Therapeutic Effect of Anti-Fas Antibody on a Collagen Induced Arthritis Model

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ABSTRACT. ***Objective.*** To investigate the therapeutic effect of anti-Fas monoclonal antibody (Mab, RK-8) in collagen induced arthritis (CIA).

Methods. CD1F1 mice were immunized with bovine type II collagen to induce CIA and were treated with RK-8 intravenously. The effect of RK-8 was monitored by visual scoring. ELISA to detect serum anti-type II collagen antibody was performed on Day 47 and 70. Histopathological analysis was performed on Days 31 and 72. Digital micrography was performed on Day 72.

Results. RK-8 treatment almost completely prevented CIA. This suppressive effect continued after RK-8 was discontinued. RK-8 significantly suppressed the serum anti-type II collagen antibody level on Day 47. Histological analysis revealed that RK-8 significantly reduced joint histopathology, as determined by the infiltration of inflammatory cells and cartilage damage, consistent with digital micrography.

Conclusion. Administration of anti-Fas Mab may be a useful therapeutic strategy for rheumatoid arthritis if used early in the disease. (J Rheumatol 2001;28:950–5)

Key Indexing Terms:
ANIMAL MODELS APOPTOSIS ANTI-FAS ANTIBODY

Rheumatoid arthritis (RA) is characterized as an autoimmune reaction of lymphocytes and a proliferative disorder of synovial tissue, accompanied by inflammatory cell infiltration[1] and bone erosion. Although the etiology of RA is not clear, effective therapy of arthritis may require elimination of various inflammatory cells and hyperplastic synovial cells.

The Fas antigen (CD95) is a cell surface receptor belonging to the tumor necrosis factor receptor/nerve growth factor receptor family, which transduces a cell death signal[2]. Recently, Fas antigen and Fas ligand (FasL) have been considered to play an important role in the development of autoimmune disease, because a mutation in the Fas antigen and Fas ligand leads to immunological disorders with lymphadenopathy and proliferative arthritis in mice and humans[3-6]. Fas is expressed on activated lymphocytes and various cells, including rheumatoid synovial cells[7,8]. Further, stimulation of the Fas antigen by FasL or agonistic anti-Fas monoclonal antibody (Mab) induces apoptosis of various cells[7-11]. Both synovial fibroblasts and mononuclear cells in the rheumatoid synovium express the functional Fas antigen and these cells are able to undergo apoptosis by anti-Fas Mab[7,8]. This latter phenomenon is thought to be specific to rheumatoid synovium since neither peripheral blood lymphocytes of patients with RA nor synovial fibroblasts of patients with osteoarthritis showed any apoptosis after treatment with anti-Fas Mab[7,12]. Thus, it is possible that the agonistic anti-Fas Mab and FasL may have therapeutic effects for RA. Indeed, gene therapy using FasL effectively ameliorates collagen induced arthritis (CIA)[13].

One serious problem of anti-Fas Mab is its reported hepatotoxicity. Intraperitoneal administration of anti-mouse Fas Mab Jo2 to mice rapidly causes death by fulminant hepatitis with hemorrhage[14]. However, adult mice given another anti-mouse Fas Mab, RK-8, which induced apoptosis both *in vivo* and *in vitro*, were not killed[15]. So it is possible to use RK-8 in treatment of the mouse disease model to evaluate the effects of anti-Fas Mab.

We investigated the therapeutic effect of RK-8 on CIA, thought to be an animal model for human RA[16-18]. It has been reported that the genes encoding the major histocompatibility complex contribute to susceptibility of CIA. Specifically, mouse strain DBA/1J, which is H-$2^{q/q}$ haplotype for MHC, is susceptible to CIA[19], but other strains are not. It has been reported that RK-8 is specific to the allotype of Fas on BALB/c and MRL mice[15]. We confirmed that RK-8 does not bind to Fas on DBA/1J, so we mated DBA/1J with BALB/c to make CD1F1 mice susceptible to CIA and recognized by RK-8. Then we performed intravenous administration of RK-8 to CD1F1 mice immunized with

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bovine type II collagen. Our results revealed that treatment with RK-8 almost completely prevented the development of arthritis, and this suppressive effect continued after the RK-8 was discontinued. These results suggest that anti-Fas Mab may represent a new therapeutic modality for RA in humans.

