteins, such as secreted protein, acidic and rich in cysteine (SPARC), SC1/hevin, testican, QR1, and follistatin-related gene protein (FLRG), have been cloned, and together, they compose the follistatin family (11). FLRG is a translated product of follistatin-related gene (*FLRG*), which was isolated from a B cell leukemia cell line (12). FRP and FLRG are different proteins (12,13) and are listed in the OMIM database under the names follistatin-like 1 (FSTL1) and follistatin-like 3 (FSTL3), respectively (13,14). Follistatin and FLRG have activin-binding activity and inhibit activin-mediated gene transcription (15). Other follistatin family proteins, including FRP, have not been demonstrated to bind activin or related TGFβ family proteins. SPARC, the best-studied of the group, is known to regulate cell–matrix interactions and thereby regulate tissue remodeling and homeostasis (11). FRP has been reported to play some roles in cell-cycle inhibition and to have negative regulatory effects on growth in human lung cancer cells (8,16). However, the exact physiologic function of FRP remains to be clarified.

We previously reported that serum autoantibodies to FRP were detected in 30% of patients with RA, and their appearance correlated with disease activity (7). Recently, our in vitro experiments demonstrated that FRP suppressed the production of matrix metalloproteinase 1 (MMP-1), MMP-3, and prostaglandin E_2 in synovial cells derived from patients with RA, and suggested that FRP could act as a joint defensive factor in RA (17).

To examine the in vivo effect of FRP on arthropathy in inflammatory joint diseases such as RA, recombinant FRP was administered in a mouse model of arthritis. In this experiment, we induced arthritis in mice with the use of anti-CII monoclonal antibodies and lipopolysaccharide (LPS) in order to achieve high reproducibility (18). We then examined the macroscopic and microscopic effects of FRP on arthritis. To obtain insight into the FRP signaling pathway, we analyzed the gene expression profiles of affected joint tissues using complementary DNA (cDNA) arrays.

MATERIALS AND METHODS

Recombinant human FRP. *Escherichia coli*–expressed glutathione S-transferase (GST)–FRP was prepared as previously described (7). The GST tag was removed from GST-FRP using a GSTrap FF column (Amersham Pharmacia Biotech, Uppsala, Sweden), and the protein was purified by an anion-exchange chromatography method with a Mono Q HR column (Amersham Pharmacia Biotech). Finally, the protein was filtered with a Zetapore Dispo filter (CUNO, Meriden, CT) to remove endotoxins.

Induction and evaluation of arthritis. Arthritis was induced in 7–8-week-old female BALB/c mice (Japan SLC, Hamamatsu, Japan) by intraperitoneal injection with 500 μl of phosphate buffered saline (PBS) containing 4 monoclonal antibodies to CII (500 μg of D1, D8, A2, and F10, respectively; Chondrex, Redmond, WA). After 72 hours, the mice were injected intraperitoneally with 100 μl of PBS containing 50 μg of LPS (Sigma, St. Louis, MO). The mice were maintained under specific pathogen-free conditions.

The severity of arthritis in each paw was estimated in a blinded manner. Paws were assessed by both visual inspection and measurement of limb swelling with calipers. Each paw was scored on a scale of 0–3, as previously described (19), where 0 = no swelling, 1 = swelling of finger joints or mild swelling of the ankle or wrist joint, 2 = severe inflammation of the entire paw, and 3 = deformity or ankylosis. The severity of arthritis in each mouse was the sum of the scores in the 4 paws (maximum possible score 12). Institutional approval of the bioethics of the study was obtained prior to these experiments (approval no. 02004).

Treatment with FRP. The mice were randomly assigned to 1 of 2 treatment groups before the onset of arthritis: FRP treatment or PBS treatment (control). The mice in the FRP treatment group were injected intraperitoneally with 20 μg (1 mg/kg) of recombinant human FRP daily for 10 days, starting 1 hour before injection of the monoclonal antibody. The mice in the PBS treatment group were injected intraperitoneally with 250 μl of PBS daily for 10 days.

Histopathologic evaluation. Pairs of arthritic hind paws from representative mice in each group (n = 6 animals, 12 paws) were removed at the end of the experiment (day 21), fixed in 10% buffered formalin, decalcified in 10% formic acid, and then embedded in paraffin. Serial sagittal sections of whole hind paws were subjected to hematoxylin and eosin staining for histologic analyses and to Safranin O and fast green/iron hematoxylin staining for estimation of the loss of cartilaginous proteoglycan. Synovial lesions were scored on a scale of 0–3, where 0 = normal synovium, 1 = mild synovial hyperplasia and/or mild synovial infiltration, 2 = moderate synovial hyperplasia and/or moderate synovial infiltration, and 3 = severe synovial hyperplasia and/or severe synovial infiltra-

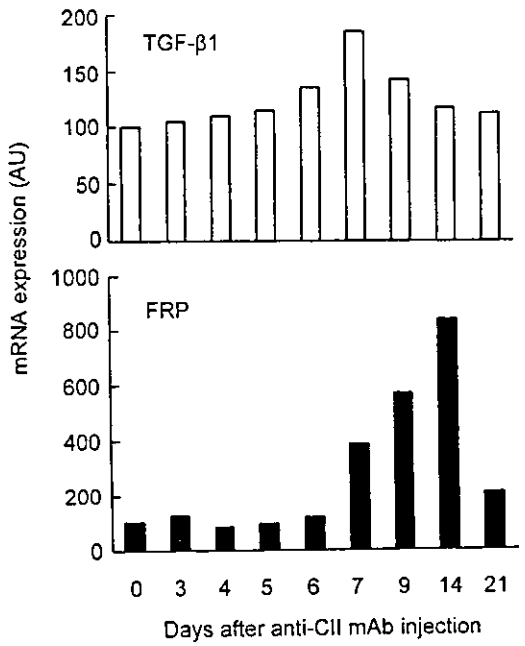

Figure 2. Changes in expression levels of transforming growth factor β1 (TGFβ1) and follistatin-related protein (FRP) mRNA during the development of arthritis. Levels of TGFβ1 and FRP mRNA expression (in arbitrary units [AU]) were quantified by real-time reverse transcription–polymerase chain reaction. Anti-CII = anti–type II collagen; mAb = monoclonal antibody.

tion. Cartilage depletion was identified by the presence of diminished Safranin O staining of the matrix and was scored on a scale of 0–3, where 0 = no cartilage destruction (full staining with Safranin O), 1 = localized cartilage erosions, 2 = more extended cartilage erosions, and 3 = severe cartilage erosions (depletion of entire cartilage). Histologic analyses were performed in a blinded manner.

Quantitative reverse transcription–polymerase chain reaction (RT-PCR) for TGFβ1 and FRP messenger RNA (mRNA) expression. Groups of mice were killed on days 0, 3, 4, 5, 6, 7, 9, 14, and 21, and total RNA was obtained from 6 arthritic hind paws in each group by mixing with TRIzol reagent (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. Five micrograms of total RNA was subjected to RT using the SuperScript First Strand Synthesis system (Invitrogen, Carlsbad, CA). Each reaction was run in duplicate using SYBR Green PCR Master Mix (PE Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The primer pairs used in these reactions were as follows: for TGFβ1, 5′-AGA-TCT-CCC-TCG-GAC-CTG-CT-3′ and 5′-GGG-TCT-CCC-AAG-GAA-AGG-TA-3′; for FRP, 5′-GGA-AGA-GAG-CGT-GAA-GGA-CA-3′ and 5′-CGG-AGG-CTC-AAA-GAA-AGG-AT-3′; and for GAPDH, 5′-CGC-TGA-GTA-CGT-CGT-GGA-GT-3′ and 5′-GGT-GCT-AAG-CAG-TTG-GTG-GT-3′. Amplification reactions, data acquisition, and analysis were performed with the ABI Prism 7900 HT instrument (PE Applied Biosystems), and relative levels of gene expression were normalized based on those of the GAPDH gene.

