Differential Effects of FK506 and Methotrexate on Inflammatory Cytokine Levels in Rat Adjuvant-Induced Arthritis

KATSUE MAGARI, SUSUMU MIYATA, FUSAKO NISHIGAKI, YOSHITAKA OHKUBO, SEITARO MUTOH, and TOSHIO GOTO

ABSTRACT. ***Objective.*** To investigate the effects of prophylactic and therapeutic treatments with FK506 (tacrolimus), an immunosuppressive drug that specifically inhibits T cell activation, and methotrexate (MTX) on inflammatory cytokines, tumor necrosis factor (TNF)-α, interleukin (IL)-1ß, and IL-6 levels in rat adjuvant-induced arthritis (AIA).

Methods. AIA was induced in female Lewis rats. Arthritis was assessed by hindpaw swelling. TNF-α, IL-1ß, and IL-6 levels in paw extracts were determined by ELISA. To assess the effects on cytokine levels, rats were treated prophylactically with FK506 (3 mg/kg) or MTX (0.1 mg/kg) from day 1 to day 17, and therapeutically with FK506 (5 mg/kg) or MTX (1 mg/kg) from day 15 to day 17 (3-day treatment) or day 15 to 20 (6-day treatment) by oral administration.

Results. TNF-α, IL-1ß, and IL-6 levels in paw tissue were found to significantly increase between day 15 and day 21 after adjuvant injection, when the arthritis was in a developed stage. Prophylactic treatment with FK506 and MTX suppressed arthritis and reduced the levels of those inflammatory cytokines. FK506 caused a marked reduction of TNF-α and IL-1ß levels in paw tissue even in short-term (3-day) therapeutic treatment. It reduced all levels of TNF-α, IL-1ß, and IL-6 in paws in 6-day therapeutic treatment. In contrast, therapeutic treatment with MTX affected neither TNF-α or IL-6 levels in paws. MTX reduced IL-1ß levels only in the 6-day treatment.

Conclusion. FK506 is more effective than MTX in reducing elevated levels of inflammatory cytokines TNF-α, IL-1ß, and IL-6 in established stages of AIA. Our findings suggest that inhibition of T cell activation results in a rapid reduction of inflammatory cytokine levels even after the arthritis is established in AIA. (J Rheumatol 2003;30:2193–200)

Key Indexing Terms:
TUMOR NECROSIS FACTOR-α INTERLEUKIN 1ß INTERLEUKIN 6 FK506
INFLAMMATORY CYTOKINE ADJUVANT INDUCED ARTHRITIS

Rheumatoid arthritis (RA) is a chronic disease characterized by inflammation of the joint with concomitant destruction of cartilage and bone. Although the cause of RA remains unknown, recent studies have revealed the key roles of T cells[1] and inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1ß, and IL-6[2] in pathogenesis of the disease. Clinical trials of anti-TNF therapy have shown marked efficacy in controlling signs and symptoms of disease[2,3]. Anti-IL-1 therapy results in less impressive control of symptoms than TNF blockade, but has shown retardation of joint destruction[4]. Anti-IL-6 therapy has also been reported to be effective as a treatment for RA, particularly in reducing acute-phase response, as indicated by the reduction of C-reactive protein (CRP)[5]. Thus, blockade of all 3 of these cytokines may be more effective than either alone in the treatment of RA.

FK506 (tacrolimus) is an immunosuppressive drug that specifically suppresses T cell activation[6,7]. FK506 exerts its immunosuppressive effects after binding to intracellular proteins termed immunophilins, i.e., FK506 binding proteins (FKBP). The drug-immunophilin complex inhibits calcineurin phosphatase, an enzyme involved in activation of transcription factor NF-AT required for the expression of cytokine genes in T cells[8]. It has been suggested that inflammatory cytokines are produced through activation of T cells and by subsequent interaction of the activated T cells and monocytes/macrophages in RA[1]. FK506 has been examined as a treatment for RA and shown to be effective in RA patients resistant to or intolerant of methotrexate (MTX)[9]. MTX, an antifolate agent, is currently the most widely used disease modifying antirheumatic drug (DMARD)[10]. The mechanism of action of MTX has not been fully characterized, even though it has been shown to produce various anti-inflammatory effects. The antifolate activity of MTX inhibits purine and pyrimidine synthesis and consequently

From Medicinal Biology Research Laboratories, Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan.

