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Study of Plasma Levels of Soluble CD40 Ligand in Systemic Lupus Erythematosus Patients Who Have Undergone Plasmapheresis

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Abstract: We studied whether soluble CD40 ligands (sCD40L) are removed by means of double filtration plasmapheresis (DFPP), and the removal may help decrease activity of systemic lupus erythematosus (SLE). We studied 10 female patients with active SLE. Double filtration plasmapheresis was conducted one or two times per week. Plasma sCD40L levels were measured before and after each round of DFPP and throughout the treatment course. The plasma sCD40L level of SLE patients was significantly higher (14.09 ± 18.88 ng/mL) than that of healthy individuals (0.19 ± 0.20 ng/mL; $P < 0.0001$). In the SLE patients, plasma sCD40L levels were significantly lower following DFPP ($P = 0.0251$). The plasma waste from DFPP of an SLE patient was subjected to gel filtration, and the sCD40L concentration in each fraction was measured. We observed a peak in the fraction corresponding to ≥60 kDa. These results indicate that trimers and higher order complexes of sCD40L are removed during DFPP. Plasma sCD40L level and SLE disease activity index (SLEDAI) were decreased following the treatment course (mean 9.3 months). sCD40L exists as both a monomer and trimer in the plasma of SLE patients. The trimer as well as higher-order compounds can be removed via DFPP. It was thought that removal of sCD40L via DFPP may be useful for improving the overall condition of SLE.
Key Words: Antibody removal, CD40 ligand, Double filtration plasmapheresis, Plasmapheresis, Systemic lupus erythematosus.

Systemic lupus erythematosus (SLE) is an autoimmune disease of unknown origin. The serum of SLE patients contains a variety of autoantibodies, including anti-DNA antibodies, which form immune complexes that precipitate in the tissues and cause various tissue disorders. While drug therapy is currently the standard treatment for SLE, concomitant plasmapheresis has been found to be effective for reducing the incidence of certain aspects of the disease, such as lupus nephritis and neuropsychiatric lupus.

CD40 ligand (CD40L) is a 39-kDa type-II glycoprotein of the tumor necrosis factor (TNF) family. CD40L primarily appears transiently on CD4-positive cells, the recipients of antigen presentation, and plays a central role in the maintenance of humoral immunity. CD40L exists in a soluble form (sCD40L) that is produced by microsomal stimulus-dependent cleavage of the membrane-bound CD40L. sCD40L is capable of inducing B-cell proliferation via CD40 present on B-cells, class switching of immunoglobulins, and production of autoantibodies. CD40 is known to be widely expressed on antigen-presenting cells such as monocytes and dendritic cells as well as on intravascular endothelial cells. By sending signals through these cells, it enhances the expression of costimulatory molecules, induces production of inflammatory cytokines, and participates in the pathogenesis of various chronic inflammatory diseases (1,2).

The ratio of CD40L-presenting T-cells is increased in the peripheral blood of SLE patients (3), and if these T-cells are stimulated in vitro, CD40L is reportedly expressed on the cell surface at higher levels and for a longer duration than in T-cells from healthy individuals (4). It has been reported that the sCD40L level in the plasma of SLE patients is high, and it

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TABLE 1. *Summary of Study Subjects*

Patient	Age	Sex	Duration of SLE	Complications	Treatment for SLE	SLEDAI	Anti-DNA Ab (IU/mL)	CH50 (u/mL)
1	32	F	1 month	APS	PSL 70 mg, mPSL 500 mg × 3 days	17	2920.9	7.5
2	19	F	3 years		PSL 30 mg	18	174.0	25.0
3	26	F	14 years	APS	PSL 15 mg	10	3.8	42.4
4	26	F	6 years	SjS	PSL 15 mg	6	56.3	29.4
5	18	F	7 months		PSL 60 mg, mPSL 500 mg × 3 days IVCY	23	1205	15.9
6	66	F	34 years	APS	PSL 15 mg, mPSL 500 mg × 3 days	14	61.5	16.3
7	12	F	3 month		PSL 50 mg, mPSL 500 mg × 3 days	14	14.8	7.0
8	32	F	1 month		PSL 40 mg	18	3.2	7.0
9	34	F	10 years		PSL 50 mg	20	163.4	17.1
10	35	F	20 years	APS	PSL 30 mg	10	8090.0	7.0

APS, antiphospholipid antibody syndrome; IVCY, intravenous cyclophosphamide; mPSL, methylprednisolone; PSL, prednisolone; SjS, Sjögren's syndrome; SLEDAI, SLE activity index.

correlates with disease activity and the level of anti-double-stranded (antiDS) DNA antibodies. Furthermore, sCD40L in the plasma of SLE patients enhances the expression of CD44 and CD95 on B-cells (5,6), and sCD40L itself is believed to have biological activity.

In light of the observations described above, we hypothesized that removal of sCD40L via plasmapheresis may help decrease disease activity. In this study, we measured the time-dependent changes in the plasma sCD40L levels in SLE patients who had undergone double filtration plasmapheresis.

MATERIALS

The subjects included 10 female patients ranging in age from 12 to 66 years (mean 30 years) who fulfilled the 1982 American College of Rheumatology (ACR) revised criteria for diagnosis of SLE. The morbidity duration ranged from 1 month to 34 years (mean 105.6 months). Elevated antiDS-DNA antibodies were detected in all subjects, and a decrease in complement was detected in seven patients. The SLE disease activity index (SLEDAI) ranged between 6 and 20 points (mean 15 points). All subjects received steroid treatment, and one subject was concomitantly treated with intravenous cyclophosphamide therapy (Table 1).

METHODS

Double filtration plasmapheresis (DFPP) was conducted one or two times per week. A secondary membrane filter with a pore size of 0.03 μm was used for DFPP. A 5% albumin solution was used as a substitution solution. For anticoagulation, 2000 U of heparin were initially administered followed by continuous administration of 2000 U/h. The plasma throughput per DFPP was 2000 mL.

Blood sample collection was conducted before and after DFPP. Whole blood was stored at 4°C and centrifuged within 30 min, and plasma was stored at –20°C.

Plasma sCD40L levels were measured before and after each round of DFPP and throughout the treatment course.

The concentration of sCD40L in plasma was determined by sandwich enzyme-linked immunosorbent assay (ELISA) using two non-cross-blocking antihuman CD40L monoclonal antibodies (Mab). Briefly, each well of a 96-well polystyrene ELISA plate (Corning Costar Corp., Cambridge, MA, USA) was coated with 5 μg/mL antihuman CD40L Mab (TRAP-1; Pharmingen, San Diego, CA, USA) in phosphate buffered saline (PBS). After washing with PBS, the plate was treated with a blocking buffer consisting of 1% bovine serum albumin in PBS at room temperature for 2 h. The plates were washed four times with 0.05% Tween-20 (SIGMA, Tokyo, Japan) in PBS (washing buffer) and incubated in washing buffer at 4°C overnight. After washing, the plates were incubated with 2 μg/mL biotinylated antihuman CD40L Mab (bio-M90, Genzyme, Cambridge, MA, USA) in washing buffer. Avidin and biotinylated horseradish peroxidase (Elite Vectastain, Vector Laboratories, Burlingame, CA, USA) were then added, and following incubation and washing, 3,3′,5,5′-tetrametylbenzidine peroxidase (Kierkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA), which served as a substrate for detection, was added. Absorbance at 450 nm was determined using a micro-ELISA reader. Serial dilutions of recombinant human sCD40L (Bender Medical Systems, Vienna, Austria) were used to construct the standard curve. In the control ELISA, isotype-matched mouse IgG (mopc-21, Sigma, St. Louis, MO, USA) was used as a coating antibody, and biotinylated mouse IgG (Ancell, Bayport, MN, USA) was used for detection.

Plasma from each SLE patient and the corresponding DFPP drainage were subjected to gel filtration. 0.05 M potassium phosphate containing 0.1 M NaCl (pH 7.5) was used as an elution buffer, and a 1 × 30 cm column filled with Sephadex G-100 (Pharmacia, Uppsala, Sweden) was used as a gel filtration column.

The plasma sCD40L levels of healthy individuals and SLE patients were compared using the Mann–Whitney non-paired non-parametric test. The plasma sCD40L levels of each SLE patient before and after DFPP were compared using the paired Student's *t*-test and the SLEDAI and plasma sCD40L levels before and after treatment were compared using the Wilcoxon signed-ranks test.

RESULTS

The plasma sCD40L levels of 15 healthy individuals and 10 SLE patients were measured. The plasma sCD40L level of SLE patients was significantly higher (14.09 ± 18.88 ng/mL) than that of healthy individuals (0.19 ± 0.20 ng/mL; $P < 0.0001$) (Fig. 1). In the SLE patients, plasma sCD40L levels were significantly lower following DFPP ($P = 0.0251$) (Fig. 2). In one of the ten SLE patients (Patient no. 5), increased sCD40L levels were observed following DFPP, but the sCD40L levels tended to decrease gradually over the course of the treatment, suggesting that the post-DFPP increase was transient. During DFPP, thrombocytes receive an activation stimulus as a result of the contact between the blood and the membrane. When thrombocytes are activated, molecules such as CD40L appear on the thrombocyte surface (7). Additionally, when thrombocytes are stimulated by thrombin, CD40L is expressed on the cell surface, and within a short time, its soluble form (sCD40L) is released (8). It seems possible therefore that a transient post-DFPP increase in sCD40L levels may be caused by thrombocyte activation.

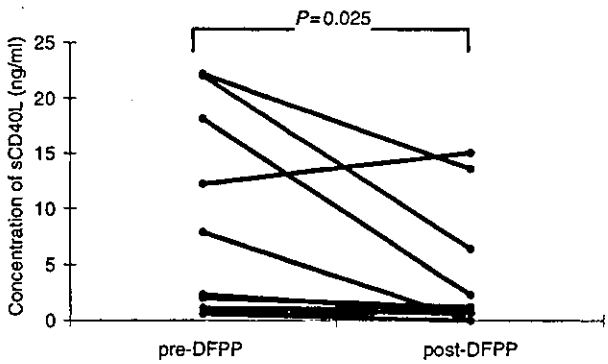

FIG. 2. Plasma sCD40L levels before and after a single round of double filtration plasmapheresis (DFPP) in systemic lupus erythematosus patients. Plasma sCD40L levels were significantly decreased following DFPP (Paired *t*-test, $P = 0.0251$).