MATERIALS AND METHODS

Animals and antibodies. Female CD1F1 (BALB/c × DBA/1J F1) mice were purchased from Charles River (Yokohama, Japan). The mice were housed in specific-pathogen-free (SPF) facilities and water and food were provided ad libitum. CD1F1 were immunized at the age of 6 weeks.

Anti-Fas Mab (RK-8; hamster IgG, MBL Co., Nagoya, Japan) or control hamster IgG Ab (ICN Pharmaceuticals-Cappel Products, Costa Mesa, CA, USA) were used as the therapeutic agents.

Induction of apoptosis in vitro by anti-Fas Mab. Thymuses were obtained from BALB/c, DBA/1J, and CD1F1 mice and single cell suspensions were obtained and washed. Cells were seeded in 96 well plates at 1×10^5 cells/well and then were incubated with RK-8 at various concentrations at 37°C for 24 h. Cell viability was measured with an assay based on a mitochondrial reduction of reagent XTT (2, 3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide).

Induction and evaluation of CIA. The mice were divided into 2 groups with 10 animals per group. Each animal was immunized by an intradermal injection at the base of the tail with 100 µg bovine type II collagen (Collagen-Gijutu-Kennsyukai, Tokyo, Japan) emulsified in complete Freund's adjuvant (Difco, Detroit, MI, USA). Seven days later (on Day 7), the animals were given booster injections with the same emulsion by the same route. One week after booster injection, antibodies were administered by intravenous injection once a week for 6 weeks (on Days 14, 21, 28, 35, 42, 49).

The mice were observed twice a week during the entire test. The arthritis lesions on each limb of every animal were scored on a scale of 0–4 as follows: 0, no change; 1, swelling of one joint; 2, swelling of 2 or more joints; 3, edema on entire paw; 4, ankylosis. The macroscopic score was the cumulative value for all paws (mean ± SD), with a maximum of 16.

On Days 47 and 70, mice were bled from an eye socket and the sera were analyzed for anti-type II collagen IgG levels by ELISA (Chondrex; Redmond, WA, USA).

On Days 31 and 72, the paw including the ankle joint was fixed for > 48 h in 10% formalin neutral buffer solution and gently decalcified for 14 days in EDTA/formalin solution. The tissue was embedded in paraffin, sectioned, and stained with hematoxylin and eosin. A microscopic evaluation was performed in a single blind manner. The histologic severity of arthritis was scored if the following events appeared: 0, no change; 1, cell infiltration; 2, pannus formation; 3, bone erosion; and 4, bony ankylosis. The microscopic scores are shown as an average of the sum of existing scores for each paw.

On Day 72, the bone structure was observed with a digital microradiography system (Fuji Film Co., Tokyo, Japan) in the tarsal bone and the heel bone.

Statistical analysis. Statistical analysis for macroscopic scores was performed by Wilcoxon's test; for the microscopic histological score and for serum anticollagen antibody levels, the unpaired t test was used. P values < 0.05 were considered significant.

RESULTS

Effect of anti-Fas antibody on apoptosis in thymocytes from various strains. CIA is mainly restricted to the H-2^q or H-2^r haplotypes[19]. In contrast, anti-murine Fas antibody, a clone of RK-8, binds to Fas in BALB/c (H-$2^{d/d}$), but does not bind to Fas in other strains such as C3H/He and C57BL/6[15]. We observed that RK-8 did not bind to Fas in DBA/1J (data not shown). We produced novel F1 mice from BALB/c and DBA/1J mice (CD1F1) to test binding of RK-8 and susceptibility to CIA. Use of CD1F1 mice to investigate CIA has not been reported.

To elucidate whether RK-8 binds to Fas in CD1F1 and acts functionally, we examined the effect of RK-8 on induction of apoptosis in thymocytes from CD1F1, BALB/c, and DBA/1J mice. As shown in Figure 1, treatment with RK-8 induced apoptosis in thymocytes from CD1F1 in a dose dependent manner as well as in those from BALB/c, but not in thymocytes from DBA/1J. These results indicate that thymocytes from CD1F1 express functional BALB/c-type Fas.

CIA in DBA/1J and CD1F1 mice. To investigate the susceptibility to CIA, CD1F1, BALB/c, and DBA/1J mice were immunized with type II collagen and development of arthritis was investigated. The first signs of arthritis were observed at 5 weeks after first immunization in CD1F1 and DBA/1J mice (Figure 2). There were no significant differences of the increase of average arthritis scores between CD1F1 and DBA/1J mice (Figure 2). CIA did not develop in BALB/c mice as reported previously[19].