Urinary deoxypyridinoline levels. Mouse urine was collected on day 9, and urinary levels of deoxypyridinoline were measured with an Osteolinks DPD enzyme-linked immunosorbent assay kit (Metra Biosystems, Mountain View, CA).

Complementary DNA array analysis. Arthritic mice treated with FRP (20 μg/day) and untreated mice were killed on day 10, and total RNA was prepared from 3 hind paws by mixture in each group as described above. Moreover, mRNA was purified from the total RNA with a MagExtractor mRNA kit (Toyobo, Osaka, Japan). The Gene Navigator cDNA Array system (Toyobo) was used in this analysis. The cDNA array consisted of 561 species of immunologically related mouse cDNA fragments (20). (The Microsoft Excel file can be downloaded from http://www.toyobo.co.jp/seihin/xr/product/mouse%20immunology.xls) The biotin-labeled probes were prepared from the sample mRNA with biotin-16-dUTP with the Gene Navigator cDNA Amplification system (Toyobo). Signals produced by the hybridized probe–avidin–biotinylated alkaline phosphatase complex and substrate (Vistra ECF substrate; Amersham Pharmacia Biotech) were detected with the Imaging High Chemifluorescence detection kit (Toyobo). The chemifluorescence image of the cDNA array filter was analyzed using ImaGene software version 4.2 (Takara Bio, Otsu, Japan). The net signal intensity of genes was calculated from the measured raw signal intensity minus that of the background. Expression of individual genes was evaluated by their net signal intensity ratio compared with the standard housekeeping gene.

Confirmation of differentially expressed genes by quantitative RT-PCR. Expression levels of mRNA were estimated by real-time quantitative PCR using SYBR Green I. We obtained cDNA from the hind paws of FRP-treated (n = 3) and PBS-treated (n = 3) mice killed on day 10 as described above. Each reaction was run in triplicate using SYBR Green PCR Master Mix according to the manufacturer's protocol. The primer pairs used in these reactions were as follows: for c-Fos, 5′-CTA-TGC-AGC-AGA-CTG-GGA-GC-3′ and 5′-ACA-GCC-TGG-TGT-GTT-TCA-CG-3′; for Ets-2, 5′-CTT-CCA-AAA-GGA-GCA-ACG-AC-3′ and 5′-GTC-CTG-GCT-GAT-GGA-ACA-GT-3′; for interleukin-6 (IL-6), 5′-CAG-AGG-ATA-CCA-CTC-CCA-ACA-3′ and 5′-CCA-GTT-TGG-TAG-CAT-CCA-TC-3′; for MMP-3, 5′-CGA-TGG-ACA-GAG-GAT-GTC-AC-3′ and 5′-CCC-TCG-TAT-AGC-CCA-GAA-CT-3′; for MMP-9, 5′-ACT-TTC-CCT-TCA-CCT-TCG-AG-3′ and 5′-GAA-CAG-GCT-GTA-CCC-TTG-GT-3′; and for GAPDH, 5′-CGC-TGA-GTA-CGT-CGT-GGA-GT-3′ and 5′-GGT-GCT-AAG-CAG-TTG-GTG-GT-3′. The amplification reactions, data acquisition, and analysis were performed with the ABI Prism 7900 HT instrument, and relative levels of gene expression were normalized against those of the GAPDH gene.

Statistical analysis. Differences between experimental groups were tested using the Mann-Whitney U test and Student's unpaired *t*-test. *P* values less than 0.05 were considered significant.

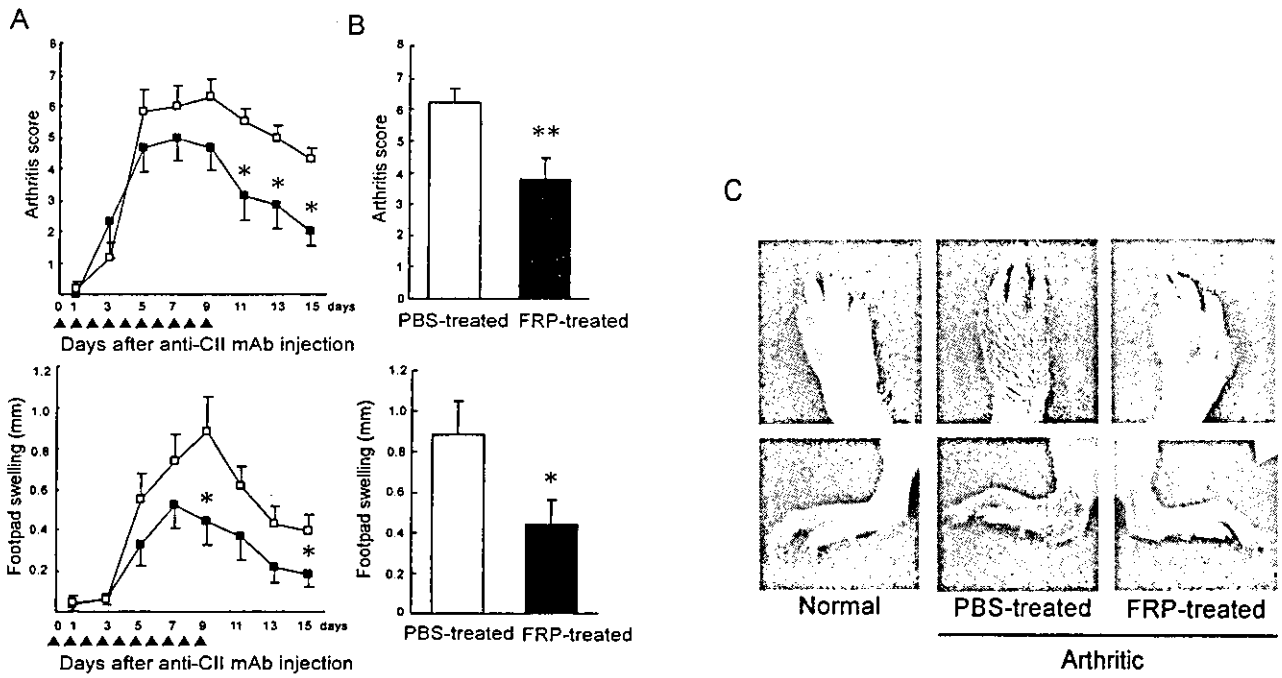

Figure 3. Amelioration of disease in arthritic mice treated with recombinant follistatin-related protein (FRP). **A,** Disease progress estimated by the arthritis score and footpad swelling (n = 6 mice per group). Anti–type II collagen (anti-CII) monoclonal antibodies (mAb) were injected on day 0, and 50 μg of lipopolysaccharide was injected on day 2. Recombinant FRP (■) or phosphate buffered saline (PBS; vehicle control) (□) was administered daily for 10 days, beginning on day 0 (arrowheads). **B,** The arthritis score and footpad swelling in both the PBS-treated (n = 12) and the FRP-treated (n = 11) groups were evaluated at the peak of arthritis (day 9). Bars show the mean and SEM. * = $P < 0.05$; ** = $P < 0.01$, by Mann-Whitney U test. **C,** Photographs showing representative paws of mice from each group on day 9.