The mean rate of sCD40L removal from the plasma of SLE patients (50.71%) during a single round of DFPP was much higher than the mean removal rates of either IgG (25.06%) or albumin (14.09%) (Fig. 3), suggesting that the molecular weight of the sCD40L removed during DFPP is higher than that of either albumin or IgG. To determine the molecular weight of the sCD40L present in the plasma of SLE patients and in the plasma waste from DFPP, corresponding samples were subjected to gel filtration, and the sCD40L concentration in each fraction was measured using ELISA. In the plasma, peaks corresponding to the expected molecular weights of sCD40L monomers (~20 kDa) and trimers (~60 kDa) were observed. In the DFPP drainage fluid, a peak was observed in the fraction corresponding to ≥60 kDa (Fig. 4). These results indicate that trimers and higher-order complexes of

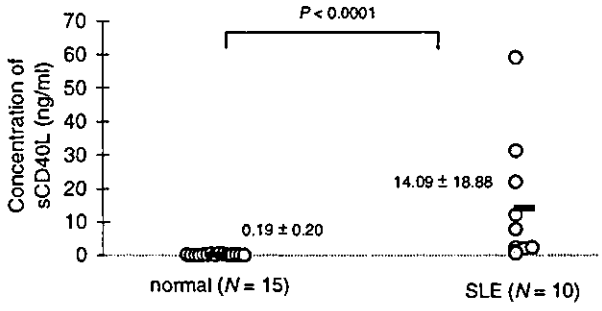

FIG. 1. Plasma sCD40L levels (ng/mL) of 15 healthy individuals and 10 systemic lupus erythematosus (SLE) patients. Mean plasma sCD40L levels were significantly higher in SLE patients(14.09 ± 18.88 ng/mL) compared to healthy individuals(0.19 ± 0.20 ng/mL) (Mann–Whitney's non-parametric non-paired test, $P < 0.0001$).

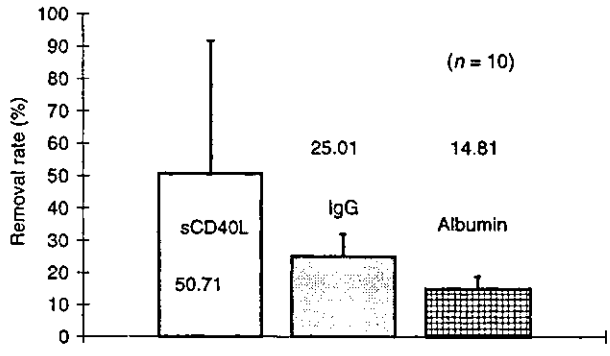

FIG. 3. The removal rates of sCD40L, IgG, and albumin from plasma of SLE patients during a single round of DFPP. The removal rates were 50.71 ± 41.09% for sCD40L, 25.01 ± 7.02% for IgG, and 14.81 ± 3.52% for albumin. The high removal rate of sCD40L suggests that it has the highest molecular weight.

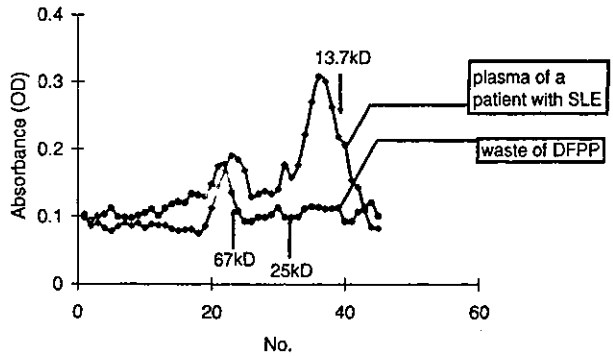

FIG. 4. Plasma from each systemic lupus erythematosus (SLE) patient and the corresponding double filtration plasmapheresis (DFPP) drainage fluid were subjected to gel filtration, and the sCD40L concentration in each fraction was measured. The concentration is shown as absorbance. (◆) indicates SLE patient plasma and (●) indicates DFPP waste fluid. In the plasma of SLE patients, peaks were observed at ~20 kDa and ~60 kDa. In the DFPP waste fluid, one peak = 60 kDa was observed. The ~20 kDa peak is thought to correspond to the expected molecular weight of the sCD40L monomer, and the ~60 kDa peak is thought to correspond to its trimer.

sCD40L are removed during DFPP. Follow-up analyses conducted in seven out of the 10 SLE patients revealed a change in plasma sCD40L level following the treatment course. The mean treatment duration was 9.3 months, and a mean of 19.2 rounds of DFPP were performed. The steroid doses remained constant or were decreased in all patients during the treatment course. The plasma sCD40L level decreased following treatment in all patients. The SLEDAI score was significantly lower following treatment, suggesting that the sCD40L level decreased with the decrease in the SLE activity (Fig. 5).

DISCUSSION

Double filtration plasmapheresis (DFPP) has been concomitantly used with drug therapy to treat intractable SLE patients. Treatment effect of DFPP is based on the non-specific removal of the medium to large molecules including immunocomplexes, antiDS-DNA antibodies, and γ-globulin from the plasma protein fraction of the treated patients. DFPP has been found to be effective for the treatment of many of the clinical conditions associated with SLE including active lupus nephritis, central nervous system (CNS) lupus, dermatological manifestations, vasculitis, and thrombocytopenia (9).

The results of this study confirm that the plasma sCD40L levels in SLE patients are significantly higher than those of the healthy individuals. Early et al. reported that in model mice of SLE, antiDS-DNA antibody production could be supported by treatment with antimouse CD40L antibody in vivo (10). It is also known that the CD40–CD40L interaction plays a significant role in SLE pathogenesis. In the recent clinical studies to treat lupus nephritis patients with humanized monoclonal antibody specific for CD40L, reduction in antiDS-DNA antibody titers, proteinuria, hematuria, and SLEDAI score were reported (11–13). In light of these findings, it was thought that the removal of sCD40L by using plasmapheresis might be useful for improving the overall condition of SLE patients.

There were, however, two questions on the removal of sCD40L by using DFPP. The first question was whether sCD40L could be removed by DFPP. A previous report has indicated that the molecular weight of sCD40L is 18–20 kDa. sCD40L exists in the body as a trimer form, and it may be biologically active by itself (14). In our study, to determine if sCD40L can be removed by DFPP, we examined the molecular weight of sCD40L by using a gel filtration method. Our results suggest that sCD40L exists as both monomer and trimer forms in the plasma of SLE patients. The trimer as well as higher-ordered compounds considered primary biological active compounds, and they can be removed by DFPP. The

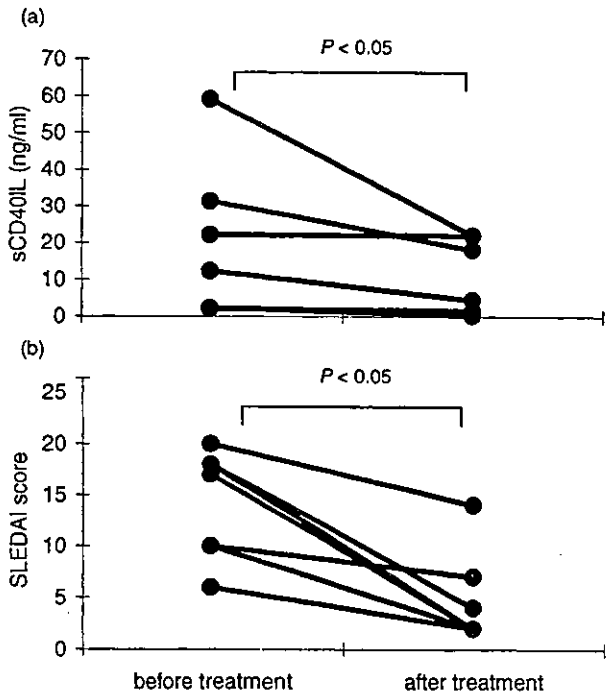

FIG. 5. Follow-up of seven patients (Patients nos. 1, 2, 3, 4, 8, 9 and 10) following the treatment course. Significant decreases in the (a) plasma sCD40L level and (b) SLEDAI score were observed following treatment in all patients (Wilcoxon sign-ranked test, $P < 0.05$).

second question was whether pathogenic substances other than sCD40L could be removed by DFPP. Because DFPP removes non-specific medium to large molecules, certain substances involved in the pathogenesis of SLE other than sCD40L can be removed. More studies should be done in order to answer this question.

It has been hoped that development of a more selective treatment against CD40-CD40L will happen in the future. It is known that compared to drug therapy, plasmapheresis is safer treatment with fewer adverse effects. In the future, we intended to study more about DFPP and the possibility of the development of selective removal of sCD40L by immunoadsorption plasmapheresis technique.

In our study, all of the seven patients we followed were taking steroid as the major drug. The degree to which DFPP contributed the overall treatment effects among the various treatments including steroid is not clear. However, it appears that the removal of sCD40L by DFPP might be one of the factors for the improvement of the symptoms we have observed.

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REFERENCES

1. Stout RD, Suttles J. The many roles of CD40 in cell-mediated inflammatory responses. *Immunol Today* 1996;17:487–92.
2. Hollenbaugh D, Mischel-Petty N, Edwards CP, Simon JC, Denfeld RW, Kiener PA, Aruffo A. Expression of functional CD40 by vascular endothelial cells. *J Exp Med* 1995;182:33–40.
3. Mehta A, Lu L, Goldman R, Datta S. Hyperexpression of CD40 ligand by B and T cells in human lupus and its role in pathogenic autoantibody production. *J Clin Invest* 1996;97:2067–73.
4. Koshy M, Berger D, Crow M. Increased expression of CD40 ligand on systemic lupus erythematosus lymphocytes. *J Clin Invest* 1996;98:826–37.
5. Kato K, Sahagun E, Rassenti L et al. The soluble CD40 ligand sCD154 in systemic lupus erythematosus. *J Clin Invest* 1999;104:947–55.
6. Vakkalanka R, Woo C, Kirou K, Koshy M, Berger D, Crow M. Elevated levels and functional capacity of soluble CD40 ligand in systemic lupus erythematosus sera. *Arthritis Rheum* 1999;42:871–81.
7. Henn V, Slupsky J, Grafe M et al. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 1998;391:591–4.
8. Henn V, Steibach S, Buchner K, Presek P, Kroczek R. The inflammatory action of CD40ligand (CD154) expressed on activated human platelets is temporally limited by coexpressed CD40. *Blood* 2001;98:1047–54.
9. Hashimoto H, Yano T, Kawanishi T, Tuda H, Nagasawa T. Outcome of Collagen Vascular Disease by Treatment with Plasmapheresis. *Ther Apher* 1998;4:268–72.
10. Early GS, Zhao W, Burns CM. Anti-CD40 ligand antibody treatment prevents the development of lupus-like nephritis in a subset of New Zealand black x New Zealand white mice. Response correlates with the absence of an anti-antibody response. *J Immunol* 1996;157:3159–64.
11. Huang W, Sinha J, Newman J et al. The effect of anti-CD40 ligand antibody on B cells in human systemic lupus erythematosus. *Arthritis Rheum* 2002;46:1554–62.
12. Boumpas DT, Furie R, Manzi S et al. A short course of BG9588 (anti-CD40 ligand antibody) improves serologic activity and decreases hematuria in patients with proliferative lupus glomerulonephritis. *Arthritis Rheum* 2003;48:719–27.
13. Grammer AC, Slota R, Fischer R et al. Abnormal germinal center reactions in systemic lupus erythematosus demonstrated by blockade of CD154–CD40 interactions. *J Clin Invest* 2003;112:1506–20.
14. Pietravalle F, Henchoz S, Blasey H et al. Human native soluble CD40L is a biologically active trimer, processed inside microsomes. *J Biol Chem* 1996;271:5965–7.