K. Magari, BS; S. Miyata, PhD; F. Nishigaki, PhD; Y. Ohkubo, PhD; S. Mutoh, PhD; T. Goto, PhD.

Address reprint requests to Dr. S. Miyata, Medicinal Biology Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 2-1-6, Kashima, Yodogawa-ku, Osaka 532-8514, Japan.
E-mail: susumu_miyata@po.fujisawa.co.jp

Submitted October 7, 2002; revision accepted February 3, 2003.

impairs proliferation of immune cells. It has also been suggested that MTX functions through the increased production of extracellular adenosine. We have shown that FK506 specifically suppresses T cell activation-mediated production of TNF-α, IL-1ß, and IL-6 *in vitro*[11,12] and that FK506 is more effective than MTX in reducing arthritis in adjuvant-induced arthritis (AIA), when treated after the disease has been established[13].

AIA has been used in preclinical studies as a standard animal model of RA in humans[14]. Anti-TNF and IL-1 therapy have been reported to be effective in this model[15-17]. Immunosuppressive agents suppress development of arthritis in longterm treatment by affecting immunopathologic events involving sensitization to antigen, proliferation, and differentiation of immunocompetent cells. Indeed, immunosuppressive agents such as MTX have been reported to suppress production of the inflammatory cytokine TNF-α in rat joints, when administered before the onset of disease in AIA[18]. However, there have been no reports showing that immunosuppressive agents are effective in reducing inflammatory cytokines in this animal model after the disease has been established. In this study, we first attempted to establish quantitative measurements of TNF-α, IL-1ß, and IL-6 levels associated with disease progression in AIA, since kinetics of inflammatory cytokines have not been fully characterized in relation to disease progression. Next, we compared the effect of prophylactic and therapeutic treatment with FK506 and MTX on inflammatory cytokines in AIA.

MATERIALS AND METHODS

Induction of arthritis. Female Lewis rats were obtained from Charles River Japan, Inc. (Kanagawa, Japan) and bred in a clean environment. Arthritis was induced by injection of 0.5 mg of *Mycobacterium tuberculosis* (Difco Laboratories, Detroit, MI, USA) in 50 µl of liquid paraffin into the right hind footpad of Lewis rats aged 7 weeks. Normal nontreated rats did not receive any injection and were used as negative controls. All experimental procedures were reviewed and approved by the Fujisawa Pharmaceutical Animal Experiment Committee.

Drug treatment. A solid dispersion formulation of FK506[19] was prepared at Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). FK506 was suspended in distilled water. MTX (Sigma, St. Louis, MO, USA) was suspended in 0.5% methylcellulose. The agents were orally administered in a volume of 5 ml/kg. Distilled water or 0.5% methylcellulose was administered as vehicle control. To assess the dose dependent effects of agents on paw swelling (n = 9–10), FK506 (1–5.6 mg/kg) or MTX (0.032–0.1 mg/kg) was prophylactically administered from day 1 to day 23, and FK506 (1–5.6 mg/kg) or MTX (0.1–1 mg/kg) was therapeutically administered from day 15 to day 24. The paw volume was measured on day 23 in the prophylactic treatment and on day 24 in the therapeutic treatment, after drug administration. For cytokine analysis (n = 5), FK506 (3 mg/kg) or MTX (0.1 mg/kg) was prophylactically administered from day 1 to day 17, and FK506 (5 mg/kg) or MTX (1 mg/kg) was therapeutically administered from day 15 to day 17 (3-day treatment) or days 15–20 (6-day treatment).

Evaluation of arthritis. Arthritis was quantified based on paw swelling. The volume of the left hind paw was measured before and after arthritis induction by a water displacement method, using a plethysmometer for rats. Paw swelling was presented as a change in the hind paw volume.

Determination of TNF-α, IL-1ß, and IL-6 levels in paw tissue. The left hind paw of each rat was dissected above the ankle joint, snap-frozen in liquid nitrogen, and stored at –80°C until use. Before homogenization for each assay, the frozen paw containing bony tissue was weighed and broken into pieces on dry ice. The paw tissues were added to 4 ml/g tissue of extraction buffer containing 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, and 0.05% Tween 20 in phosphate buffered saline. Tissues were homogenized on ice with a polytron and centrifuged at 5000 g for 15 min. Supernatants were stored at –80°C until analysis. TNF-α, IL-1ß, and IL-6 levels in the supernatants were determined using ELISA kits specific for rat TNF-α (Genzyme, Cambridge, MA, USA), IL-1ß, and IL-6 (both from Endogen, Woburn, MA, USA). The sensitivity of the assays for TNF-α, IL-1ß, and IL-6 was 5, 12, and 16 pg/ml, respectively. Percentage of reduction was calculated using the following formula: % reduction = (1 – B/A) × 100; where A = vehicle treated – normal, B = drug treated – normal.