RESULTS

First, to assess the regulation of FRP and its synovial inducer TGFβ1 in arthritis, we examined time course changes in their mRNA expression levels in arthritic paws from monoclonal antibody–injected mice (Figure 2). Depending on arthritis activity, both TGFβ1 and FRP were elevated temporarily. The elevation of TGFβ1 preceded up-regulation of FRP. Interestingly, after TGFβ1 began to decrease, FRP continued to rise for a time. Thus, some molecule besides TGFβ1 may regulate FRP expression.

To investigate the antiarthritic properties of FRP in vivo, we studied its effect on mouse arthritis using anti-CII antibodies and LPS. In this mouse model, severe arthritis occurs about 24 hours after LPS injection and persists for more than 3 weeks (18). As shown in Figure 3, FRP treatment demonstrated a significant ameliorative effect on the arthritis by reducing the clinical score and preventing the development of footpad swelling. On day 9, we could not detect a significant difference in the arthritis score in the small number of mice shown in Figure 3A (n = 6), but a significant difference was detected in the larger sample shown in Figure 3B (n = 11). Macroscopically, redness and swelling of the fore paws and hind paws were milder in the FRP-treated mice than in the PBS-treated mice (Figure 3C). The 10 daily 20-μg doses of FRP produced no significant change in body weight and no macroscopically abnormal findings in FRP-treated mice.

Tarsocrural joints from normal mice had no synovial inflammation or cartilage erosion (Figure 4a). Joints from PBS-treated mice with an arthritis score of 2 had severe synovial inflammation, cartilage erosion, and bone resorption (Figure 4b), while joints from FRP-treated mice with an arthritis score of 1 had mild synovial inflammation and no cartilage or bone erosion (Figure 4c). The weak staining by Safranin O in the articular cartilage of the PBS-treated mice indicated a loss of proteoglycans from the cartilage matrix (Figure 4e). In contrast, Safranin O staining of articular cartilage from FRP-treated mice was equivalent to that in cartilage from normal mice (Figures 4f and d). Histologic

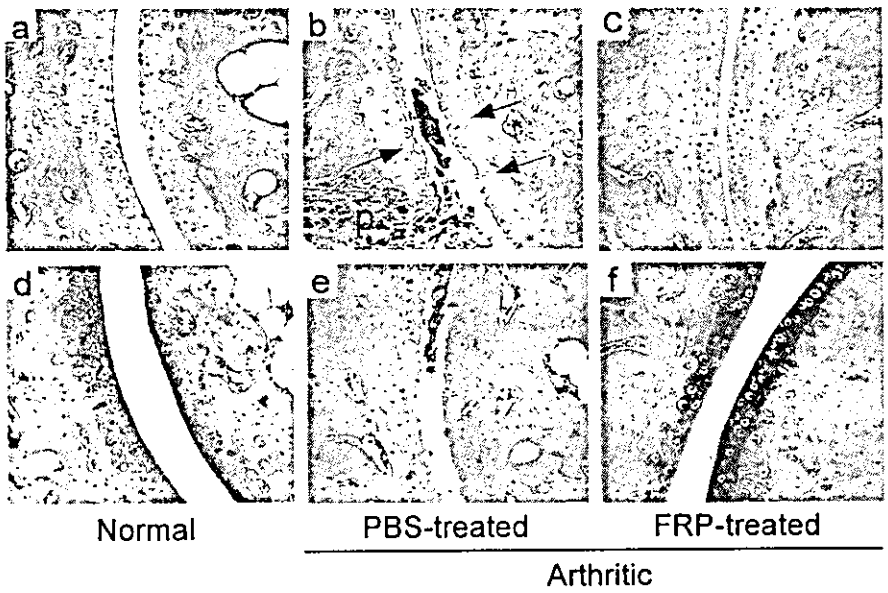

Figure 4. Histologic analysis of tarsocrural joints from normal and arthritic BALB/c mice. All specimens from normal, phosphate buffered saline (PBS)–treated, and follistatin-related protein (FRP)–treated mice were collected on day 21 after monoclonal antibody injection and stained with either hematoxylin and eosin (**a**, **b**, and **c**) or Safranin O (**d**, **e**, and **f**). **a** and **d**, Joints from normal mice exhibit normal histologic features, with intact articular cartilage and no evidence of inflammation or erosion. **b** and **e**, Affected joints from PBS-treated mice show severe synovitis, pannus formation (**p**), and cartilage/bone erosion (**arrows**). Absence of Safranin O staining indicates depletion of proteoglycans. **c** and **f**, In contrast, synovitis, cartilage/bone erosion, and loss of Safranin O staining of the cartilage were mild in FRP-treated arthritic mice. (Original magnification × 200.)

scores assessed on day 21 were significantly lower in the FRP-treated mice than in the PBS-treated mice. This confirmed the ameliorative effects of FRP on joint inflammation and cartilage destruction (Figure 5). Thus, FRP seemed to prevent cartilage breakdown in arthritis.

Because deoxypyridinoline is one of the common markers of bone resorption, we measured urinary levels of deoxypyridinoline on day 9 in arthritic mice treated with recombinant FRP or PBS. The urinary levels of deoxypyridinoline were markedly higher in the arthritic mice than in the normal mice. However, those in the FRP-treated arthritic mice were significantly lower than those in the PBS-treated mice (Figure 6). This suggested that FRP worked against the bone destruction in arthritis.

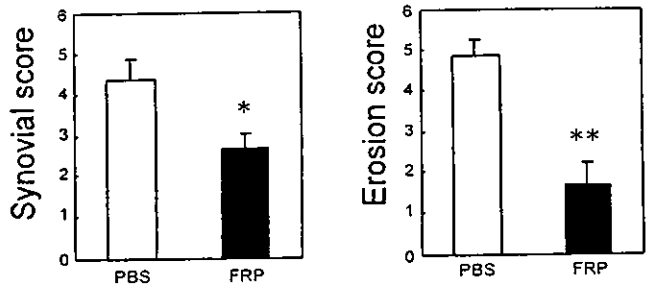

Figure 5. Comparison of histologic scores in phosphate buffered saline (PBS)–treated and follistatin-related protein (FRP)–treated mice on day 21. Bars show the mean and SEM (n = 6 mice per group). * = $P < 0.05$; ** = $P < 0.01$, by Mann-Whitney U test.