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Treatment of Rheumatoid Arthritis With Humanized Anti–Interleukin-6 Receptor Antibody

A Multicenter, Double-Blind, Placebo-Controlled Trial

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Objective. **Interleukin-6 (IL-6) is a pleiotropic cytokine that regulates the immune response, inflammation, and hematopoiesis. Overproduction of IL-6 plays pathologic roles in rheumatoid arthritis (RA), and the blockade of IL-6 may be therapeutically effective for the disease. This study was undertaken to evaluate the safety and efficacy of a humanized anti–IL-6 receptor antibody, MRA, in patients with RA.**

Methods. **In a multicenter, double-blind, placebo-controlled trial, 164 patients with refractory RA were randomized to receive either MRA (4 mg/kg body weight or 8 mg/kg body weight) or placebo. MRA was administered intravenously every 4 weeks for a total of 3 months. The clinical responses were measured using the American College of Rheumatology (ACR) criteria.**

Results. **Treatment with MRA reduced disease activity in a dose-dependent manner. At 3 months, 78% of patients in the 8-mg group, 57% in the 4-mg group, and 11% in the placebo group achieved at least a 20% improvement in disease activity according to the ACR criteria (an ACR20 response) ($P < 0.001$ for 8-mg group versus placebo). Forty percent of patients in the 8-mg group and 1.9% in the placebo group achieved an ACR50 response ($P < 0.001$). The overall incidences of adverse events were 56%, 59%, and 51% in the placebo, 4-mg, and 8-mg groups, respectively, and the adverse events were not dose dependent. A blood cholesterol increase was observed in 44.0% of the patients. Liver function disorders and decreases in white blood cell counts were also observed, but these were mild and transient. There was no increase in antinuclear antibodies or anti-DNA antibodies. Anti-MRA antibodies were detected in 2 patients.**

Conclusion. **Treatment with MRA was generally well tolerated and significantly reduced the disease activity of RA.**

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by persistent synovitis and progressive joint damage, and it is often associated with the presence of antiimmunoglobulin autoantibodies, rheumatoid factors (1). Although the causes of RA are not fully understood, proinflammatory cytokines are involved in the development of the disease (2,3). These cytokines and their actions may be potential targets to block for the treatment of RA. Interleukin-6 (IL-6) is a pleiotropic cytokine that regulates the immune response, inflammation, hematopoiesis, and bone metabolism (2). Constitutive overproduction of IL-6 is thought to play pathologic roles in RA. Elevation of IL-6 levels was observed in both serum and synovial fluid in patients with RA (4–6), and serum IL-6 levels correlated with disease activity and radiographic joint damage (7–11).

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Drs. Nishimoto, Yoshizaki, Miyasaka, Yamamoto, Kawai, Takeuchi, Hashimoto, and Azuma have served as consultants to and/or received honoraria from Chugai Pharmaceutical, the manufacturer of MRA.

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Administration of mouse monoclonal anti–IL-6 antibody to 5 patients with RA was shown to ameliorate disease activity, although the effect was transient (12). Therefore, interference with the action of IL-6 may be effective for treating RA.

MRA is a humanized anti–human IL-6 receptor (anti–IL-6R) monoclonal antibody that inhibits the binding of IL-6 to IL-6R (13). The antibody was humanized by grafting the complementarity-determining regions from the murine anti–IL-6R antibody into human IgG1, thereby creating a functioning antigen-binding site in a reshaped human antibody. Safety studies in healthy adult male volunteers revealed that intravenous administrations of MRA were well tolerated. There were trends toward reductions in disease activity in 2 safety and dose-finding studies, 1 of single-dose MRA (14) and 1 of repetitive treatment for 6 months (15), involving small numbers of RA patients in the UK and Japan, respectively. On the basis of these findings, we conducted a multicenter, double-blind, placebo-controlled trial of MRA in patients with established and active RA.

PATIENTS AND METHODS

Patients. The study began in March 2001, and patients were enrolled from April 16, 2001 to November 26, 2001. Eligible patients were age ≥20 years, fulfilled the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) 1987 revised criteria for RA (16), had a history of >6 months of disease activity, and were in functional class I, II, or III according to the Steinbrocker criteria (17). All candidates had been treated unsuccessfully (due to lack of efficacy) with at least 1 disease-modifying antirheumatic drug (DMARD) or immunosuppressant.

No DMARDs, immunosuppressants, parenteral and/or intraarticular use of corticosteroids, plasma exchange therapies, or surgical treatments were allowed during a 4-week washout period before the first dose and throughout the study period. Patients receiving prednisolone (maximum of 10 mg/day) and/or nonsteroidal antiinflammatory drugs (NSAIDs) were eligible if the dosage had not increased during the washout period. Both medications remained stable during the study period. In addition, eligible patients had white blood cell counts of at least 3,500/mm^3 and platelet counts of at least 100,000/mm^3 at enrollment. The necessary degree of disease activity at enrollment was confirmed by a finding of ≥6 swollen joints, ≥6 tender joints, and 1 of the following 2 criteria: a Westergren erythrocyte sedimentation rate (ESR) of at least 30 mm/hour or a C-reactive protein (CRP) level >1.0 mg/dl.

Sexually active premenopausal women were required to use effective contraception during the study period. Women in this category also had to have a negative urine pregnancy test result before enrollment. Patients were excluded if they had a medical history of a serious allergic reaction, significant cardiac, blood, respiratory system, neurologic, endocrine, renal, hepatic, or gastrointestinal diseases, or an active intercurrent infection requiring medication. Patients were not screened for purified protein derivative of tuberculin because most Japanese receive BCG vaccine. Chest radiographs specifically to rule out either active tuberculosis or old granulomatous disease were not required in the study protocol, but were often done to screen for active intercurrent respiratory infections.

Study protocol. The study protocol was approved by the Ministry of Health, Labor and Welfare of Japan and by the ethics committee at each participating center. Before enrollment, patients gave written informed consent, had a complete medical history taken, and underwent a complete examination. The examination consisted of a physical examination, measures of disease activity, and laboratory tests. In addition, serum was obtained for testing blood IL-6, soluble IL-6R (sIL-6R), anti-DNA antibodies, antinuclear antibodies (ANAs), and bone metabolism markers (blood osteocalcin, C-terminal type I procollagen propeptide [PICP]) on day 0 and at week 12 or 4 weeks after the last dose of the study drug. Serum for testing antibodies to MRA (neutralizing antibodies and IgE antibodies) was collected on day 0, every 2 weeks after the administration day, 1 month after the last infusion, and before the extension study when MRA was no longer detectable in the serum.

Baseline clinical assessments included the following: complete count of swollen and tender joints (49 joints evaluated; cervical spine and hips evaluated only for tenderness), physician's and patient's global assessment of disease status on a visual analog scale (VAS) from 0 (asymptomatic) to 100 (severe symptoms), patient's assessment of pain on a VAS from 0 (no pain) to 100 (severe pain), functional disability measured with a modified Health Assessment Questionnaire (18), Westergren ESR, and CRP level (19). Disease activity assessments were repeated on day 0 and every 4 weeks throughout the study. Each patient was assessed by the same rheumatologist at each visit.

Treatment. Patients were randomly assigned to 1 of 3 treatment groups: placebo, 4 mg of MRA per kg of body weight (4-mg group), or 8 mg of MRA per kg of body weight (8-mg group). Patients were administered an allocated study drug 3 times at 4-week intervals for 3 months. MRA was supplied as a sterile liquid formulation with 200 mg of MRA per 10-ml vial. The placebo was a liquid formulation not containing active substance and indistinguishable from the MRA vial. The appropriate amount of MRA was diluted to a total volume of 250 ml in sterile saline and administered over a period of 1 hour by intravenous drip infusion using a 0.2-μm in-line filter under careful monitoring.

Statistical analysis. The primary end point was the incidence of a 20% improvement in disease activity according to the ACR criteria (ACR20) (19) at week 12 with the last observation carried forward (LOCF) method. The closed testing procedure for multiple comparisons was used in the primary dose-response analysis. The first- and second-step comparisons were those for 8 mg/kg MRA versus placebo and 4 mg/kg MRA versus placebo, respectively. Secondary end points included the Disease Activity Score in 28 joints (DAS28) (20), the incidences of 50% and 70% improvement in disease activity according to the ACR criteria (ACR50 and ACR70, respectively), the presence of overall improvement in the ACR criteria from week 0 to week 12, and the different

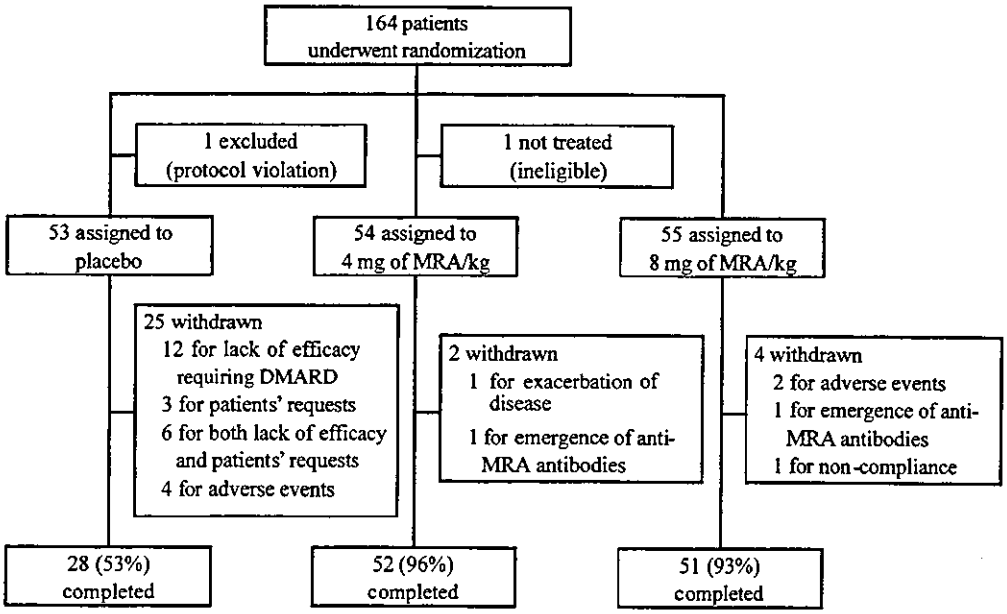

Figure 1. Randomization, reasons for withdrawal, and numbers of patients who completed the trial. MRA = humanized anti–interleukin-6 receptor antibody; DMARD = disease-modifying antirheumatic drug.

variables within the ACR core set (21). Statistical analyses were performed with SAS version 8.2 TS2M0 (SAS Institute, Cary, NC). The incidences of improvements were analyzed by use of the chi-square test. The differences among groups of DAS28 scores and the individual variables of the ACR core set were analyzed by use of Student's *t*-test.