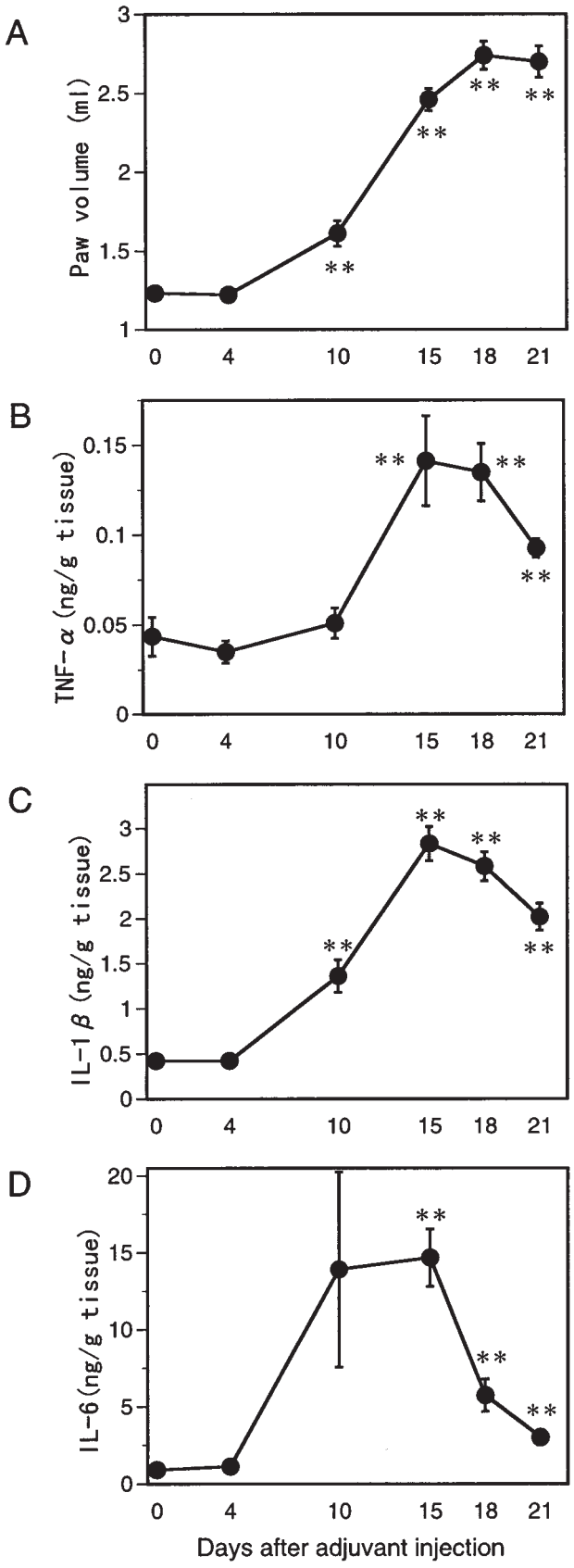
Determination of TNF-α, IL-1ß, and IL-6 levels in plasma. Peripheral blood anticoagulated with EDTA was obtained from the abdominal artery of rats under ether anesthesia. Plasma was collected by centrifugation and frozen until analysis. TNF-α, IL-1ß, and IL-6 levels in the plasma were determined using ELISA kits specific for rat TNF-α (Genzyme), IL-1ß, and IL-6 (both from Endogen).

Statistical analysis. Results are presented as mean ± SE. Differences between vehicle and drug treatment groups were determined using Dunnett's multiple comparison test. Differences before and after adjuvant injection were determined using Student's t test. P values < 0.05 were considered statistically significant.

RESULTS

Changes in TNF-α, IL-1ß, and IL-6 levels in paw tissue during development of arthritis. An increase in paw volume was observed 10 days after adjuvant injection and the arthritis as assessed by paw swelling was fully developed after day 15 (Figure 1A). TNF-α levels in paw tissue significantly increased on day 15 and the increase was sustained through to day 21 during development of arthritis (Figure 1B). IL-1ß levels in arthritic paws significantly increased on day 10 after adjuvant injection and the increase was sustained through to day 21 (Figure 1C). IL-6 levels also significantly increased between day 15 and day 21, although the level after day 18 was considerably lower than the peak level (Figure 1D). These data indicate that upregulation of TNF-α, IL-1ß, and IL-6 levels in paw tissue is associated with the development of arthritis.