To identify the genes that are affected by FRP administration in this mouse model of arthritis, we examined the gene expression profiles by cDNA array techniques in joint tissues from normal, PBS-treated, and FRP-treated mice (n = 3 per group). We studied the expression of 561 genes blotted on the cDNA array, including 9 housekeeping genes and 2 negative control genes (see Materials and Methods). As a result, we found a change of >1.5-fold in the expression levels of 170 genes in the FRP-treated group versus the PBS-treated group. Five of these 170 genes (c-*fos*, *ets*-2, *IL6*, *MMP3*, and *MMP9*) were down-regulated in the FRP-treated group (Figure 7A); these genes have been

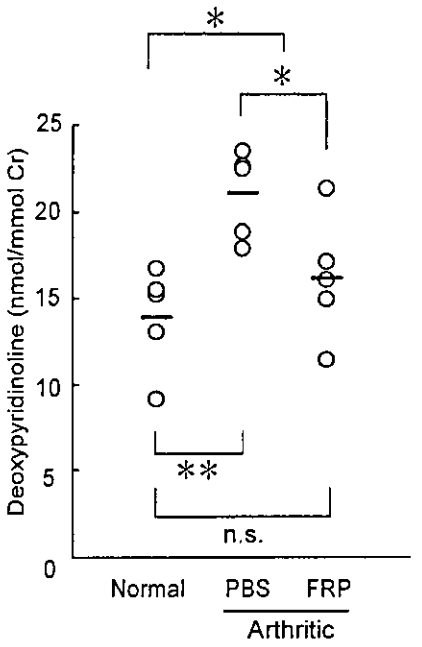

Figure 6. Urinary deoxypyridinoline levels in normal and arthritic mice. Urinary levels of deoxypyridinoline (expressed in nmoles/mmole of creatinine [Cr]) were significantly decreased in the follistatin-related protein (FRP)–treated arthritic mice compared with the phosphate buffered saline (PBS)–treated arthritic mice. Deoxypyridinoline levels were measured by enzyme-linked immunosorbent assay in samples collected on day 9 (n = 5 mice per group). * = $P < 0.05$; ** = $P < 0.01$, by Mann-Whitney U test. n.s. = not significant.

reported to be responsible for the destructive joint inflammation in RA (21–24).

To confirm whether the change in the expression of the 5 genes was in fact significant, we performed a real-time RT-PCR analysis using the same cDNA sources as those for the cDNA array. We found that the expression levels of the c-*fos*, *ets*-2, *IL6*, *MMP3*, and *MMP9* genes were significantly lower in paws from the FRP-treated mice than in the paws from the PBS-treated mice (Figure 7B). Therefore, FRP seemed to exert its preventive effect on the arthritis, in part, by down-regulating disease-promoting genes such as the 5 we identified.

DISCUSSION

In this study, we demonstrated that FRP had therapeutic effects on murine arthritis. Because of its rapid induction and high reproducibility, we used a mouse model of acute, destructive arthritis (resembling human RA) induced by anti-CII monoclonal antibodies and LPS. The expression of endogenous FRP and its synovial inducer TGFβ1 was higher in the arthritic paws of this model than in the normal paws. In addition, the expression of FRP was maintained at a high level even if the arthritis had peaked. A recent study also showed that the expression of FRP was increased at the synovium–bone erosion interface in early and late-phase collagen-

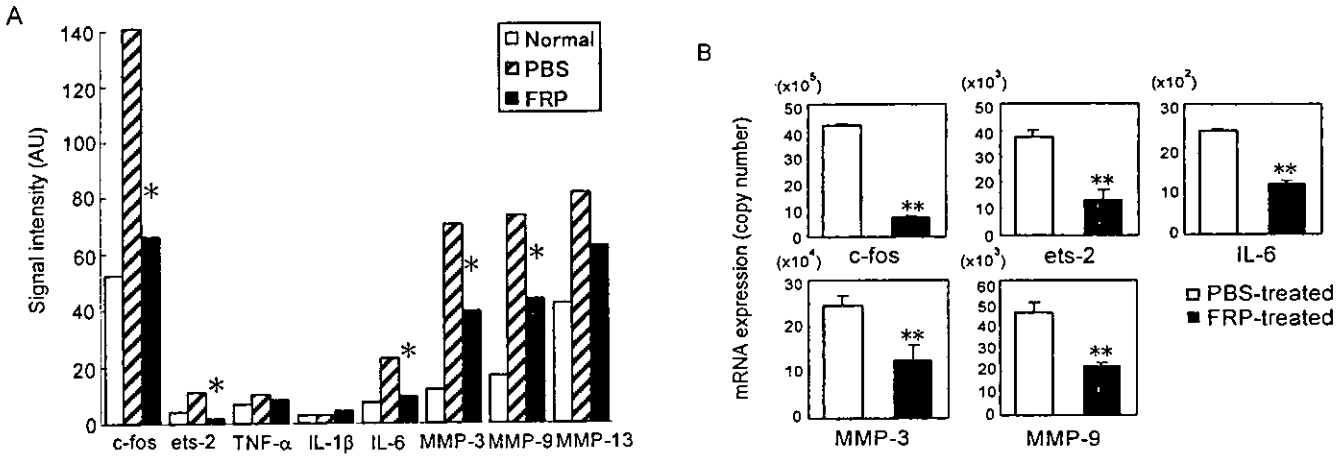

Figure 7. Gene expression in joint tissues of normal, phosphate buffered saline (PBS)–treated, and follistatin-related protein (FRP)–treated mice, as analyzed by cDNA arrays and real-time quantitative reverse transcription–polymerase chain reaction (RT-PCR). In both experiments, mRNA was extracted from joint tissues on day 10 of the experiment (n = 3 mice per group). **A**, The results of cDNA arrays analysis showed that the gene expression levels of c-*fos*, *ets*-2, *IL6*, *MMP3*, and *MMP9* were lower in the joints of FRP-treated mice than in the joints of PBS-treated mice. * = ratio of signal intensity (in arbitrary units [AU]) in PBS-treated mice to that in FRP-treated mice >1.5. TNF-α = tumor necrosis factor α. **B**, Real-time quantitative RT-PCR studies confirmed the significantly reduced levels of gene expression in FRP-treated mouse joints compared with PBS-treated mouse joints (control) for c-*fos*, *ets*-2, *IL6*, *MMP3*, and *MMP9*. ** = $P < 0.01$, by Student's unpaired *t*-test.

induced arthritis (25). Thus, we presumed that endogenous FRP was induced by TGFβ1 mainly from the synovium as the arthritis developed and may contribute to the regulation of arthritis. Our previous in vitro experiments suggested that FRP could prevent destructive arthritis (17). Therefore, we postulated that abundant exogenous FRP, in addition to feedback-regulated FRP, may improve arthritis.

FRP is a genetically highly conserved protein, having 92% amino acid sequence identity between humans and mice (9), and our previous in vitro study strongly suggested that recombinant human FRP may function in other species (17). Accordingly, we used recombinant human FRP for the present in vivo experiments. Treatment of mice with recombinant FRP just before the onset of arthritis brought about a significant improvement in the clinical parameters of arthritis (i.e., arthritis score and footpad swelling), indicating that FRP had preventive effects on joint inflammation. In addition, the striking benefit of FRP administration was the prevention of joint destruction. Postmortem histologic examination of tarsocrural joints from the FRP-treated mice revealed not only reduced synovial cellular infiltration, but also retained cartilage proteoglycan. Moreover, the reduction in urinary levels of deoxypyridinoline, one of the metabolic markers of bone resorption in RA (26), confirmed that FRP protected joints against destructive arthritis by preventing cartilage degradation and bone resorption. Therefore, FRP has antidestructive as well as antiinflammatory effects, and therapeutic potential in inflammatory joint diseases such as RA.