We determined that a sample size of 39 patients per group was needed in order to obtain 80% power to detect a

Table 1. Characteristics of patients at study entry*

	Treatment group		
	Placebo (n = 53)	4 mg/kg MRA (n = 54)	8 mg/kg MRA (n = 55)
Age, median (range) years	53.0 (31–73)	53.5 (21–74)	56.0 (25–74)
No. of men/no. of women	14/39	14/40	9/46
Functional class†			
I	3	3	3
II	35	36	35
III	15	15	17
IV	0	0	0
RA stage†			
I	0	3	0
II	13	14	12
III	21	20	24
IV	19	17	19
Duration of disease, median (range) years	8.4 (0.7–52.7)	7.3 (0.6–35.8)	8.3 (1.3–45.7)
No. of failed DMARDs, median (range)	5 (1–10)	4 (2–8)	5 (1–11)
Tender joint count, mean ± SD	18.2 ± 8.4	19.1 ± 9.0	17.8 ± 9.8
Swollen joint count, mean ± SD	14.1 ± 6.1	16.0 ± 9.1	13.6 ± 6.9
ESR, mean ± SD mm/hour	68.7 ± 31.2	71.2 ± 29.1	67.4 ± 30.9
CRP level, mean ± SD mg/dl	5.5 ± 4.2	4.7 ± 2.9	4.5 ± 3.3

* MRA = humanized anti–interleukin-6 receptor antibody; RA = rheumatoid arthritis; DMARDs = disease-modifying antirheumatic drugs; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein.
† RA functional status determined by American College of Rheumatology criteria. RA stage determined by Steinbrocker criteria.

statistically significant ($P < 0.05$) difference in incidences between the placebo group and the 8-mg group by use of the two-sided chi-square test, where response rates in the population were assumed to be 20% and 50% in the placebo and 8-mg groups, respectively. We decided to recruit 45 patients per group to allow for anticipated withdrawals.

ANAs, anti-DNA antibodies, serum MRA levels, and anti-MRA antibodies. ANAs were measured indirectly by fluorescent antibody with HEp-2 cell substrate; a titer of ≥1:40 was considered positive. Anti-DNA antibodies were measured by ^{125}I radiobinding assay; a value of ≥6.0 IU/ml was considered positive.

Serum MRA levels were assessed by enzyme-linked immunosorbent assay (ELISA). Briefly, 100 μl of recombinant human sIL-6R (1 μg/ml) was added to the wells of an immunoplate precoated with MT18 and incubated at room temperature for 2 hours. After washing, bound MRA was measured using alkaline phosphatase (AP)–conjugated goat anti-human IgG. The calorimetric reaction was measured with a microplate reader.

Serum anti-MRA antibodies neutralizing MRA activities were measured by ELISA. Briefly, serum was added to wells coated with 100 μl of Fab fragment of MRA (0.2 μg/ml) and incubated for 2 hours. After washing, biotin-conjugated Fab fragment of MRA was added and developed with AP conjugated to streptavidin. IgE-type anti-MRA antibodies were also measured by ELISA. In this case, whole MRA was used because an antigen coated each cup, and enzyme-linked anti-IgE antibodies were used as second antibodies.

RESULTS

Characteristics of the patients. One hundred sixty-four patients were enrolled in the study (Figure 1). After enrollment, 1 patient was found to be ineligible (due to exacerbation of renal disease) for entry into the study and was withdrawn before administration of drug or placebo. Another patient was judged to be ineligible because of intravenous infusion of prednisolone during the washout period. A total of 162 patients (37 men and 125 women) were included in the full analysis set. The baseline demographic and clinical data are summarized in Table 1.

The groups were well matched according to their pretreatment characteristics or baseline levels of disease activity. The median age of the groups was 54 years, and the median duration of disease was 7.6 years. A median of 4–5 DMARDs had previously been tried unsuccessfully in the patient population. Furthermore, all patients had active disease in terms of high counts of swollen and tender joints and increased ESRs and CRP levels. Therefore, the population of included patients probably had relatively severe disease. Ninety-five percent of the patients completed MRA treatment (96% in the 4-mg group and 93% in the 8-mg group) compared with 53% of the patients receiving placebo. Among the 109 patients receiving MRA, 6 withdrew (2 for emergence of anti-MRA antibodies, 2 for adverse events, 1 for exacerbation of disease, and 1 for noncompliance because of changing an NSAID). Among the 53 patients receiving placebo, 25 withdrew (12 for lack of efficacy that required introduction of a DMARD at the discretion of the treating physician, 3 at the patients' requests, 6 for both lack of efficacy and patients' requests, and 4 for adverse events).

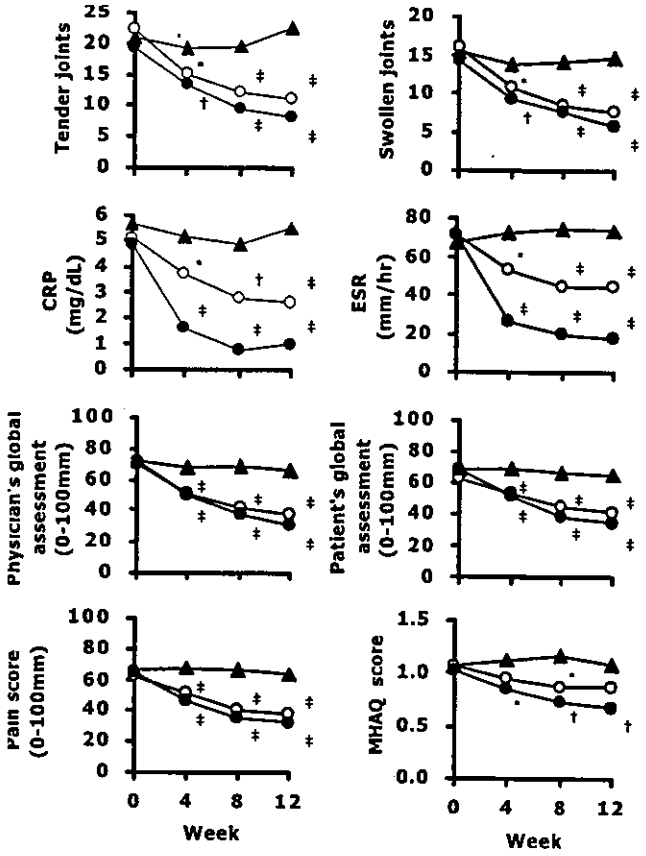

Figure 2. Rheumatoid arthritis disease activity assessments. Solid triangles indicate placebo-treated group. Open circles indicate group treated with 4 mg/kg humanized anti–interleukin-6 receptor antibody (MRA). Solid circles indicate 8 mg/kg MRA–treated group. Values are the mean for each group at each time point. * = $P < 0.05$; † = $P < 0.01$; ‡ = $P < 0.001$ versus placebo, by Student's t-test. CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; M-HAQ = modified Health Assessment Questionnaire.

Efficacy. MRA treatment significantly improved all measures of disease activity in the ACR core set, and a dose-response relationship was observed between the 4-mg and 8-mg groups (Figure 2). The efficacy was apparent at week 4 and became most pronounced at the

Table 2. Percentage of responders according to the American College of Rheumatology (ACR) improvement criteria and the Disease Activity Score in 28 joints (DAS28)*

Response criteria	Treatment group			P		
	Placebo (n = 53)	4 mg/kg MRA (n = 54)	8 mg/kg MRA (n = 55)	Placebo vs. 8 mg/kg MRA	Placebo vs. 4 mg/kg MRA	4 mg/kg MRA vs. 8 mg/kg MRA
ACR improvement†						
ACR20	11.3	57.4	78.2	<0.001	<0.001	0.020
ACR50	1.9	25.9	40.0	<0.001	<0.001	0.118
ACR70	0.0	20.4	16.4	0.002	0.001	0.589
DAS28‡						
Good	0.0	5.6	18.2	0.001	0.085	0.042
Good or moderate	18.9	72.2	90.9	<0.001	<0.001	0.012

* Except where indicated otherwise, values are the percentage of patients achieving a given response. MRA = humanized anti–interleukin-6 receptor antibody; ACR20 = 20% improvement in disease activity according to ACR criteria.
† See ref. 19.
‡ See refs. 20 and 22.

end of treatment (week 12). In particular, complete normalization of the CRP level was observed in 76% and 26% of the patients in the 8-mg and 4-mg groups, respectively, while the CRP level was normalized in only 1.9% of patients in the placebo group. Seventy-eight percent of the 8-mg group achieved at least an ACR20 response compared with 57% of the 4-mg group ($P = 0.02$) and 11% of the placebo group ($P < 0.001$) (Table 2). There were also significantly more ACR50 and ACR70 responses in the 8-mg group than in the placebo group ($P < 0.001$ and $P = 0.002$, respectively). The efficacy was also confirmed by the percentages of patients in the DAS28 categories (20); the incidence of "good or moderate" (22) was 91% in the 8-mg group compared with 72% in the 4-mg group ($P = 0.012$) and 19% in the placebo group ($P < 0.001$) (Table 2).

In addition to the ACR core set of disease activity measures, considerable improvement occurred in platelet counts and in levels of hemoglobin, fibrinogen, serum amyloid A, and albumin (Table 3). Interestingly, fibrinogen levels and platelet counts remained in the low-to-normal range. Rheumatoid factors were positive in 102 of 109 patients (94%) in the MRA groups at baseline. Their titers decreased significantly in the 8-mg group (Table 3), and 3 patients became negative for rheumatoid factors at week 12. However, there was no statistically significant correlation between the decrease in rheumatoid factor titer and ACR response rate in this 3-month study. MRA treatment also significantly increased serum levels of the bone formation markers osteocalcin and PICP and simultaneously decreased levels of the bone absorption markers urinary pyridinoline and deoxypyridinoline.

Safety. Treatment tolerance of MRA was good. The incidences of adverse events were 56%, 59%, and 51% of the patients in the placebo, 4-mg, and 8-mg groups, respectively, and were not dose dependent. Most of the adverse events were mild and acceptable relative to the benefit provided. Table 4 shows the adverse events appearing in >3% of patients in this study. These adverse events did not require cessation of MRA treatment. Upper respiratory infection (common cold) was the most common adverse event overall, but the incidences were similar among the groups. Skin eruptions were reported in 5 patients in the MRA groups. Skin eruptions in 2 of these patients were classified as unrelated to MRA. The other 3 patients had mild and transient redness or papule of the skin. All 5 patients continued MRA treatment, and no exacerbation was observed. Infusion reactions, defined as any adverse experience occurring during or after the infusion on the treatment day, were found in 15%, 13%, and 16% of the patients in the placebo, 4-mg, and 8-mg groups, respectively. Most of them were mild and transient. Sleepiness (2.8%), mild headaches (2.8%), and increases in blood pressure (2.8%) were observed. Three patients had transient increases in blood pressure after infusion (138/75 mm Hg before, 165/80 mm Hg after; 134/82 mm Hg before, 140/90 mm Hg after; 150/88 mm Hg before, 192/104 mm Hg after), and 2 of these patients had had hypertension before entry into the study.

Five serious adverse events were reported in this study: 3 in the MRA group (2.8%) and 2 in the placebo group (3.8%). One patient died of reactivation of chronic active Epstein-Barr virus (EBV) infection and consequent hemophagocytosis syndrome 61 days after receiving a single 8-mg/kg dose of MRA. She showed fluctuating liver function and CRP levels that were inversely correlated with white blood cell counts, and she had increased EBV DNA in plasma before enrollment.