Effects of prophylactic and therapeutic treatment with FK506 and MTX on paw swelling in AIA. In a previous study, we showed that FK506 was more effective than MTX in suppressing arthritis in therapeutic treatment of AIA[13]. Here, the effect of prophylactic treatment with FK506 and MTX was compared with the therapeutic effects of these agents. The agents were orally administered from days 1–23 (prophylactic treatment) or from days 15–24 (therapeutic treatment). As shown in Figure 2A, FK506 suppressed paw swelling dose-dependently in both the prophylactic and therapeutic treatments. Effective doses of 50% suppression (ED_{50}) in prophylactic and therapeutic treatment were 1.5 mg/kg and 3.9 mg/kg, respectively. MTX also suppressed paw swelling with an ED_{50} value of 0.055 mg/kg when the agent was administered prophylactically (Figure 2B).



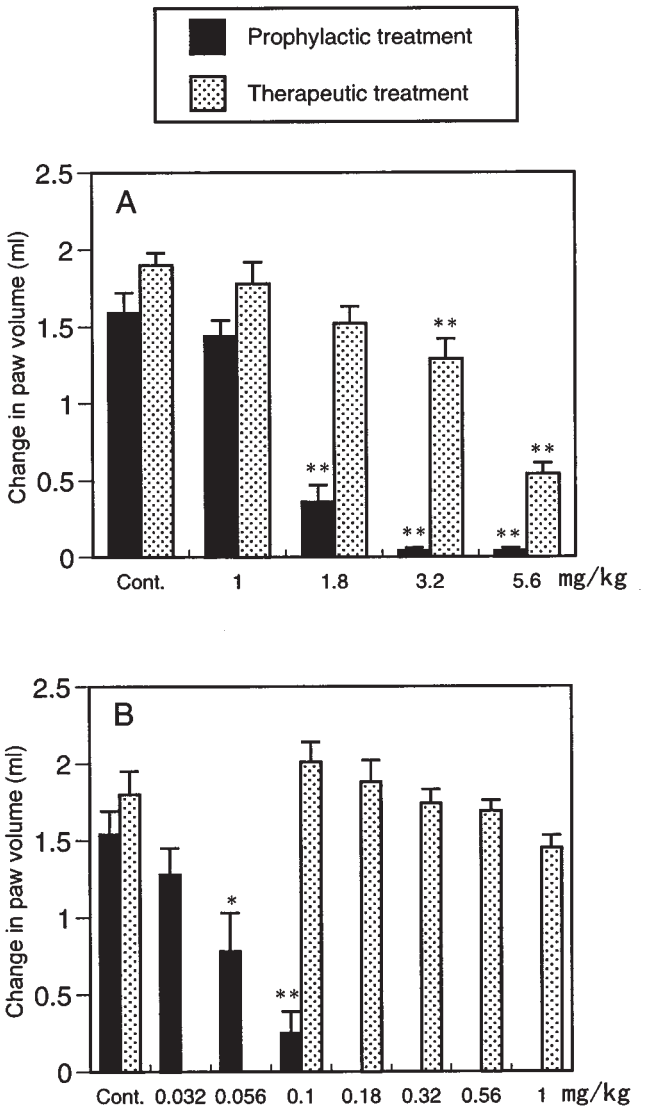


Figure 2. Effects of prophylactic and therapeutic treatment of FK506 and MTX on paw swelling in AIA. Rats were orally administered with 1–5.6 mg/kg of FK506 from days 1–23 (prophylactic treatment) or from days 15–24 (therapeutic treatment). (A) Rats were orally administered with 0.032–0.1 mg/kg of MTX (prophylactic treatment) or 0.1–1 mg/kg of MTX (therapeutic treatment). (B) Data represent mean ± SE of 9–10 animals per group. *p < 0.05, **p < 0.01 compared to the vehicle controls (Cont).

However, therapeutic treatment of MTX did not affect paw swelling significantly even at 1 mg/kg, more than a 10-fold higher dose of the effective dose in prophylactic treatment.

Effects of prophylactic treatment with FK506 and MTX on TNF-α, IL-1β, and IL-6 levels in paw tissue. FK506 (3

Figure 1. Changes in TNF-α, IL-1ß, and IL-6 levels during development of AIA. (A) Paw volume before (day 0) and after adjuvant injection. TNF-α (B), IL-1ß (C), and IL-6 (D) levels in the paw extract before (day 0) and after adjuvant injection. Cytokine levels were determined by ELISA. Data represent mean ± SE of 10 (A) or 5 (B-D) animals per group. **p < 0.01 compared to before adjuvant injection.

mg/kg) or MTX (0.1 mg/kg), at which dose paw swelling was almost completely suppressed (Figure 2), was administered to rats from day 1 to day 17 and cytokine levels in paw tissue were determined on day 18. As shown in Figure 3, FK506 and MTX suppressed the elevation of TNF-α level by 84.8% and 82.1%, respectively. Both agents completely suppressed IL-1ß and IL-6 level to normal levels.