FRP is considered to be an orphan molecule because its receptor has not yet been identified, and there has been no information about the signaling system of FRP. Thus, to investigate the molecular mechanisms through which FRP treatment ameliorated murine arthritis, we compared the gene expression profiles in FRP-treated and PBS (vehicle)–treated joints. Among 170 genes with significant changes in expression levels, 5 down-regulated genes, c-*fos*, *ets*-2, *IL6*, *MMP3*, and *MMP9*, have been reported to be closely associated with immune response and joint destruction in inflammatory arthritis. Their reduced expression in FRP-treated mice was confirmed by a real-time quantitative RT-PCR method.

The expression of *IL6* was significantly reduced in joints from FRP-treated mice, suggesting that inhibition of *IL-6* could be one of the reasons for the antiinflammatory effects of FRP. IL-6 is thought to be one of the proinflammatory cytokines responsible for disease progression in RA, together with TNFα and IL-1 (23). Thus, FRP could be expected to have beneficial effects on joint inflammation in other animal models of arthritis and still greater effects in human diseases such as RA. However, a recent study indicated that in this mouse model of arthritis induced by anti-CII monoclonal antibodies and LPS, IL-6 was not as responsible for the progression of arthritis as TNFα and IL-1 were (27). There may be other mechanisms by which FRP ameliorates joint inflammation than by antagonism of IL-6.

The inhibitory effect of FRP on *MMP3* and *MMP9* was notable because these MMPs are thought to play a crucial role in the joint destruction of RA, where they are major direct effectors that degrade the extracellular matrix components of the joint structure, such as collagen and proteoglycan (24). Serum levels of MMP-3 in particular have been reported to be correlated with joint damage in RA (28). Synovial down-regulation of MMP-3 by FRP was also observed in our previous in vitro experiments (17). Gene expression of these MMPs is strongly up-regulated by proinflammatory cytokines, such as TNFα and IL-1, in the RA synovium (24), and it is considered that conditions in which the activities of the MMPs overwhelm the activities of the tissue inhibitors of metalloproteinases and MMP inhibitors, the joint destructive process is facilitated, as in RA (29). For these reasons, inhibition of MMPs has been considered as a new therapeutic approach to rheumatoid joint destruction (24). It has been reported that specific inhibition of individual MMPs has almost no effect in the treatment of murine arthritis, possibly due to another MMP compensation (30), but broad-spectrum MMP inhibition was shown to successfully reduce the clinical severity and cartilage destruction in a mouse model of RA (31,32). In our study, FRP inhibited the expression of *MMP3*, *MMP9*, and *MMP13*. The decrease ratio was significant in *MMP3* and *MMP9* (>1.5), but slight in *MMP13* (<1.5). Therefore, we hypothesized that one of the major mechanisms by which FRP prevented joint destruction was the down-regulation of several MMPs, such as MMP-3 and MMP-9.

How does FRP regulate MMP gene transcription? We found that 2 transcription factor genes, c-*fos* and *ets*-2, were down-regulated in FRP-treated joints. Their expression has been associated with a proliferative role in many tissues (21,33). It has been reported that c-*fos* and *ets*-2 expression was higher in rheumatoid synovial tissue than that in normal synovial tissue, suggesting that they may play an important role in the progression of RA (22,34,35). The nuclear oncogene c-*fos* and its product c-Fos bind to activator protein 1

sites on matrix-degrading genes, such as *MMP3* and *MMP9*. The gene *ets*-2 is one of the Ets family of transcription factors that binds to polyomavirus enhancer 3 (PEA-3) sites (36). Genes involved in the degradation of the extracellular matrix, such as *MMP3* and *MMP9*, contain PEA-3 sites in their regulatory sequences, and the binding of Ets-2 to PEA-3 sites accelerates their transcription (37). Moreover, Ets-2 cooperates with other transcription factors, including c-Fos, to stimulate the transcription of matrix-degrading proteases (37). Therefore, it is likely that FRP negatively regulates c-*fos* and *ets*-2 gene expression and, as a result, the transcription of *MMP3* and *MMP9* is suppressed.

In this series of experiments, for the limited amount of available recombinant FRP and the relatively short disease duration in this mouse model of arthritis, we performed preventive therapy for the arthritis by administering FRP just before the onset of disease. However, for further estimation of the therapeutic potential of FRP in arthritis, another series of experiments conducted according to the controlled curative therapy protocol will be required, involving the administration of FRP and control drugs during disease progression in other mouse models of chronic arthritis.

In conclusion, we demonstrated that the progression of murine arthritis can be inhibited by recombinant FRP treatment. Its beneficial effect was the prevention of joint destruction, which seemed to be mainly due to the reduction in MMP activities at the transcription level. In inflammatory joint diseases such as RA, FRP may be one of the key molecules that prevents the disease progression and a candidate as a new therapeutic biologic agent that may block the disease process in a manner different from that of TNFα, IL-1, and IL-6 antagonists.

ACKNOWLEDGMENTS

We wish to thank Mr. Norihiro Nishimura (Genetic Lab., Sapporo, Japan) and colleagues for preparing and analyzing cDNA arrays, and Ms Shio Kobayashi for technical assistance.