Table 3. Laboratory parameters*

	Treatment group		
	Placebo (n = 53)	4 mg/kg MRA (n = 54)	8 mg/kg MRA (n = 55)
Hemoglobin, gm/dl			
Baseline	11.3 ± 1.5	11.3 ± 1.6	11.3 ± 1.1
Week 12	11.2 ± 1.5	12.2 ± 1.5†	12.8 ± 1.3†
Platelets, $\times 10^4/\mu l$			
Baseline	36.6 ± 13.0	36.4 ± 8.7	32.6 ± 9.2
Week 12	38.5 ± 11.9	29.6 ± 8.8†	21.8 ± 6.5†
Fibrinogen, mg/dl			
Baseline	470.2 ± 118.1	480.0 ± 110.0	463.8 ± 102.1
Week 12	487.9 ± 130.1	396.2 ± 130.3†	231.6 ± 103.3†
Serum amyloid A, mg/ml			
Baseline	383.3 ± 343.0	404.9 ± 355.6	364.8 ± 288.6
Week 12	391.8 ± 375.2	202.0 ± 267.9†	75.0 ± 259.9†
Albumin, gm/dl			
Baseline	3.6 ± 0.4	3.6 ± 0.4	3.5 ± 0.4
Week 12	3.7 ± 0.4†	4.0 ± 0.4†	4.2 ± 0.4†
Rheumatoid factors, IU/ml			
Baseline	337.6 ± 364.8	297.9 ± 377.7	345.5 ± 436.2
Week 12	348.2 ± 402.6	261.2 ± 415.5	235.6 ± 365.6†
Osteocalcin, ng/ml			
Baseline	5.8 ± 2.8	5.7 ± 2.8	5.1 ± 2.2
Week 12	6.2 ± 3.1	6.6 ± 3.1†	6.5 ± 2.7†
PICP, ng/ml			
Baseline	135.4 ± 65.7	127.0 ± 63.5	117.2 ± 47.7
Week 12	144.3 ± 77.8	146.4 ± 70.6†	166.4 ± 78.7†
Urinary pyridinoline, μmoles/mole creatinine			
Baseline	60.3 ± 32.6	58.8 ± 32.7	58.5 ± 29.8
Week 12	62.6 ± 30.9	49.0 ± 29.5†	47.7 ± 24.2†
Deoxypyridinoline, μmoles/mole creatinine			
Baseline	7.8 ± 3.4	7.6 ± 3.5	7.4 ± 3.1
Week 12	7.6 ± 3.8	6.6 ± 3.2†	6.9 ± 3.1†

* Values are the mean ± SD. MRA = humanized anti–interleukin-6 receptor antibody; PICP = C-terminal type I procollagen propeptide.
† $P < 0.05$ versus baseline of each assessment, by paired t-test.

Two weeks after injection of the study drug, her liver function became worse in association with the EBV DNA increase in the plasma, followed by the hemophagocytosis syndrome. Another patient in the 8-mg group was hospitalized because of allergic pneumonitis after completion of the 3 doses of MRA. One patient in the 4-mg group was hospitalized because of infection secondary to a grade 2–3 burn on the leg, but she continued MRA treatment. Both patients were cured by medication. In the placebo group, a subarachnoid hemorrhage and a fracture of the neck of the femur were reported as serious adverse events.

Table 4. Adverse events observed in at least 3% of patients*

	Treatment group		
	Placebo (n = 53)	4 mg/kg MRA (n = 54)	8 mg/kg MRA (n = 55)
Common cold	7 (13.0)	9 (16.7)	5 (9.1)
Headache	1 (1.9)	2 (3.7)	3 (5.5)
Pruritus	3 (5.6)	3 (5.6)	2 (3.6)
Skin eruption	1 (1.9)	2 (3.7)	3 (5.5)
Stomatitis	2 (3.7)	3 (5.6)	4 (7.3)
Fever	1 (1.9)	3 (5.6)	3 (5.5)

* Values are the number (%) of patients. MRA = humanized anti–interleukin-6 receptor antibody.

For the laboratory profiles, abnormalities were observed in 41%, 57%, and 76% of patients in the placebo, 4-mg, and 8-mg groups, respectively. Lipid metabolism–related reactions such as increases in total cholesterol, triglycerides, and high-density lipoprotein (HDL) cholesterol were common in the MRA groups (Figure 3). A blood cholesterol increase was observed in 48 of 109 patients (44.0%) in the MRA groups. Total cholesterol levels did not continue increasing, but became stable at a certain level in the extension study (data not shown). No cardiovascular complications were observed in association with the increase in total cholesterol. Liver function disorders were observed in 14 of

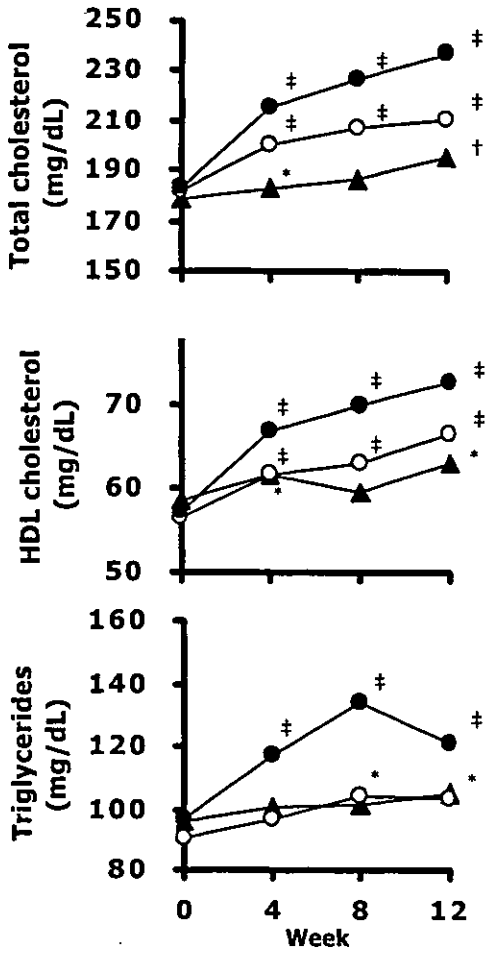

Figure 3. Changes from baseline in serum levels of total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides in patients with rheumatoid arthritis. Solid triangles indicate placebo-treated group. Open circles indicate group treated with 4 mg/kg humanized anti–interleukin-6 receptor antibody (MRA). Solid circles indicate 8 mg/kg MRA–treated group. Each parameter was compared with its baseline value. Values are the mean for each group at each time point. * = $P < 0.05$; † = $P < 0.01$; ‡ = $P < 0.001$ versus baseline, by paired t-test.

109 patients (12.8%) in the MRA groups. An increase to grade 2 in alanine aminotransferase was observed in 2 patients, and the others had grade 1 according to the World Health Organization (WHO) guideline. An increase to grade 1 in aspartate aminotransferase was observed in 8 patients. These increases were transient and normalized with repeated administration of MRA. Decreases in white blood cell counts were observed in 17 of 109 patients (15.6%) (to grade 3 in 1 patient, to grade 2 in 5 patients, and to grade 1 in the other patients according to the WHO guideline). The decreases in white blood cell counts recovered without any treatment in all patients, mostly within 2 weeks. Only 1 patient stopped taking the study drug. There was no serious infection associated with transient neutropenia.

ANAs were positive in 69 of 109 patients (63.3%) at baseline. Eight patients became negative for ANAs at week 12 (according to the LOCF method), while 6 patients became positive. Anti-DNA antibodies were positive in 7 of 109 patients (6.4%) at baseline. Five patients became negative for anti-DNA antibodies at week 12 (according to the LOCF method), and only 1 patient became positive. Anti-MRA antibodies were detected in 2 patients who received MRA (1.8%), and although these patients were asymptomatic, they were withdrawn according to the study protocol.

DISCUSSION

This randomized, double-blind, placebo-controlled trial provided evidence for a rapid reduction in disease activity in response to MRA in patients with active RA. The efficacy was dose related, and 8 mg/kg of MRA provided marked clinical benefit. The success in the treatment of RA with MRA confirmed that IL-6 plays important pathologic roles in RA.

Recently, biologic agents targeting tumor necrosis factor (TNF) have been used successfully to treat RA (23–26). However, ~30% of patients failed to respond to the TNF inhibitors. Further study will be required to establish whether MRA is effective for those patients.

MRA showed benefit in some safety profiles. MRA did not induce anti-DNA antibodies, while anti-TNF therapy induced anti–double-stranded DNA antibodies in 16% of patients (23). The benefit of a humanized antibody was also demonstrated in the repetitive treatment, because anti-MRA antibodies were detected in <2% of MRA-treated patients without requiring the use of immunosuppressive agents such as methotrexate. Although serious infections were rare in repetitive treatment with MRA, 1 patient died of reactivation of chronic active EBV infection and consequent hemophagocytosis syndrome. After that event, we screened all patients who were receiving MRA in this and other studies (>200 patients) for plasma EBV DNA, and the patient who died was the only one with detectable EBV DNA in the plasma (data not shown).

We have detected EBV DNA in whole blood (including white blood cells) in some other patients; however, those patients have not experienced any severe adverse events during MRA treatment for more than 1 year in the extension study. Furthermore, blood EBV

DNA became undetectable during MRA treatment in some patients. Although we do not currently know the exact mechanism for the reactivation of chronic active EBV infection, we can exclude such a patient if we carefully examine eligibility, especially regarding existing infection. A long-term safety study is also required, since some serious infections associated with TNF inhibitors were reported after they became commercially available (27,28).

MRA markedly improved inflammation markers, such as CRP levels and ESRs, in a manner similar to that of TNF and IL-1 inhibitors (23,29,30). Specifically, MRA completely normalized CRP levels in 76% of patients in the 8-mg group. These results indicate that IL-6 is a major cytokine responsible for acute-phase protein production in RA. IL-6 was also proven to be a potent regulator of lipid metabolism in vivo. Dose-dependent increases in total cholesterol, HDL cholesterol, and triglycerides were observed and were above the normal range in some patients. This finding is concordant with previous reports that administration of recombinant IL-6 decreased serum cholesterol in cancer patients (31,32) and that IL-6–deficient mice showed an increase in triglycerides and very-low-density lipoprotein in the blood in association with suppressed energy expenditure and increased food intake, with no increase in HDL cholesterol (33). However, MRA treatment also increased HDL cholesterol. The precise mechanisms of this phenomenon are uncertain, but total cholesterol levels did not continue increasing, and no cardiovascular complications were observed. Therefore, the findings do not preclude further use of MRA for RA. Since RA patients reportedly have an increased risk of developing cardiovascular complications (34), we are examining the safety issue of cardiovascular complications after long-term treatment in the extension study.

A mild and transient decrease in white blood cell counts was sometimes observed in the MRA groups. A similar phenomenon was reported in clinical studies with other biologic agents such as anti-CD20 antibody (35) and anti-TNFα antibody (36). Therefore, the phenomenon is not specific to MRA treatment. Most of the liver function disorders observed in the MRA groups were also mild and transient. However, we should be careful when we use MRA in combination with methotrexate.

This clinical trial indicates an acceptable safety profile relative to the clinical benefit. Further studies are therefore required to determine the long-term safety and efficacy of MRA as well as the feasibility of preventing joint damage in RA patients.