Effects of therapeutic treatment with FK506 and MTX on TNF-α, IL-1ß, and IL-6 levels in paw tissue. FK506 (5 mg/kg) and MTX (1 mg/kg) were administered to rats from day 15-17 (3-day treatment) or day 15-20 (6-day treatment) and cytokine levels in paw tissue were determined on day 18 or day 21, respectively. FK506 significantly reduced the elevated levels of TNF-α (59.2%) and IL-1ß (68.9%) in arthritic paws, even in 3-day treatment (Figure 4). FK506 also reduced IL-6 level by 39.4%, although the suppressive effect was not significant. The less potent suppression of IL-6 level may be ascribed to the progressive decrease of the cytokine during development of arthritis after day 15, as shown in Figure 2D. Cytokine levels in arthritic paws were not affected by short term treatment with MTX. FK506 significantly reduced the levels of TNF-α (45.8%), IL-1ß (58.5%), and IL-6 (58.1%) in paws in 6-day treatment (Figure 5). MTX did not affect paw swelling, TNF-α and IL-6 levels but significantly reduced IL-1ß level by 43.2% in 6-day treatment.

Effects of therapeutic treatment with FK506 and MTX on IL-6 levels in plasma. Therapeutic treatment with MTX appeared to slightly increase IL-6 level in paw tissue, although the effect was not significant. We further examined the effects of therapeutic treatments of the agent on systemic IL-6 levels. After 3-day and 6-day treatment with FK506 (5 mg/kg) or MTX (1 mg/kg), the IL-6 level in plasma was determined on day 18 and day 21, respectively. IL-6 levels in plasma of AIA rats was elevated on day 18 and day 21 (Figure 6). IL-6 levels in plasma of some normal rats were under or near the detection limit (< 10 pg/ml), therefore, a normal level is not indicated in the figure. FK506 reduced the IL-6 level in plasma after 3-day and 6-day treatment. In contrast, 3-day and 6-day treatment with MTX significantly increased IL-6 levels in plasma to 2.6 and 3.2-fold, respectively. We could not detect significantly elevated levels of