REFERENCES

1. Feldmann M, Brennan FM, Maini RN. Rheumatoid arthritis. Cell 1996;85:307–10.
2. Rowley M, Tait B, Mackay IR, Cunningham T, Phillips B. Collagen antibodies in rheumatoid arthritis. Arthritis Rheum 1986;29:174–84.
3. Sharif M, Worrall JG, Singh B, Gupta RS, Lydyard PM, Lambert C, et al. The development of monoclonal antibodies to the human mitochondrial 60-kd heat-shock protein and their use in studying the expression of the protein in rheumatoid arthritis. Arthritis Rheum 1992;35:1427–33.
4. Mimori T, Suganuma K, Tanami Y, Nojima T, Matsumura M, Fujii T, et al. Autoantibodies to calpastatin (an endogenous inhibitor for calcium-dependent neutral protease, calpain) in systemic rheumatic diseases. Proc Natl Acad Sci U S A 1995;92: 7267–71.
5. Sekine T, Masuko-Hongo K, Matsui T, Asahara H, Takigawa M, Nishioka K, et al. Recognition of YKL-39, a human cartilage related protein, as a target antigen in patients with rheumatoid arthritis. Ann Rheum Dis 2001;60:49–54.
6. Shaller M, Burton DR, Ditzel HJ. Autoantibodies to GPI in rheumatoid arthritis: linkage between an animal model and human disease. Nat Immunol 2001;2:746–53.
7. Tanaka M, Ozaki S, Osakada F, Mori K, Okubo M, Nakao K. Cloning of follistatin-related protein as a novel autoantigen in systemic rheumatic diseases. Int Immunol 1998;10:1305–14.
8. Shibanuma M, Mashimo J, Mita A, Kuroki T, Nose K. Cloning from a mouse osteoblastic cell line of a set of transforming growth factor β1 regulated genes, one of which seems to encode a follistatin-related protein. Eur J Biochem 1993;217:13–9.
9. Zwijsen A, Blockx H, Van Arnhem W, Willems J, Fransen L, Devos K, et al. Characterization of a rat C6 glioma-secreted follistatin-related protein (FRP): cloning and sequence of the human homologue. Eur J Biochem 1994;225:937–46.
10. Esch FS, Shimasaki S, Mercado M, Cooksey K, Ling N, Ying S, et al. Structural characterization of follistatin: a novel follicle-stimulating hormone release-inhibiting polypeptide from the gonad. Mol Endocrinol 1987;1:849–55.
11. Brekken RA, Sage EH. SPARC, a matricellular protein: at the crossroads of cell-matrix communication. Matrix Biol 2001;19: 816–27.
12. Hayette S, Gadoux M, Martel S, Bertrand S, Tigaud I, Magaud JP, et al. FLRG (follistatin-related gene), a new target of chromosomal rearrangement in malignant blood disorders. Oncogene 1998;16:2949–54.
13. National Library of Medicine. OMIM database. FSTL1. URL: http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=605547.
14. National Library of Medicine. OMIM database. FSTL3. URL: http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=605343.
15. Tsuchida K, Arai KY, Kuramoto Y, Yamakawa N, Hasegawa Y, Sugino H. Identification and characterization of a novel follistatin-like protein as a binding protein for the TGF-β family. J Biol Chem 2000;275:40788–96.
16. Sumitomo K, Kurisaki A, Yamakawa N, Tsuchida K, Shimizu E, Sone S, et al. Expression of a TGFβ1 inducible gene, TSC-36, causes growth inhibition in human lung cancer cell lines. Cancer Lett 2000;155:37–46.
17. Tanaka M, Ozaki S, Kawabata D, Kishimura M, Osakada F, Okubo M, et al. Potential preventive effects of follistatin-related protein on joint destruction and antagonistic modulation of its autoantibodies in rheumatoid arthritis. Int Immunol 2003;15:71–7.
18. Terato K, Harper DS, Griffiths MM, Hasty DL, Ye XJ, Cremer MA, et al. Collagen-induced arthritis in mice: synergistic effect of E. coli lipopolysaccharide bypasses epitope specificity in the induction of arthritis with monoclonal antibodies to type II collagen. Autoimmunity 1995;22:137–47.
19. Tada Y, Ho A, Matsuyama T, Mak TW. Reduced incidence and severity of antigen-induced autoimmune diseases in mice lacking interferon regulatory factor-1. J Exp Med 1997;185:231–8.
20. Toyobo. Mouse cDNA array. URL: http://www.toyobo.co.jp/seihin/xr/. In Japanese.
21. Shiozawa S, Shimizu K, Keiichi T, Hino K. Studies on the contribution of c-fos/AP-1 to arthritic joint destruction. J Clin Invest 1997;99:1210–6.
22. Dooley S, Herlitzka I, Hanselmann R, Ermis A, Henn W, Remberger K, et al. Constitutive expression of c-fos and c-jun, overex-

pression of ets-2, and reduced expression of metastasis suppressor gene nm23-H1 in rheumatoid arthritis. Ann Rheum Dis 1996;55: 298–304.
23. Nishimoto N, Kishimoto T, Yoshizaki K. Anti-interleukin 6 receptor antibody treatment in rheumatic disease. Ann Rheum Dis 2000;59:21–7.
24. Mengshol JA, Mix KS, Brinckerhoff EB. Matrix metalloproteinases as therapeutic targets in arthritic diseases: bull's-eye or missing the mark? Arthritis Rheum 2002;46:13–20.
25. Thornton S, Sowders D, Aronow B, Witte DP, Brunner HI, Giannini EH, et al. DNA microarray analysis reveals novel gene expression profiles in collagen-induced arthritis. Clin Immunol 2002;105:155–68.
26. Seibel MJ, Duncan A, Robins SP. Urinary hydroxy-pyridinium crosslinks provide indices of cartilage and bone involvement in arthritic diseases. J Rheumatol 1989;16:964–70.
27. Kagari T, Doi H, Shimozato T. The importance of IL-1β and TNF-β, and the noninvolvement of IL-6, in the development of monoclonal antibody-induced arthritis. J Immunol 2002;169:1459–66.
28. Yamanaka H, Matsuda Y, Tanaka M, Sendo W, Nakajima H, Taniguchi A, et al. Serum matrix metalloproteinase 3 as a predictor of the degree of joint destruction during the six months after measurement, in patients with early rheumatoid arthritis. Arthritis Rheum 2000;43:852–8.
29. Yoshihara Y, Nakamura H, Obata K, Yamada H, Hayakawa T, Fujikawa K, et al. Matrix metalloproteinases and tissue inhibitors of metalloproteinases in synovial fluids from patients with rheumatoid arthritis or osteoarthritis. Ann Rheum Dis 2000;59:455–61.
30. Mudgett JS, Hutchinson NI, Chartrain NA, Forsyth AJ, McDonnell J, Singer II, et al. Susceptibility of stromelysin 1–deficient mice to collagen-induced arthritis and cartilage destruction. Arthritis Rheum 1998;41:110–21.
31. Conway JG, Wakefield JA, Brown RH, Marron BE, Sekut L, Stimpson SA, et al. Inhibition of cartilage and bone destruction in adjuvant arthritis in the rat by a matrix metalloproteinase inhibitor. J Exp Med 1995;182:449–57.
32. Lewis EJ, Bishop J, Bottomley KM, Bradshaw D, Brewster M, Broadhurst MJ, et al. Ro 32-3555, an orally active collagenase inhibitor, prevents cartilage breakdown in vitro and in vivo. Br J Pharmacol 1997;121:540–6.
33. Bhat NK, Fisher RJ, Fujiwara S, Ascione R, Papas TS. Temporal and tissue-specific expression of mouse ets genes. Proc Natl Acad Sci U S A 1987;84:3161–5.
34. Trabandt A, Aicher WK, Gay RE, Sukhatme VP, Fassbender HG, Gay S. Spontaneous expression of immediately-early response genes c-fos and egr-1 in collagenase-producing rheumatoid synovial fibroblasts. Rheumatol Int 1992;12:53–9.
35. Asahara H, Fujisawa K, Kobata T, Hasunuma T, Maeda T, Asanuma M, et al. Direct evidence of high DNA binding activity of transcription factor AP-1 in rheumatoid arthritis synovium. Arthritis Rheum 1997;40:912–8.
36. Lan ZJ, Lye RJ, Holic N, Labus JC, Hinton BT. Involvement of polyomavirus enhancer activator 3 in the regulation of expression of gamma-glutamyl transpeptidase messenger ribonucleic acid-IV in the rat epididymis. Biol Reprod 1999;60:664–73.
37. Buttice G, Kurkinen M. A polyomavirus enhancer A-binding orotein-3 site and Ets-2 protein have a major role in the 12-O-tetradecanoylphorbol-13-acetate response of the human stromelysin gene. J Bio Chem 1993;268:7196–204.