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REFERENCES

1. Harris ED Jr. Rheumatoid arthritis: pathophysiology and implications for therapy. N Engl J Med 1990;322:1277–89.
2. Nishimoto N, Kishimoto T, Yoshizaki K. Anti-interleukin 6 receptor antibody treatment in rheumatic disease. Ann Rheum Dis 2000;59:121–7.
3. Feldmann M, Brennan FM, Foxwell BM, Maini RN. The role of TNFα and IL-1 in rheumatoid arthritis. Curr Dir Autoimmun 2001;3:188–99.
4. Guerne PA, Zuraw BL, Vaughan JH, Carson DA, Lotz M. Synovium as a source of interleukin 6 in vitro: contribution to local and systemic manifestations of arthritis. J Clin Invest 1989;83: 585–92.
5. Hirano T, Matsuda T, Turner M, Miyasaka N, Buchan G, Tang B, et al. Excessive production of interleukin 6/B cell stimulatory factor-2 in rheumatoid arthritis. Eur J Immunol 1988;18:1797–801.
6. Houssiau FA, Devogelaer JP, van Damme J, de Deuxchaisnes CN, van Snick J. Interleukin-6 in synovial fluid and serum of patients with rheumatoid arthritis and other inflammatory arthritides. Arthritis Rheum 1988;31:784–8.
7. Dasgupta B, Corkill M, Kirkham B, Gibson T, Panayi G. Serial estimation of interleukin 6 as a measure of systemic disease in rheumatoid arthritis. J Rheumatol 1992;19:22–5.
8. Madhok R, Crilly A, Watson J, Capell HA. Serum interleukin 6

levels in rheumatoid arthritis: correlations with clinical and laboratory indices of disease activity. Ann Rheum Dis 1993;52:232–4.
9. Sack U, Kinne RW, Marx T, Heppt P, Bender S, Emmrich F. Interleukin-6 in synovial fluid is closely associated with chronic synovitis in rheumatoid arthritis. Rheumatol Int 1993;13:45–51.
10. Uson J, Balsa A, Pascual-Salcedo D, Cabezas JA, Gonzalez-Tarrio JM, Martin-Mola E, et al. Soluble interleukin 6 (IL-6) receptor and IL-6 levels in serum and synovial fluid of patients with different arthropathies. J Rheumatol 1997;24:2069–75.
11. Kotake S, Sato K, Kim KJ, Takahashi N, Udagawa N, Nakamura I, et al. Interleukin-6 and soluble interleukin-6 receptors in the synovial fluids from rheumatoid arthritis patients are responsible for osteoclast-like cell formation. J Bone Miner Res 1996;11: 88–95.
12. Wendling D, Racadot E, Wijdenes J. Treatment of severe rheumatoid arthritis by anti-interleukin 6 monoclonal antibody. J Rheumatol 1993;20:259–62.
13. Sato K, Tsuchiya M, Saldanha J, Koishihara Y, Ohsugi Y, Kishimoto T, et al. Reshaping a human antibody to inhibit the interleukin 6-dependent tumor cell growth. Cancer Res 1993;53: 851–6.
14. Choy EH, Isenberg DA, Garrood T, Farrow S, Ioannou Y, Bird H, et al. Therapeutic benefit of blocking interleukin-6 activity with an anti–interleukin-6 receptor monoclonal antibody in rheumatoid arthritis: a randomized, double-blind, placebo-controlled, dose-escalation trial. Arthritis Rheum 2002;46:3143–50.
15. Nishimoto N, Maeda K, Kuritani T, Deguchi H, Sato B, Imai N, et al. Toxicity, pharmacokinetics, and dose finding study of repetitive treatment with the humanized anti-interleukin 6 receptor antibody, MRA, in rheumatoid arthritis. Phase I/II clinical study. J Rheumatol 2003;30:1426–35.
16. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315–24.
17. Hochberg MC, Chang RW, Dwosh I, Lindsey S, Pincus T, Wolfe F. The American College of Rheumatology 1991 revised criteria for the classification of global functional status in rheumatoid arthritis. Arthritis Rheum 1992;35:498–502.
18. Pincus T, Summey JA, Soraci SA Jr, Wallston KA, Hummon NP. Assessment of patient satisfaction in activities of daily living using a modified Stanford Health Assessment Questionnaire. Arthritis Rheum 1983;26:1346–53.
19. Felson DT, Anderson JJ, Boers M, Bombardier C, Furst D, Goldsmith C, et al. American College of Rheumatology preliminary definition of improvement in rheumatoid arthritis. Arthritis Rheum 1995;38:727–35.
20. Prevoo ML, van 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight–joint counts: development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. Arthritis Rheum 1995;38:44–8.
21. Felson DT, Anderson JJ, Boers M, Bombardier C, Chernoff M, Fried B, et al. The American College of Rheumatology preliminary core set of disease activity measures for rheumatoid arthritis clinical trials. Arthritis Rheum 1993;36:729–40.
22. Van Gestel AM, Prevoo ML, van 't Hof MA, van Rijswijk MH, van de Putte LB, van Riel PL. Development and validation of the European League Against Rheumatism response criteria for rheumatoid arthritis: comparison with the preliminary American College of Rheumatology and the World Health Organization/International League Against Rheumatism criteria. Arthritis Rheum 1996;39:34–40.
23. Maini R, St Clair EW, Breedveld F, Furst D, Kalden J, Weisman M, et al. Infliximab (chimeric anti-tumour necrosis factor α monoclonal antibody) versus placebo in rheumatoid arthritis patients receiving concomitant methotrexate: a randomised phase III trial. Lancet 1999;354:1932–9.
24. Moreland LW, Schiff MH, Baumgartner SW, Tindall EA, Fleischmann RM, Bulpitt KJ, et al. Etanercept therapy in rheumatoid arthritis. Ann Intern Med 1999;130:478–86.
25. Lipsky PE, van der Heijde DM, St Clair EW, Furst DE, Breedveld FC, Kalden JR, et al. Infliximab and methotrexate in the treatment of rheumatoid arthritis. N Engl J Med 2000;343:1594–602.
26. Bathon JM, Martin RW, Fleischmann RM, Tesser JR, Schiff MH, Keystone EC, et al. A comparison of etanercept and methotrexate in patients with early rheumatoid arthritis. N Engl J Med 2000; 343:1586–93.
27. Keane J, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwieterman WD, et al. Tuberculosis associated with infliximab, a tumor necrosis factor α-neutralizing agent. N Engl J Med 2001;345:1098–104.
28. Lee JH, Slifman NR, Gershon SK, Edwards ET, Schwieterman WD, Siegel JN, et al. Life-threatening histoplasmosis complicating immunotherapy with tumor necrosis factor α antagonists infliximab and etanercept. Arthritis Rheum 2002;46:2565–70.
29. Charles P, Elliott MJ, Davis D, Potter A, Kalden JR, Antoni C, et al. Regulation of cytokines, cytokine inhibitors, and acute-phase proteins following anti-TNF-α therapy in rheumatoid arthritis. J Immunol 1999;163:1521–8.
30. Bresnihan B, Alvaro-Gracia JM, Cobby M, Doherty M, Domljan Z, Emery P, et al. Treatment of rheumatoid arthritis with recombinant human interleukin-1 receptor antagonist. Arthritis Rheum 1998;41:2196–204.
31. Weber J, Yang JC, Topalian SL, Parkinson DR, Schwartzentruber DS, Ettinghausen SE, et al. Phase I trial of subcutaneous interleukin-6 in patients with advanced malignancies. J Clin Oncol 1993;11:499–506.
32. Van Gameren MM, Willemse PH, Mulder NH, Limburg PC, Groen HJ, Vellenga E, et al. Effects of recombinant human interleukin-6 in cancer patients: a phase I-II study. Blood 1994;84: 1434–41.
33. Wallenius V, Wallenius K, Ahren B, Rudling M, Carlsten H, Dickson SL, et al. Interleukin-6-deficient mice develop mature-onset obesity. Nat Med 2002;8:75–9.
34. Del Rincon I, Williams K, Stern MP, Freeman GL, Escalante A. High incidence of cardiovascular events in a rheumatoid arthritis cohort not explained by traditional cardiac risk factors. Arthritis Rheum 2001;44:2737–45.
35. Tobinai K, Kobayashi Y, Narabayashi M, Ogura M, Kagami Y, Morishima Y, et al, the IDEC-C2B8 Study Group. Feasibility and pharmacokinetic study of a chimeric anti-CD20 monoclonal antibody (IDEC-C2B8, rituximab) in relapsed B-cell lymphoma. Ann Oncol 1998;9:527–34.
36. Paleolog EM, Hunt M, Elliott MJ, Feldmann M, Maini RN, Woody JN. Deactivation of vascular endothelium by monoclonal anti–tumor necrosis factor α antibody in rheumatoid arthritis. Arthritis Rheum 1996;39:1082–91.

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Expression of Interleukin-22 in Rheumatoid Arthritis

Potential Role as a Proinflammatory Cytokine

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Objective. **Interleukin-22 (IL-22) is a novel cytokine of the IL-10 family. Although its pathophysiologic function is largely unknown, induction of acute-phase responses by IL-22 has suggested proinflammatory properties. In this study, we sought to examine whether IL-22 plays a role in the pathogenesis of rheumatoid arthritis (RA).**

Methods. **Expression of IL-22 and IL-22 receptor 1 (IL-22R1) was examined by reverse transcription–polymerase chain reaction (RT-PCR), Western blot, and immunohistochemical analysis. The effects of recombinant IL-22 (rIL-22) on cultured synovial fibroblasts derived from RA patients (RASF), with regard to the proliferation of synovial fibroblasts and production of monocyte chemoattractant protein 1 (MCP-1), were examined by alamer blue assay and enzyme-linked immunosorbent assay, respectively.**

Results. **IL-22 messenger RNA was detected by RT-PCR in RA synovial tissues and mononuclear cells isolated from RA synovial fluid samples. High levels of IL-22 were expressed both in the lining and the sublining layers of RA synovial tissues. Staining for vimentin and CD68, as markers of synovial fibroblasts or macrophages, respectively, showed that the majority of IL-22–positive cells were synovial fibroblasts and macrophages. IL-22R1 was also expressed in both the lining and the sublining layers of RA synovial tissues. The majority of cells expressing IL-22R1 were positive for vimentin, but not for CD68. Expression of IL-22 and IL-22R1 in RASF was confirmed by RT-PCR and Western blot analysis. According to the in vitro findings, rIL-22 significantly increased proliferation of RASF and production of MCP-1 by RASF above the value of medium controls. Moreover, MAPK activation was induced in RASF in response to IL-22 stimulation.**

Conclusion. **These data suggest that IL-22, produced by synovial fibroblasts and macrophages, promotes inflammatory responses in RA synovial tissues by inducing the proliferation and chemokine production of synovial fibroblasts.**

Rheumatoid arthritis (RA) is a systemic disease that causes progressive joint damage and disability. Rheumatoid synovium is histologically characterized by prominent infiltration of inflammatory mononuclear cells, such as T cells and macrophages, and the proliferation of synovial fibroblasts. Inflammatory cytokines, including tumor necrosis α (TNFα), interleukin-1β (IL-1β), and IL-6, mainly produced by macrophages, play a central role in the development of such synovitis (1,2). For example, TNFα is shown to directly induce synovial fibroblast proliferation, which leads to pannus formation (3). TNFα is also critical for the expression of inflammatory chemokines and adhesion molecules, which, in combination, facilitate further recruitment of inflammatory leukocytes and perpetuation of inflammatory responses (2).