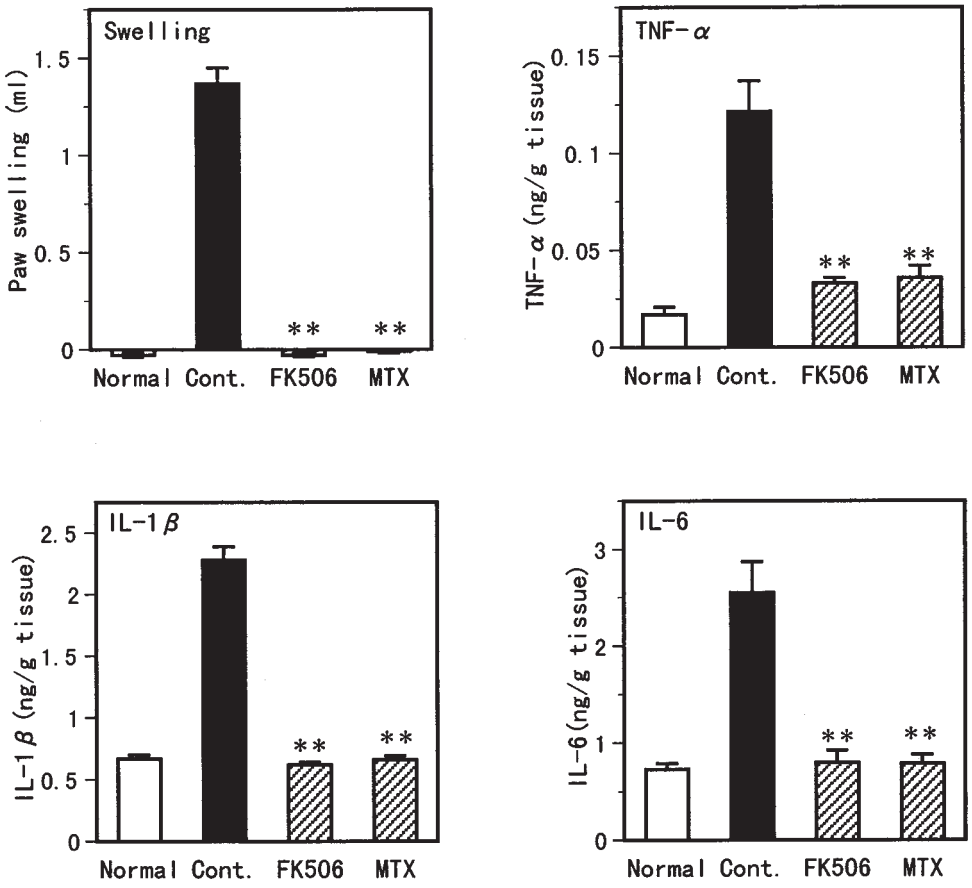


Figure 3. Effects of prophylactic treatment with FK506 and MTX on TNF-α, IL-1ß, and IL-6 levels in paw tissue. Rats were orally administered FK506 (3 mg/kg), MTX (0.1 mg/kg), or distilled water (Cont) from days 1–17 after adjuvant injection. Paw tissues were dissected on day 18. Cytokine levels in paw extracts were determined by ELISA. Data represent mean ± SE of 5 animals per group. **p < 0.01 compared to the vehicle controls.

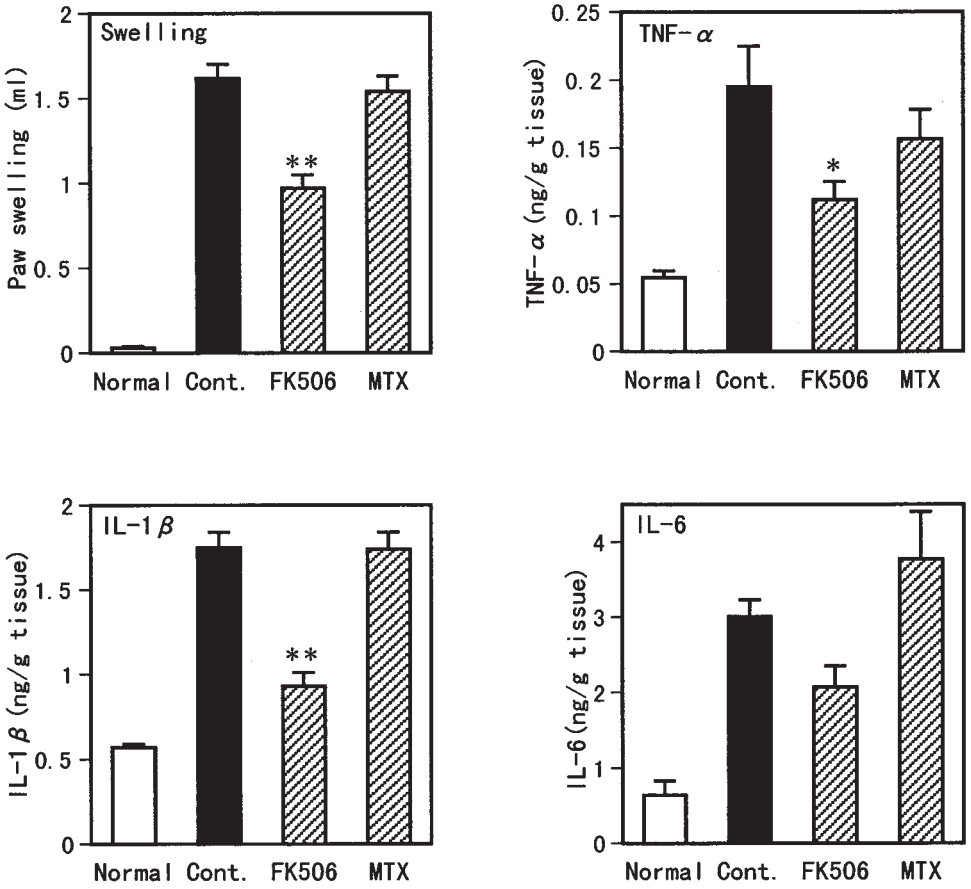


Figure 4. Effects of 3-day therapeutic treatment with FK506 and MTX on TNF-α, IL-1ß, and IL-6 levels in paw tissue. Arthritic rats were orally administered FK506 (5 mg/kg), MTX (1 mg/kg), or distilled water (Cont) from days 15–17 after adjuvant injection. Paw tissues were dissected on day 18. Cytokine levels in the paw extracts were determined by ELISA. Data represent mean ± SE of 5 animals per group. *$p < 0.05$, **$p < 0.01$ compared to the vehicle controls.

TNF-α and IL-1ß in plasma in established stage of AIA (data not shown).