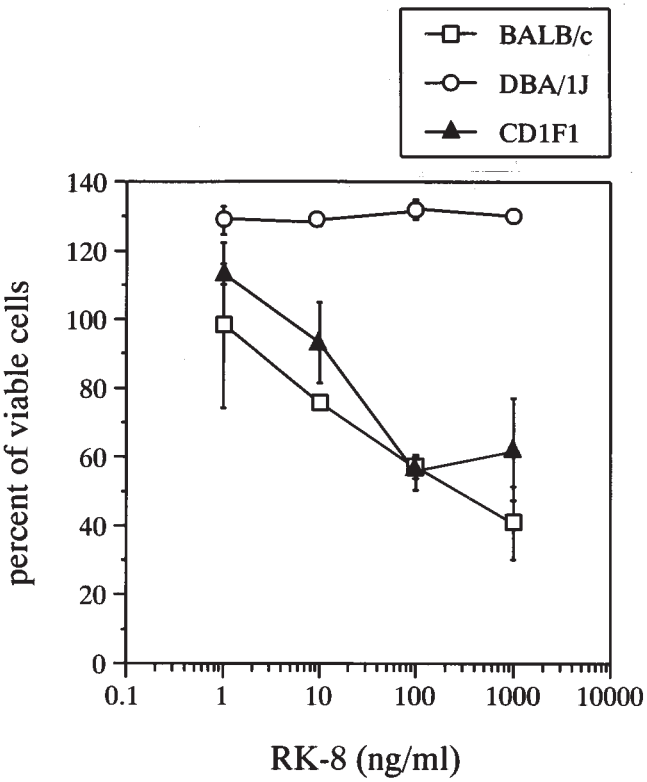

Figure 1. Effect of anti-mouse Fas antibody (RK-8) on the viability of thymocytes from various strains. Indicated concentrations of anti-mouse Fas antibody (RK-8) were incubated with thymocytes from BALB/c, DBA/1J, or CD1F1 mice for 24 h. Ability to reduce XTT reagent was measured in the last 6 h.

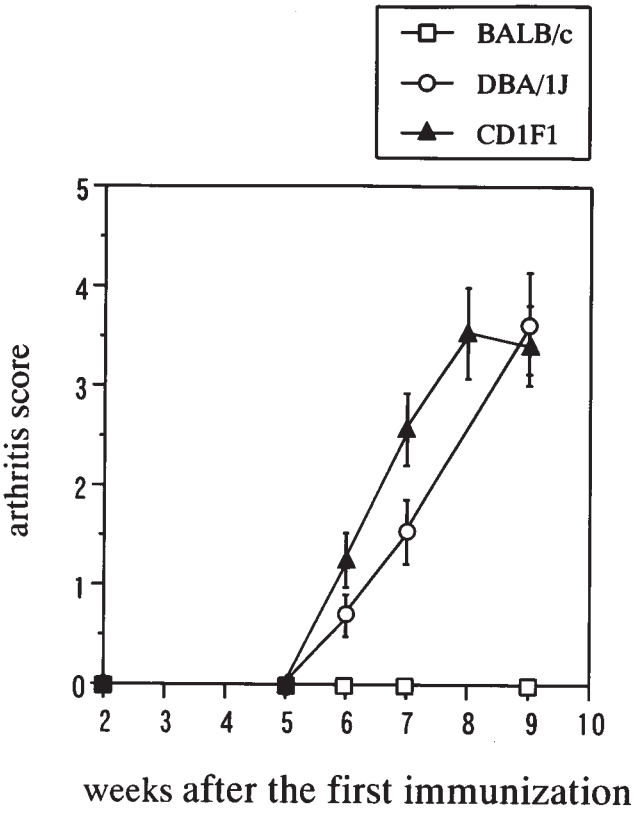

Figure 2. CIA in DBA/1J and BALB/c and CD1F1 mice. Ten mice per group were immunized with 100 µg type II collagen at the base of the tail on Day 0 and boosted with the same concentration on Day 7. Arthritis severity score is described in Materials and Methods. Immunized CD1F1 mice developed arthritis as well as DBA/1J mice. Immunized BALB/c mice did not develop arthritis. Data are represented as average ± SE.