Mod Rheumatol (2004) 14:82–86
DOI 10.1007/s10165-003-0272-3

CASE REPORT

**Hideyuki Murata · Yoshihiro Adachi · Takehiko Ebitsuka
Yusuke Chino · Reiko Takahashi · Taichi Hayashi
Daisuke Goto · Isao Matsumoto · Akito Tsutsumi
Hideyuki Akaza · Takayuki Sumida**

Reiter's syndrome following intravesical bacille bilié de Calmette–Guérin treatment for superficial bladder carcinoma: report of six cases

Received: April 18, 2003 / Accepted: August 8, 2003

Abstract We report the cases of six patients who developed acute Reiter's syndrome following intravesical bacille bilié de Calmette–Guérin (BCG) immunotherapy for superficial bladder cancer. After the third to eighth BCG intravesical injection, the patients developed conjunctivitis, aseptic urethritis, and polyarthritis consistent with a diagnosis of Reiter's syndrome. HLA-B27 antigen was negative in five of the patients examined. Two of the patients responded to nonsteroidal anti-inflammatory drugs for polyarthritis, and the other four responded to steroids (prednisolone 5–10 mg/day). The frequent use of intracavitary BCG may increase the incidence of BCG-induced Reiter's syndrome. Further analysis of the relationship between HLA-B and -DR alleles and arthritis should shed light on the mechanism of BCG-induced Reiter's syndrome.

Key words Bladder carcinoma · Intravesical bacille bilié de Calmette–Guérin (BCG) · Reiter's syndrome · Therapy

Introduction

Intravesical bacille bilié de Calmette–Guérin (BCG) immunotherapy is an effective treatment for superficial bladder cancer and carcinoma in situ.[1] The most common adverse effects are persistent fever, malaise, and irritable urination. Arthralgia and arthritis are reported to occur in 0.5% of patients treated with intravesical BCG.[2] We report six cases of BCG-induced Reiter's syndrome, discuss the possible pathogenesis, and review the literature.

H. Murata · Y. Adachi · T. Ebitsuka · Y. Chino · R. Takahashi · T. Hayashi · D. Goto · I. Matsumoto · A. Tsutsumi · T. Sumida (✉)
Division of Rheumatology, Department of Internal Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba 305-8575, Japan
Tel. +81-29-853-3105; Fax +81-29-853-3222
e-mail: tsumida@md.tsukuba.ac.jp

H. Akaza
Department of Urology, University of Tsukuba, Tsukuba, Japan

Case reports

Patient 1

A 79-year-old man with a history of pulmonary tuberculosis at 18 years of age was admitted to the hospital because of persisting macrohematuria. Endoscopic examination revealed a superficial bladder carcinoma, which was confirmed histopathologically to be a transitional cell carcinoma (Grade 2), stage T1N0M0 according to the TNM classification. The bladder carcinoma was removed by endoscopic resection, which was followed by four courses of weekly intravesical BCG therapy to prevent a relapse of the bladder cancer. After each BCG injection, the patient reported self-limiting fever and ureteral pain for a few days. Five days after the fourth injection, he developed bilateral conjunctivitis (Fig. 1a), and 7 days later he developed soft tissue swelling and effusions in the right shoulder joint, and both wrists and knees (Fig. 1b), bilateral first metacarpophalangeal (MCP) joints (Fig. 1c), and the left ankle (Fig. 1d).

Hematological investigations showed leukocytosis of 9100/mm^3, increased erythrocyte sedimentation rate (ESR) of 106 mm/h, and high C-reactive protein (CRP) of 11.3 mg/dl. Blood chemistry was within normal limits. Antinuclear antibody (ANA) was positive at 1:1280, with a speckled pattern. Anti-DNA antibody, anti-Sm antibody, anti-RNP antibody, anti-SS-A antibody, and anti-SS-B antibody were negative. Rheumatoid factor (RF) and HLA-B27 antigen were negative (HLA B46, B61), and DR4 and 8 were positive. A tuberculin test was positive at 12 mm in diameter. A chest X-ray was normal, and an X-ray of the knees revealed the presence of bilateral joint effusion. Thirty-five millilitres of yellowish-white synovial fluid (SF) was aspirated from the right knee. The SF was aseptic and the leukocyte count was normal. Urinary and blood cultures were negative. Serological tests for *Chlamidia trachomatis* were negative, and PCR for *Chlamidia trachomatis* was also negative. An ophthalmological examination confirmed the presence of bilateral conjunctivitis.

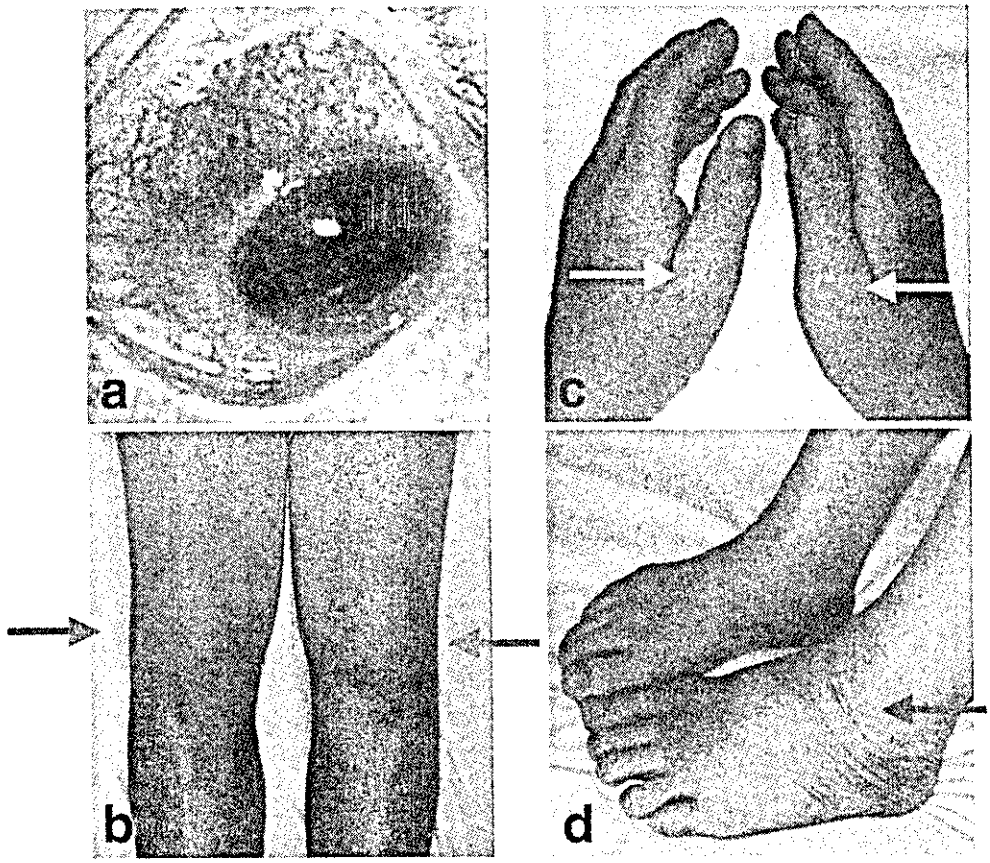

Fig. 1. **a** Bilateral conjunctivitis after intravesical bacillus Calmette–Guérin (BCG) therapy. **b** Swelling of the knee joints. **c** Swelling of bilateral first metacarpophalangeal joints. **d** Swelling of the left foot