DISCUSSION

We studied the effect of prophylactic and therapeutic treatments of FK506 compared to MTX on inflammatory cytokine levels in AIA to investigate their antiarthritic potential. We first attempted to measure more quantitatively the levels of these cytokines associated with disease progression in AIA, since kinetics of inflammatory cytokines have not been fully characterized in relation to disease progression. For systemic levels of TNF-α and IL-1ß, results vary according to the report. Szekanecz, *et al* reported that serum levels of inflammatory cytokines were correlated with clinical symptoms of arthritis[20]. However, Silva, *et al* concluded that serum TNF-α and IL-1ß levels might not reflect the efficacy of drug treatment[21]. Also, serum TNF-α levels in AIA have been reported to elevate in the acute phase and do not appear to correlate with disease progression[22]. We could not detect significantly elevated levels of TNF-α and IL-1ß in plasma in established stage of AIA, probably due to the detection limit of ELISA assays. In contrast, local levels of inflammatory cytokines seem to be correlated more to disease progression. TNF-α and IL-1ß levels in ankle joints have been reported to increase during development of arthritis[20]. However, the IL-6 level in the ankle joint was found to be elevated only in the late stage of AIA. TNF-α and IL-1ß in the subcutaneous tissue of arthritic paws have been examined to evaluate drug effects on these cytokines, but it was not clear whether IL-1ß in the arthritic rats increased compared to normal rats[23]. Prostaglandin E_2 or cyclooxygenase-2 levels in whole paw extracts have been reported to increase with good correlation to disease development[24]. In our study, extracts from whole paw tissue were employed to analyze the level of inflammatory cytokines. Consequently, all levels of TNF-α, IL-1ß, and IL-6 in the paw extracts were found to significantly increase in the established stage of AIA (days 15–21), although the IL-6 level decreased progressively after day 18. The analysis of inflammatory cytokine levels in whole paw

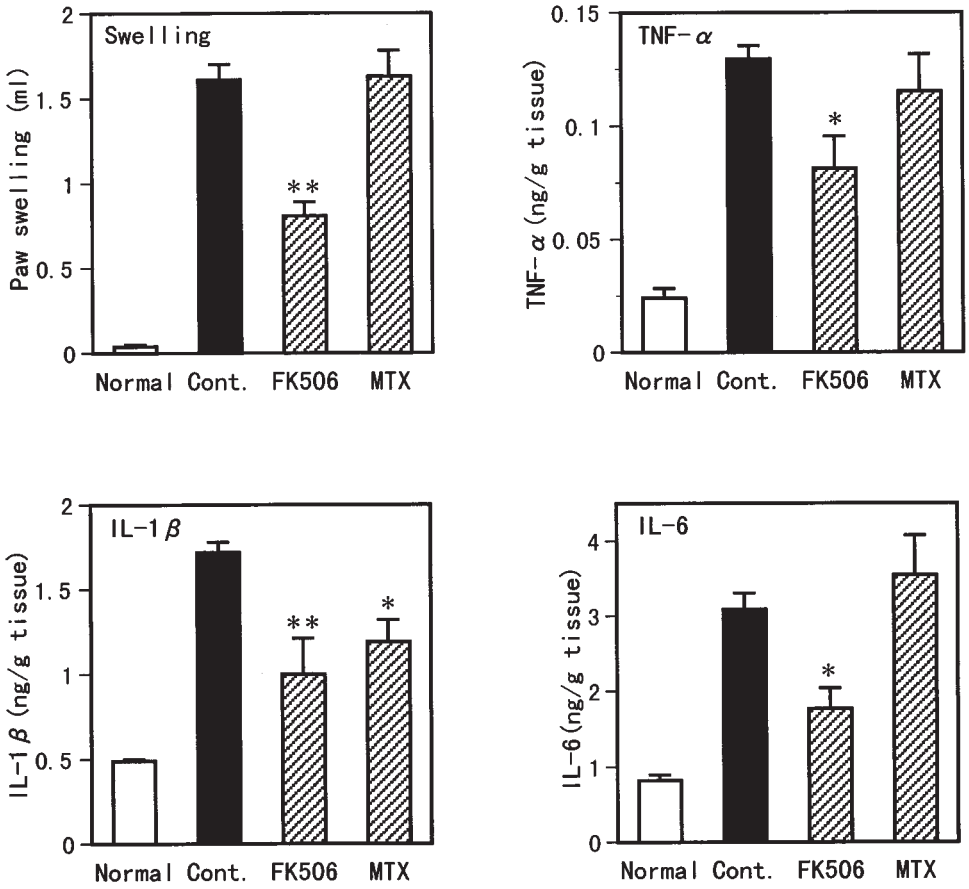


Figure 5. Effects of 6-day therapeutic treatment with FK506 and MTX on TNF-α, IL-1ß, and IL-6 levels in paw tissue. Arthritic rats were orally administered FK506 (5 mg/kg), MTX (1 mg/kg), or distilled water (Cont) from days 15–20 after adjuvant injection. Paw tissues were dissected on day 21. Cytokine levels in the paw extracts were determined by ELISA. Data represent mean ± SE of 5 animals per group. *$p < 0.05$, **$p < 0.01$ compared to the vehicle controls.

extracts may be useful for studying the pathogenic role of cytokines and also the effects of drug treatments on AIA, as the kinetics seem to be associated with the development of AIA.