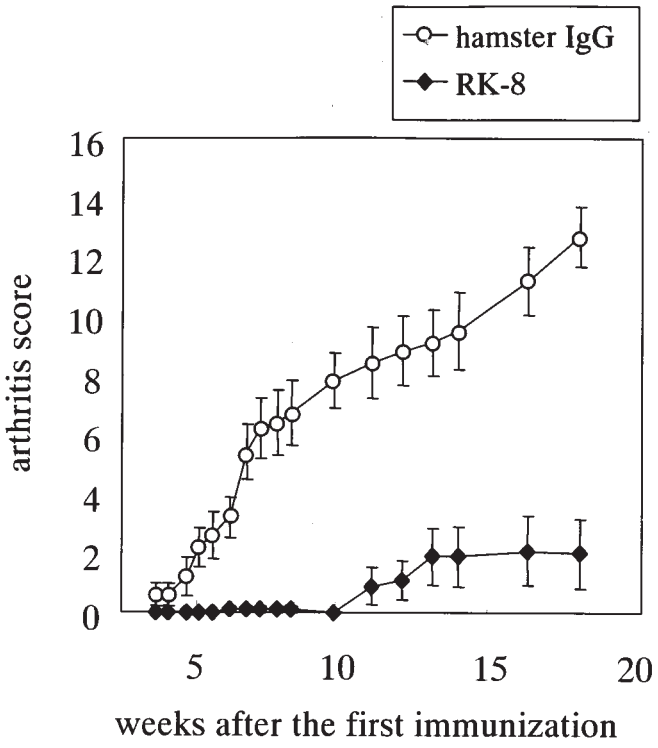

Figure 3. Effect of anti-mouse Fas antibody (RK-8) treatment on CIA. Two groups of CD1F1 mice, 10 mice per group, were immunized with 100 µg of type II collagen at the base of the tail on Day 0 and boosted with the same concentration on Day 7. The mice were injected with 20 µg of hamster IgG or 20 µg of RK-8 on Days 14, 21, 28, 35, 42, 49. Severity of arthritis, measured by macroscopic scores, in RK-8 treated mice was statistically significant ($p < 0.01$) compared to control mice from 5 weeks after the first immunization.

These results indicate that this CD1F1 CIA model could be used to investigate the effect of RK-8 on CIA *in vivo*.

Effect of RK-8 treatment on development of arthritis. To examine the effect of anti-Fas Mab on the development of arthritis, one group of immunized CD1F1 mice was treated once a week with 20 µg of RK-8 intravenously for 6 weeks (2 to 7 weeks after first immunization) and another group of mice was administered 20 µg of hamster IgG as a control. Figure 3 summarizes the disease course of 10 animals, presented as average arthritis score per mouse. In the control group, first signs of arthritis were observed within 4 weeks after first immunization, and polyarthritis was observed in almost all animals within 10 weeks. On the other hand, as shown in Figure 3, no animal exhibited any sign of arthritis in any joint of the limbs in the group treated with RK-8 during the treatment (7 weeks after first immunization). Further, this complete suppressive effect lasted at least 3 more weeks after the treatment with RK-8 was discontinued, and a significant suppressive effect lasted for at least 18 weeks. Essentially the same results were obtained with 3 independent experiments (data not shown).

Effect of RK-8 on the suppression of humoral response to type II collagen. To ascertain whether the immune response to collagen in mice is suppressed by treatment with RK-8, serum anti-type II collagen antibody levels were measured. Induction of CIA caused elevation of serum anti-type II collagen antibody levels (3260 ± 998 µg/ml; n = 10) compared with normal mice (17 ± 15 µg/ml; n = 5). Administration of RK-8 significantly suppressed serum anti-type II collagen antibody levels in an early stage (Day 47) of arthritis (Table 1). At a late stage (Day 70) of arthritis, serum anti-type II collagen antibody levels in the hamster IgG group and the RK-8 group were almost similar.

Table 1. Effect of anti-mouse Fas antibody (RK-8) treatment on the suppression of humoral response to type II collagen. The values represent mean ± SD of 10 mice per group.

Group	Serum Anti-Type II Collagen Antibody Level (µg IgG/ml) Day 47	Day 70
Hamster IgG	3260 ± 998	1794 ± 695
RK-8	881 ± 573*	1444 ± 963

* $p < 0.001$ vs hamster IgG group, by unpaired t test.