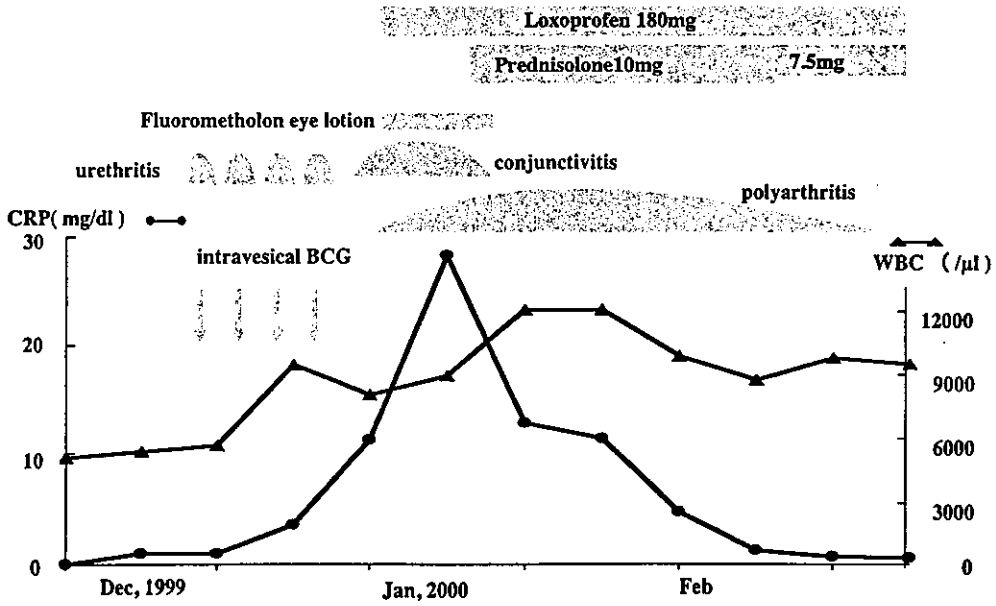

Fig. 2. The clinical course of patient 1. *CRP*, C-reactive protein

The diagnosis established was Reiter's syndrome based on polyarthritis, aseptic urethritis, and conjunctivitis. Discontinuation of the intravesical BCG resulted in a complete recovery from urethritis in 7 days. Bilateral conjunctivitis was treated with steroidal eye ointment for a few days, resulting in complete recovery. However, the administration of loxoprofen (180 mg/day) failed to resolve the arthritis. Oral prednisolone at 10 mg/day was started (Fig. 2). However, the polyarthritis and serological inflammatory findings gradually resolved to normal within about 2 months. The patient has remained asymptomatic, without any joint symptoms or medication, during the last 2 years of follow-up.

Patient 2

A 54-year-old man was diagnosed with a recurrence of transitional cell carcinoma of the bladder. After the second intravesical injection of BCG, he developed ureteral pain for a day. Two days after the fourth injection, he presented with arthritis affecting both knees, and 6 days later presented with bilateral conjunctivitis. Laboratory tests showed increased ESR (56mm/h) and CRP (11.5mg/dl). Blood chemistry was normal. ANA and RF were negative. HLA-B27 was negative, but HLA-DR was not checked. Blood and urine cultures were negative. Treatment with etodolac (400mg/day) caused liver dysfunction. The patient was then switched to prednisolone at 5mg/day, which resulted in an improvement in his symptoms. Prednisolone was discontinued after 2 months, and he remains asymptomatic 1 year later.

Patient 3

A 58-year-old woman was diagnosed with a recurrence of transitional cell carcinoma of the bladder. One day after the third injection of BCG intravesically, she developed hematuria and ureteral pain, followed by arthritis of the costotransverse and left fourth metatarsophalangeal (MTP) joints at 10 days, and bilateral conjunctivitis at 17 days. Laboratory tests showed increased ESR of 82mm/h, and increased CRP of 1.8mg/dl. Blood chemistry was normal. ANA and RF were negative. HLA-B27 was negative (HLA B39, B61), but HLA-DR was not checked. Blood and urine cultures were negative. Discontinuation of the intravesical BCG resulted in a complete recovery from urethritis in a few days. Bilateral conjunctivitis was treated with pranoprofen eye ointment for 5 days, resulting in a complete recovery. The joint symptoms disappeared after 2 months of etodolac treatment (400mg/day). The patient remains asymptomatic 1 year later.

Patient 4

A 68-year-old woman was diagnosed with a recurrence of transitional cell carcinoma of the bladder. One day after the second intravesical injection of BCG, she developed pollakiuria and ureteric pain. Two days after the fifth BCG injection, she presented with arthritis of the ribs and both knees, and bilateral conjunctivitis. After the sixth BCG injection, the joint symptoms had disappeared, but the ureteric pain persisted after the eighth BCG injection. ESR, CRP, and blood chemistry were normal. ANA, RF, and HLA-B and HLA-DR were not checked. Blood and urine cultures were negative. Bilateral conjunctivitis was treated with steroidal eye ointment for a few days, resulting in a complete recovery. The administration of loxoprofen (120mg/day) resulted in a gradual improvement in the ureteric pain, which disappeared after 23 months of loxoprofen treatment.

Patient 5

A 47-year-old man was diagnosed with a recurrence of transitional cell carcinoma of the bladder. One day after the sixth intravesical injection of BCG, he complained of ureteric pain, and 5 days after the eighth BCG injection he developed arthritis of the ribs and bilateral conjunctivitis. Eleven days later, he developed arthritis of the right wrist, knee, and foot. Laboratory tests showed high ESR (48mm/h) and CRP (1.2mg/dl). Blood chemistry was normal. ANA and RF were negative. HLA-B27 was negative (HLA B61, B70), but HLA-DR was not checked. Blood and urine cultures were negative. Treatment with diclofenac (75mg/day) resulted in liver dysfunction. He was then treated with prednisolone at 5mg/day, which resulted in an improvement in his symptoms. One year later, prednisolone was discontinued. He has remained asymptomatic during the last 6 months of follow-up.

Patient 6

A 42-year-old man was diagnosed with a recurrence of transitional cell carcinoma of the bladder. One day after the fourth intravesical injection of BCG, he developed ureteric colic. Four days after the sixth BCG injection, he presented with arthritis of the right knee, the right second proximal interphalangeal (PIP) joint and the left fourth PIP joint, and bilateral conjunctivitis. One day after the seventh BCG injection, he presented with arthritis of the left knee. Laboratory tests showed an increased CRP of 3.8mg/dl. Blood chemistry was normal. ANA and RF were negative. HLA-B27 was negative (HLA B46, B52), but HLA-DR was not checked. Fifty millilitres of yellowish-white SF was aspirated from the left knee. The SF was aseptic and his leukocyte count was normal. Blood and urine cultures were negative. The administration of diclofenac (75mg/day) provided only partial relief. Daily treatment with sulfasalazine at 1000mg caused severe headache. He was then switched to prednisolone at 5mg/day, which resulted in an improvement in his symptoms. Five months later, prednisolone was discontinued. He has remained asymptomatic during the last 6 months of follow-up.

Discussion

Most patients with synovitis occurring after intravesical BCG immunotherapy show the infiltration of mononuclear cells without granulomas in their synovial membrane.[3] Immunohistochemical studies, after intravesical BCG therapy, of serial bladder biopsies from patients with bladder carcinoma in situ have shown a large number of infiltrating mononuclear cells, consisting mainly of CD4+ T cells and macrophages, into the bladder. The antitumoral effect of BCG is thought to be mediated by T cell immunity. Although the antitumoral effect of BCG injection is considered to be limited to the urinary bladder, systemic reactions,