Neutralization of these inflammatory cytokines is proven to be effective in treating human RA (4). However, many patients do not respond to such anticytokine therapy, indicating heterogeneity of the disease. Different cytokines may play a dominant role in these patients. In this regard, efforts should be continued to search for

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any novel cytokines that are critically involved in the pathogenesis of RA.

In contrast to proinflammatory cytokines such as TNFα, IL-10 is regarded as an immunomodulatory cytokine with a broad spectrum of biologic activities, including antiinflammatory and immunosuppressive effects. Collagen-induced arthritis can be ameliorated by administration of IL-10 (5). In the rheumatoid synovium, large amounts of IL-10 are produced, and neutralization with anti–IL-10 antibodies increases the production of the proinflammatory cytokines, including TNFα and IL-1β (6). These data have established the antiinflammatory role of IL-10 in RA, although the results of clinical trials with administration of IL-10 to RA patients have not been encouraging.

Recently, a novel IL-10 family cytokine, IL-22, was identified. By use of the complementary DNA (cDNA) subtraction method, IL-22 was cloned as a molecule induced by IL-9 in murine T cells. IL-22 encoded 180 amino acids, showing 22% amino acid identity with IL-10 (7). Subsequently, a human homolog was cloned and found to have 25% identity with human IL-10. Initial studies have shown that IL-22 is expressed by thymic lymphocytes, mast cells, T cells activated with anti-CD3, or concanvalin A (8). Another study has shown that in peripheral blood mononuclear cells (PBMCs), expression of IL-22 was exclusively detected in T cells, especially upon Th1 polarization, and natural killer cells (9). In vivo, IL-22 was shown to be expressed in the thymus and the brain (8).

A receptor for IL-22, IL-22 receptor 1 (IL-22R1), was cloned and the functional IL-22 receptor was then identified as a complex of IL-22R1 and IL-10R2 (the second chain of the IL-10 receptor complex) (10). IL-22R1 is expressed in tissues such as those from the liver, kidney, pancreas, and skin, but not in PBMCs (11). Upon lipopolysaccharide (LPS) stimulation, IL-22R1 messenger RNA (mRNA) expression is highly upregulated in the liver, in contrast to IL-10R2, which is expressed constitutively in a variety of tissues (11). A soluble receptor that binds to IL-22 was also cloned as a way of identifying the class II cytokine receptor family, and this was designated as IL-22 binding protein (IL-22BP). IL-22BP has 34% amino acid identity with the extracellular domain of the IL-22R1 and has been shown to neutralize the effects of IL-22 (12,13).

Although the pathophysiologic function of IL-22 is largely unknown, the following data suggest that it has proinflammatory properties, in contrast to IL-10. IL-22 induced up-regulation of serum amyloid A (SAA) in HepG2 cells. In vivo, IL-22 also increased SAA expression in the liver, and in turn, LPS injection induced up-regulation of IL-22 in various tissues, including those of the kidney and the liver (14). In contrast to IL-10, IL-22 neither inhibited production of proinflammatory cytokines by macrophages nor induced immunoglobulin production by activated human B cells (15). IL-22 activated STAT-3 and, to a lesser extent, STAT-5 in MES13 cells, and STAT-1 and STAT-3 in HepG2 cells. Of interest, a recent study has shown that IL-22 in a rat hepatoma cell line, H4IIE, activated not only STAT, but also kinases such as ERK, JNK, and p38 MAPK (16). Since p38 MAPK is regarded to be an important kinase for the inflammatory responses, these data support the proinflammatory property of IL-22.

In this study, we examined whether IL-22 plays a role in the pathogenesis of RA. To this aim, we analyzed the expression of IL-22 at both the mRNA and the protein level, using samples derived from RA patients. We further examined the effects of recombinant IL-22 (rIL-22) on cultured synovial fibroblasts derived from RA patients (RASF). Our results demonstrate that high levels of IL-22 are expressed in RA synovial tissues, and that rIL-22 induces synovial fibroblast proliferation and production of chemokines by RASF.

PATIENTS AND METHODS

Antibodies and cytokines. Goat anti-human IL-22 polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-human IL-22R1 polyclonal antibodies were from Prosci Incorporated (Poway, CA). Mouse anti-CD3 monoclonal antibodies were from Nichirei (Tokyo, Japan). Mouse antivimentin, anti-CD20, anti-CD68 monoclonal antibodies, horseradish peroxidase (HRP)–conjugated rabbit anti-goat polyclonal antibodies, and HRP-conjugated goat anti-rabbit polyclonal antibodies were from Dako (Glostrup, Denmark). Rabbit anti–ERK-1/2, anti–phospho-ERK-1/2, anti-p38, and anti–phospho-p38 were from Cell Signaling Technology (Frankfurt, Germany). Recombinant human IL-10, IL-20, and IL-22 were obtained from R&D Systems (Minneapolis, MN).

Patients' samples. Synovial tissues from either RA or osteoarthritis (OA) patients were obtained after joint replacement surgery. All synovial tissues were fixed in 4% parafolmaldehyde overnight and then paraffinized. Synovial fluids were isolated from RA patients during therapeutic arthrocenthesis. Mononuclear cells from the synovial fluid or peripheral blood of normal donors were isolated by Ficoll-Hypaque density gradient centrifugation. All samples were obtained after the patients had given their informed consent.

Immunohistochemical analysis. Three-micrometer–thick paraffinized synovial tissues were deparaffinized, placed in 0.01*M* of sodium citrate buffer (pH 6.0), and heated 2 times for 5 minutes in a microwave oven. After inactivation of endogenous peroxidase with 0.5% metaperiodic acid in phos-

phate buffered saline (PBS) for 10 minutes, sections were incubated with 10% horse serum in PBS for 1 hour. Sections were incubated at 4°C overnight with optimally diluted primary antibodies (goat anti-human IL-22 antibodies, rabbit anti-human IL-22R1 antibodies, mouse anti-human CD3, CD20, CD68, and vimentin antibodies). Sections were incubated with the Dako Envision Plus system to amplify the signal after washing with PBS. The signals were finally developed with diaminobenzidine (Nichirei)

For dual-labeling immunofuorescense, tissue sections were deparaffinized and blocked as described above. Sections were then incubated with a mixture of primary antibodies at 4°C overnight. After washing in PBS, slides were next reacted with HRP-conjugated anti-goat or anti-rabbit IgG. After amplification by the TSA-biotin system, sections were finally incubated with a mixture of anti-mouse IgG labeled with phycoerythrin, streptavidin—fluorescein isothiocyanate, and 4'6-diamidino-2-phenylindole (Roche Molecular Biochemicals, Indianapolis, IN) for 1 hour. Images were acquired and processed digitally.

Cell culture. RASFs were isolated and cultured as previously described (17). Briefly, synovial tissues were minced into 2–3-mm pieces and treated for 2 hours with 2 mg/ml of type I collagenase in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) at 37°C under continuous shaking. After removal of tissue debris through a nylon mesh, the cells were washed with DMEM and then harvested in 75-cm^2 flasks in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS) (Sigma). Cells were cultured until reaching 80% confluence, before being used in experiments. All in vitro experiments were carried out using primary RASF cultures between passages 3 and 8.

Reverse transcription–polymerase chain reaction (RT-PCR) of synovial tissues, RASF, and mononuclear cells. Homogenized RA synovial tissues, mononuclear cells in synovial fluid, and PBMCs were stimulated with or without 10 ng/ml of phytohemagglutinin-leukoagglutinin (PHA-L) (Sigma) for 8 hours, and RASF were prepared. Total RNA was obtained with an RNeasy mini kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. Single-stranded cDNA was synthesized by reverse transcription of 2 μg total RNA, using oligo(dT) primer (Invitrogen, San Diego, CA) and the Omniscript RT kit (Qiagen), to a final volume of 20 μl. Amplification of the cDNA template was performed in a 50-μl PCR mix containing 1 μl cDNA template, 200 μM dNTP, 5 μl PCR Gold Buffer (Applied Biosystems, Foster City, CA), 2.5 mM $MgCl_2$, 1.25 units AmpliTaq Gold DNA polymerase (Roche Laboratories, Nutley, NJ), and 0.5 μM of each primer. Amplification conditions for β-actin and IL-22 were as follows: 10 minutes at 94°C followed by 35 cycles, each consisting of 30 seconds at 94°C, 30 seconds at 60°C, and 45 seconds at 72°C, and finally followed by 7 minutes at 72°C. For IL-22 receptors, conditions were 10 minutes at 95°C followed by 30 cycles, each consisting of 30 seconds at 94°C, 30 seconds at 60°C, and 45 seconds at 72°C, and finally 7 minutes at 72°C. The sequences of oligonucleotide primers used for PCR were as follows: for β-actin, 5'-GAACTTTGGGGGATGCTCGC-3' and 5'-CGGGAAATCGTGCGTGACAT-3' (18); for IL-22, 5'-ACAACACAGACGTTCGTCTCATTG-3'and5'-GAACAG-CACTTCTTCAAGGGTGA-3' (9); and for IL-22R1, 5'-CCT-GAGCTACAGATATGTCACCAAG-3' and 5'-GGCTGG-AAAGTCAGGACTCG-3' (9).

Quantitative real-time PCR. To determine the mRNA expression of monocyte chemoattractant protein 1 (MCP-1), quantitative real-time PCR was performed as previously described, using the ABI Prism 7700 Sequence Detection System (Perkin Elmer Applied Systems, Foster City, CA) and the SYBR Green I PCR kit (Qiagen) (18). Each reaction contained 25 μl of 2× SYBR Green Master Mix, 5 μl of a 1:10 dilution of the prepared cDNA and 300 nM primers, and water to 50 μl. The sequences of primers used for MCP-1 were as follows: 5'-GATCTCAGTGCAGAGGCTCG-3' and 5'-TGCTTGTCCAGGTGGTCCAT-3' (18). For MCP-1 and β-actin, conditions were 15 minutes at 94°C followed by 40 cycles, each consisting of 30 seconds at 94°C, 30 seconds at 58°C, and 30 seconds at 72°C, and finally 7 minutes at 72°C. The ABI Prism 7700 Sequence Detection System software was used to determine the mRNA expression of MCP-1 relative to β-actin in each sample. The final MCP-1 mRNA expression levels in each sample were determined after correction for β-actin expression.