Table 2. Effect of anti-mouse Fas antibody treatment on the histology of collagen induced arthritis. Microscopic evaluation was performed in a single blind manner. Histologic severity of arthritis was scored for intercarpal or intertarsal joints and interphalangeal joints if the following events appeared: 0, no change; 1, cell infiltration; 2, pannus formation; 3, bone erosion; 4, bone ankylosis. The microscopic scores are the sum of existing scores with a maximum value of 10. Values represent mean ± SD of 8 different joints.

Group	Day 31	Day 72
Hamster IgG	9.0 ± 1.2	7.5 ± 2.4
RK-8	1.5 ± 2.1*	0.1 ± 0.2*

* $p < 0.001$ vs hamster IgG group, by unpaired t test.

Histological examination of synovium. To determine the effect of RK-8 on the histological features of arthritis, we examined the ankle joints of immunized CD1F1 mice treated with RK-8 or hamster IgG by a single blind scoring test (Table 2). The joints of mice treated with hamster IgG showed marked arthritis symptoms, including erosion of the bone and cartilage and formation of a pannus-like granulation tissue with proliferation of fibroblasts and infiltrating mononuclear cells (Figure 4). In contrast, joints of mice treated with RK-8 showed limited signs of arthritis on Days 31 and 72 after immunization (Table 2). Joints of normal mice showed no signs of arthritis (score = 0.0). The histological observations were almost consistent with the macroscopic arthritis scores.

Examination of bone structure using digital microradiography. We used a digital microradiography system to examine the effect of RK-8 on bone structure in late stage arthritis in CD1F1 CIA mice; Figure 5 compares the front limb of a collagen arthritic mouse with a mouse treated with RK-8. On Day 72 after immunization, remarkable bone destruction was recognized in the tarsal bone and the heel bone of mice treated with the hamster IgG. However, bone tissue was observed as a clear image in RK-8 treated mice. These results were consistent with the macroscopic arthritis scores and histological observations.

DISCUSSION

There is increasing evidence that RA is characterized by synovial hyperplasia and an autoimmune type overactivation of lymphocytes or inflammatory cells[1]. There is also increasing evidence that the Fas/FasL apoptosis system is involved in the development and maintenance of RA[7,8]. For example, synovial fibroblasts and infiltrating cells from

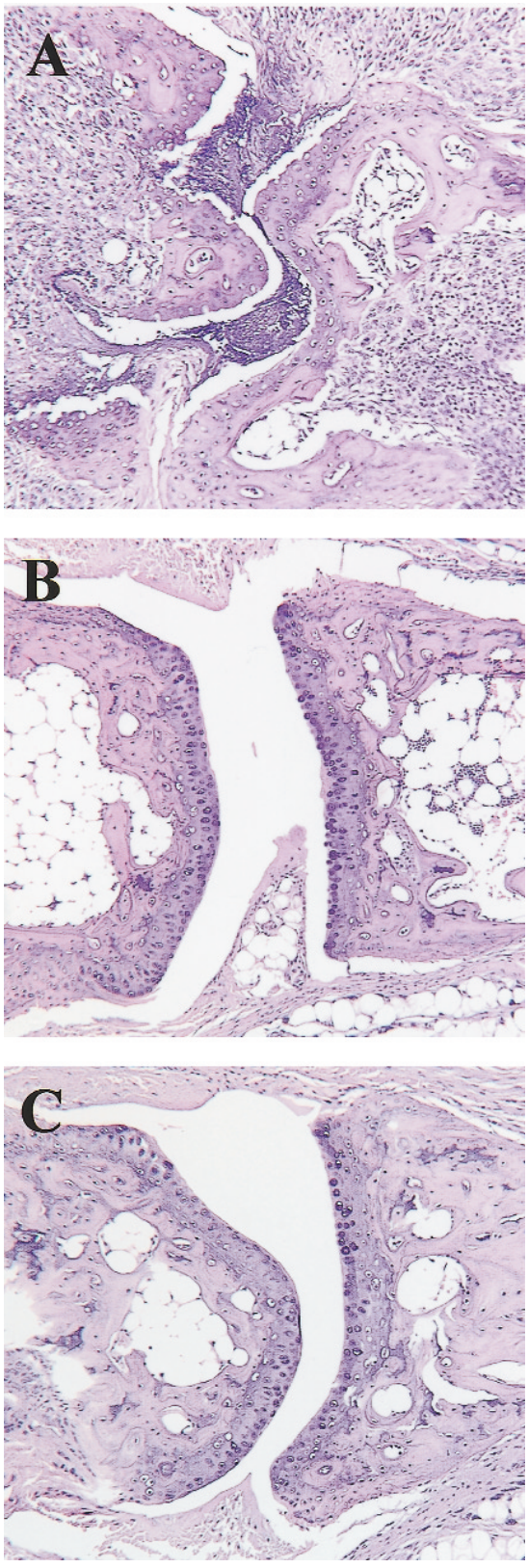

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Figure 4. Histopathological evaluation of arthritis in arthritic mice and RK-8 treated mice. A. Severe arthritis in CIA mouse with hamster IgG. Note the formation of pannus-like granulation tissue, synovial fibroblasts, bone erosion. B. Drastic improvement of arthritis in mice treated with RK-8. A remarkable improvement in arthritis was observed in all mice examined. C. Untreated CD1F1 mice.