Western blot analysis. Cells were washed with PBS and homogenized in lysis buffer containing 1.5% Triton X-100, 150 mM Tris HCl, pH 7.2, 150 mM NaCl, 15 mM EDTA, 3 mM phenylmethylsulfonyl fluoride, 30 mg/ml aprotinin, 30 mg/ml pepstatin A, 3 mM sodium orthovanadate, and 15 mM pyrophosphate. After centrifugation at 14,000 revolutions per minute for 10 minutes, the protein concentration of the supernatants was determined using a micro BCA protein assay kit (Pierce, Rockford, IL). Protein samples containing 20 μg total protein were separated on 10% denaturing sodium dodecyl sulfate–polymacrylamide gel and transferred onto a polyvinyl difluoride membrane. Blots were initially incubated in Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat dry milk powder (T-TBS milk) to block nonspecific binding. This was followed by further incubation overnight with specific antibodies optimally diluted in T-TBS milk at 4°C. Signals were detected by HRP-conjugated anti-rabbit immunoglobulins (Dako). For visualization, SuperSignal West Dura Extended Duration Substrate (Pierce) was used in accordance with the manufacturer's protocol.

Enzyme-linked immunosorbent assay (ELISA) and cell proliferation assay. The concentration of MCP-1 in cell-culture supernatants was determined by an ELISA obtained from R&D Systems. Cell proliferation was assessed by alamer blue assay. Briefly, RASF were seeded at 1×10^4 cells/well in 24-well plates. After 3 days of incubation with IL-22, 50 μl of alamer blue (Biosource, Camarillo, CA) was added to each well. After 4 hours, the optical density of each well was determined using a microplate reader set at 544 nm (excitation) and 590 nm (emission).

Detection of ERK-1/2 and p38 MAPK activation by Western blot. RASF were grown to semiconfluence in 6-well plates, and cell growth was rested for 24 hours in DMEM containing 1% FCS. Cells were then stimulated with 100 ng/ml rIL-22 for the indicated periods. Cells were harvested and Western blot was performed using specific antibodies against phosphorylated ERK-1/2 and phosphorylated p38 MAPK in accordance with the manufacturer's manual of instructions.

Statistical analysis. All experiments were repeated at least 3 times. Results reported are representative of several

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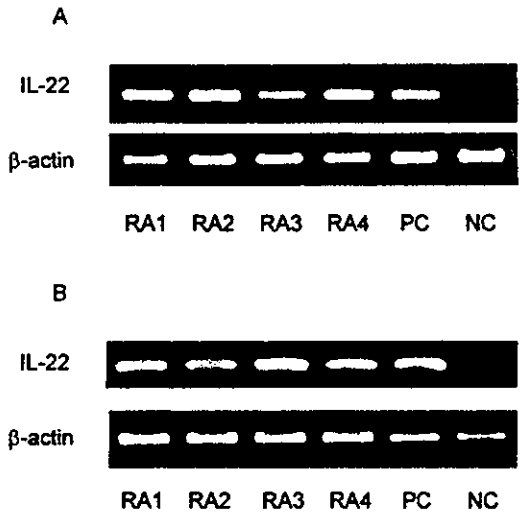

Figure 1. Interleukin-22 (IL-22) mRNA expression in rheumatoid arthritis (RA) synovial tissues (**A**) and mononuclear cells in RA synovial fluid (**B**). Reverse transcription–polymerase chain reaction for IL-22 or β-actin was performed using mRNA extracted from synovial tissues (**A**) or mononuclear cells in synovial fluid (**B**) from 4 RA patients (RA1–RA4). Peripheral blood mononuclear cells from normal donors, stimulated with phytohemagglutinin (PC) or without phytohemagglutinin (NC), were used as positive and negative controls, respectively.

experiments. All results are expressed as the mean ± SEM of 3 separate experiments. Statistical significance was determined by one-way analysis of variance followed by Scheffe's F test. A P value of less than 0.05 was considered to represent a statistically significant difference between group means.

RESULTS

IL-22 expression in RA synovial tissues and mononuclear cells in RA synovial fluid. To examine whether IL-22 is involved in the pathogenesis of RA, we first examined the mRNA expression of IL-22 in RA synovial tissues and mononuclear cells isolated from RA synovial fluid samples. To this end, RT-PCR was performed. As shown in Figure 1A, significant levels of IL-22 mRNA were detected in all RA synovial tissues. PBMCs from normal donors, incubated with or without PHA-L, were used as positive or negative controls, respectively. IL-22 mRNA was also expressed in mononuclear cells in RA synovial fluid (Figure 1B). IL-22 mRNA expression in synovial tissues and mononuclear cells from the synovial fluid was examined in 8 patients with active RA. The results were consistent in all patients examined, and representative results from 4 patients are shown in Figure 1.

We next sought to examine the localization of IL-22 in RA synovial tissues and performed immunohistochemical analysis with a specific antibody against IL-22. As shown in Figure 2A, IL-22 was expressed in both the synovial lining and the sublining layers. In the negative control, no significant signals were detected with nonspecific immunoglobulins (Figure 2E). We also examined IL-22 in synovium from patients with OA (compared with noninflammatory arthropathy as a control). As shown in Figure 2D, modest expression of IL-22 was observed in the synovial lining layers of OA tissue. However, expression of IL-22 was relatively weaker in the sublining layers in OA synovium, which is in contrast with the high expression levels in the respective areas in RA synovium.

To identify the cell types expressing IL-22, vimentin or CD68 were stained in sequential slices as markers of synovial fibroblasts or macrophages, respectively. Because the distribution of cells positive for IL-22 and vimentin was similar, the majority of IL-22–positive cells were thought to be synovial fibroblasts (Figures 2A and B). CD68-positive macrophages were detected in the lining and sublining layers; however, it was difficult to determine whether these cells were positive for IL-22. Therefore, to further examine the cell types expressing IL-22, immunofluorescence double-staining with anti–IL-22 and anti-CD68 antibodies was performed. In the same section, IL-22–positive cells were stained in green (Figure 3A) and CD68-positive cells were in red (Figure 3B). The merged image showed substantial numbers of CD68 cells, but not all were also positive for IL-22 (Figure 3D).

A previous study has shown that T cells produce IL-22 in response to IL-9 (7). Using a double-staining method, we further examined whether infiltrating T cells expressed IL-22 in the rheumatoid synovial tissues. As shown in Figure 3H, the majority of, but not all, CD3-positive cells were negative for IL-22. Taken together, these results show that high levels of IL-22 were expressed in rheumatoid synovial tissues. The major source of IL-22 was thought to be the synovial fibroblasts and macrophages, but predominantly the synovial fibroblasts.

IL-22R1 expression in RA synovial tissues. A functional IL-22 receptor was recently identified as a complex of IL-22R1 and IL-10R2 (10). We therefore examined IL-22R1 mRNA expression in the rheumatoid synovial tissues. RT-PCR with specific primers against IL-22R1 revealed that significant levels of IL-22R1

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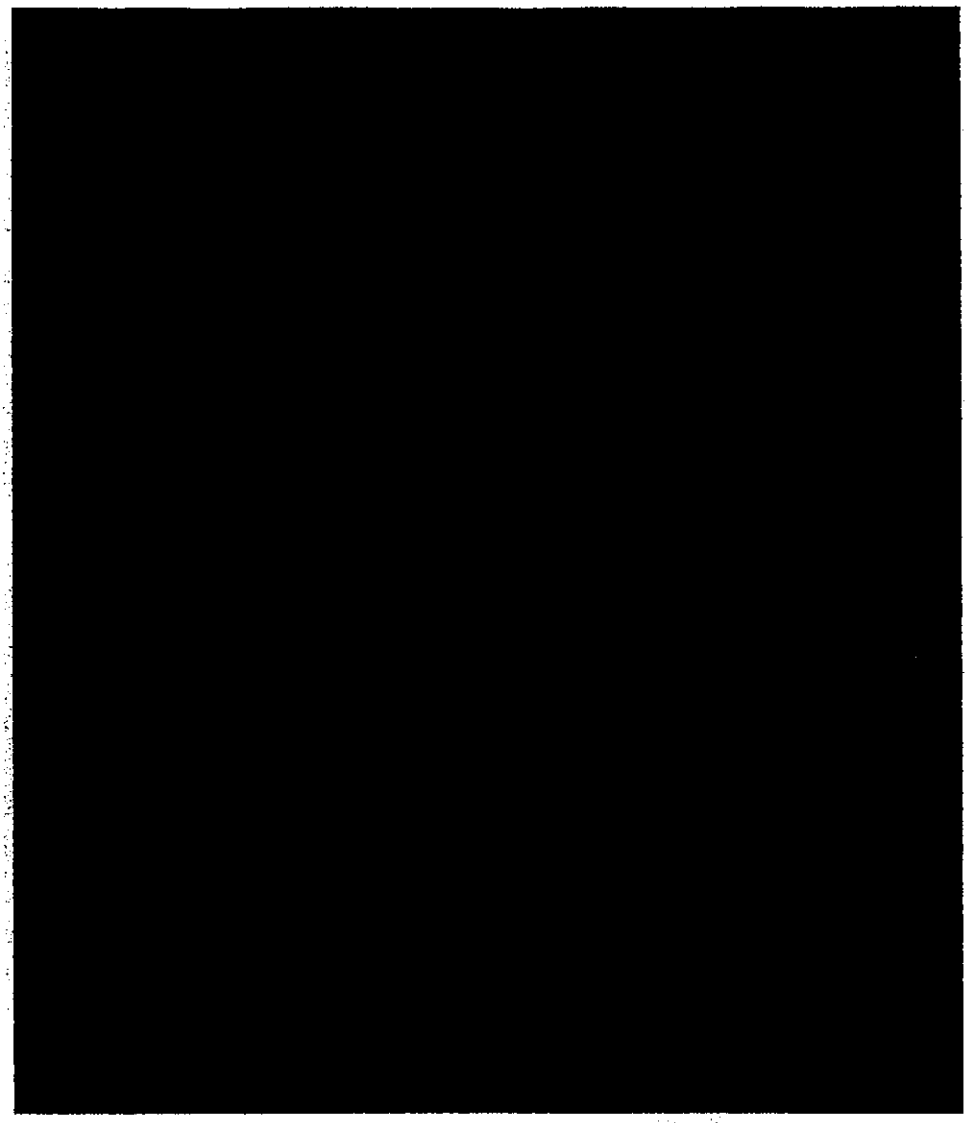

Figure 2. Immunohistologic localization of interleukin-22 (IL-22) in rheumatoid synovial tissues. Immunohistochemical analysis in sequenced slices of the rheumatoid synovium was performed using specific antibodies against IL-22 (**A**), vimentin (**B**), CD68 (**C**), or control IgG (**E**). Synovial tissue from osteoarthritis patients was also stained with anti–IL-22 antibodies (**D**). Sections were counterstained with hematoxylin (original magnification × 200).

F4 mRNA were expressed in RA synovial tissues (Figure 4). As a positive control, mRNA isolated from HepG2, known as the IL-22–responsive human hepatoma cell line in a previous study (8), was used.

In the next experiment, we determined the cellular distribution of IL-22R1 in the rheumatoid synovium, by immunohistochemical analysis. As shown in Figure F5 5A, high levels of IL-22R1 expression were observed in both the lining and the sublining layers. In immunofluorescence staining, IL-22R1 expression was also detected in both the lining and the sublining layers (Figure 5C, green). Vimentin was expressed in a very similar manner (Figure 5D, red). In the merged image, the majority of cells expressing IL-22R1 were also stained with antivimentin antibodies, indicating that the dominant cell type for IL-22R1 is the synovial fibroblast