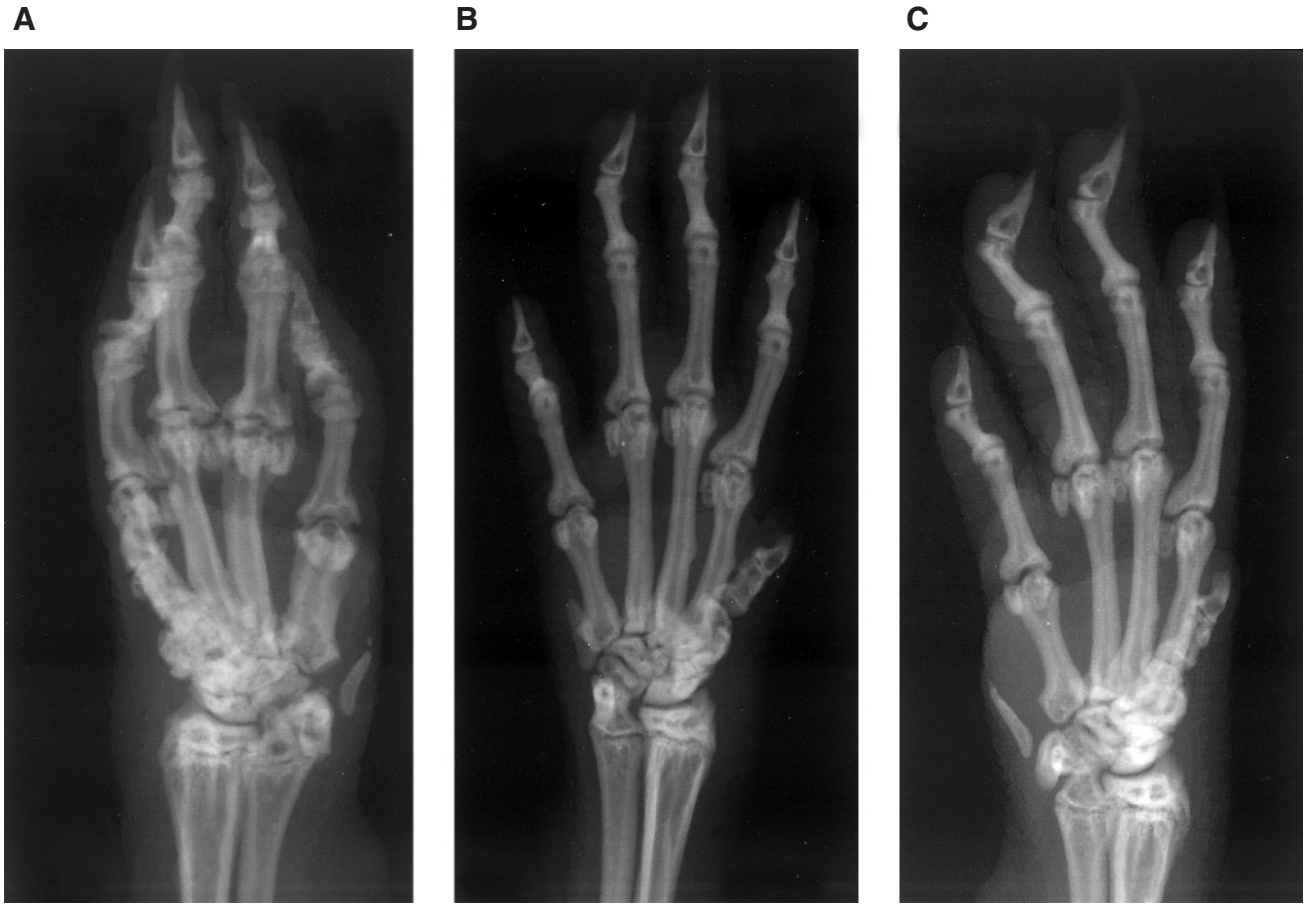

Figure 5. Digital microradiographs of mice on Day 72: A. forelimb of CIA mouse with hamster IgG. B. Forelimb of an RK-8 treated mouse. C. Forelimb of an untreated mouse.

patients with RA were susceptible to apoptosis induction by anti-Fas antibody *in vitro*[7]. Intraarticular injection of RK-8 decreased the arthritis symptoms induced in HTLV-I tax transgenic mice[20], and intraperitoneal injection of RK-8 suppressed the arthritis symptoms induced in FasL deficient MRL-*gld*/*gld* mice[14]. We investigated the effect of anti-murine Fas antibody RK-8 on the CIA model with CD1F1 mice, which we have newly developed.

As described, RK-8 recognizes BALB/c type murine Fas and not other strains[21], probably because BALB/c type murine Fas has a point mutation with a single amino acid change near the N-terminal and RK-8 may recognize the epitope including the mutation or conformation caused by the mutation. As RK-8 does not bind to thymocytes from DBA/1J mice, conventional CIA with DBA/1J mice is not a suitable model to investigate the effect of RK-8. We hypothesized that CD1F1 mice, produced by mating a female BALB/c mouse and a male DBA/1J mouse, expressing BALB/c type murine Fas and DBA/1J type murine Fas simultaneously, are susceptible to RK-8 induced apoptosis and are also susceptible to CIA. This hypothesis appeared to be true, as thymocytes from CD1F1 mice were susceptible to RK-8 induced apoptosis as well as BALB/c thymocytes, and CD1F1 mice developed CIA as well as DBA/1J mice.

In this CD1F1 CIA model, intravenous administration of RK-8 almost completely suppressed the development of CIA evidenced by macroscopic scores during the dosing period and 3 additional weeks. RK-8 also suppressed serum anti-type II collagen antibody levels during the dosing period, and histopathological changes at the joints were limited. However, 4 weeks after the final RK-8 treatment, 30% of these mice developed CIA and serum anti-type II collagen antibody levels were increased. This is probably because not all lymphocytes activated by type II collagen were eliminated and the remaining lymphocytes proliferated after the disappearance of RK-8.

A possible mechanism of suppression of CIA by RK-8 treatment is as follows: T and B lymphocytes are activated with type II collagen immunization to produce serum anti-type II collagen antibody and to cause inflammation at the joint where type II collagen is abundant. Activated lymphocytes express Fas molecule on their cell surface and RK-8 induces apoptosis in activated lymphocytes, so that immune

response to type II collagen and inflammation are suppressed. RK-8 probably acts systemically and locally.

Intraperitoneal administration of anti-Fas Mab Jo2 was lethal to mice due to severe liver damage. Previous studies suggested that 100 µg of RK-8 did cause mild, transient, and histologically reversible liver damage to BALB/c mice[15]. In this CIA model, liver enzyme levels were not significantly elevated in mice treated with 20 µg of RK-8. For example, the ALT level of CIA mice was 33.3 ± 15.3 IU/l and that of RK-8 treated mice was 73.3 ± 32.5 IU/l, while the ALT level of Jo2 treated mice was 663.3 ± 270.0 IU/l. No systemic toxicity (weight loss, etc.) was ever observed during the study. Also, administration of 20 µg of RK-8 did not elevate AST and ALT in BALB/c mice. The reason for the different toxicity between Jo2 and RK-8 may be due to different epitopes on Fas antigen recognized by the Mab[21].

Our results open a novel therapeutic possibility to use anti-human Fas antibody with the characteristics of RK-8 — agonistic and low toxicity — for the treatment of human rheumatoid arthritis.

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