FK506 suppressed TNF-α, IL-1ß, and IL-6 production in both prophylactic and therapeutic treatments. In particular, FK506 was found to be effective in reducing TNF-α and IL-1ß, even in short-term (3-day) therapeutic treatment. We have shown that FK506 specifically suppresses T cell activation-triggered inflammatory cytokine production *in vitro*, although it does not affect lipopolysaccharide-induced cytokine production[11,12]. It has been suggested that inflammatory cytokines are produced through continuous activation of T cells and interaction of the activated T cells and monocytes/macrophages in RA[1]. AIA as well as human RA have been reported to be T cell dependent diseases[1,25]. It is therefore likely that FK506 suppresses T cell activation-triggered production of TNF-α, IL-1ß, and IL-6 in AIA, thus the agent is effective in reducing the elevated level of these cytokines even in advanced stages of AIA. Recently, down-regulation of the inflammatory cytokines has been focused on as an efficacious therapy for RA. The present findings suggest that inhibition of T cell activation results in a rapid reduction of inflammatory cytokines even after the arthritis is established in AIA. This study is, to our knowledge, the first showing that a drug treatment is effective in reducing inflammatory cytokine levels after the arthritis is established in animal models of RA.

MTX has been reported to suppress TNF-α production in joints of AIA rats, when administered before the onset of arthritis[18]. In this study, MTX was found to potently suppress not only TNF-α but also IL-1ß and IL-6 levels in paw tissue in prophylactic treatments. It has been reported that MTX inhibits proliferation of various cells, including immune cells[10]. Inhibition of clonal expansion of T cells has been proposed as one of the mechanisms by which MTX suppresses RA[26]. In prophylactic treatment, it is thought that MTX suppresses differentiation and proliferation of immune cells, including T cells, and consequently reduces inflammatory cytokine levels. In contrast, MTX failed to

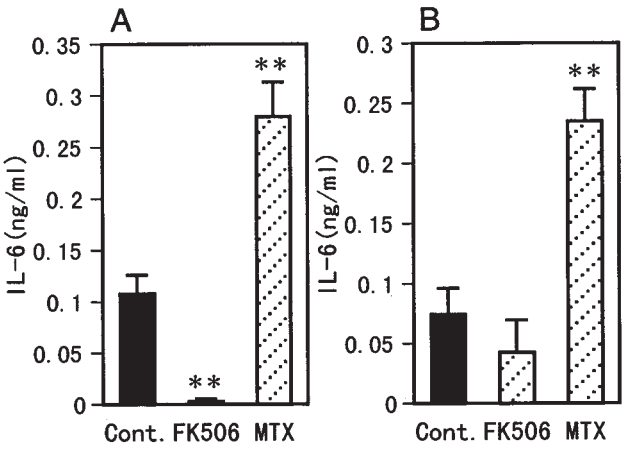


Figure 6. Effects of therapeutic treatment with FK506 and MTX on IL-6 levels in plasma. Arthritic rats were orally administered FK506 (5 mg/kg), MTX (1 mg/kg), or distilled water (Cont) from days 15–17 (A: 3-day treatment) or days 15–20 (B: 6-day treatment) after adjuvant injection. Plasma was obtained on day 18 or day 21. IL-6 levels in plasma were determined by ELISA. Data represent mean ± SE of 5 animals per group. **$p < 0.01$ compared to the vehicle controls.

reduce elevated levels of TNF-α and IL-6 in arthritic paws in therapeutic treatment. The antiproliferative action of MTX is not considered to result in reduction of T cell activation-triggered inflammatory cytokine production, as T cells and other inflammatory cells have already been activated in inflammatory sites at this stage. MTX reduced only IL-1ß levels in longer term (6-day) treatment. TNF-α and IL-6 are produced from both T cells and monocytes/macrophages, although IL-1ß is not derived from T cells. MTX is reported to affect differentiation of monocytes/macrophages *in vitro*[27,28]. It is probable that IL-1ß, a monocyte/macrophage product, may be selectively affected by treatment with MTX, provided that a large part of TNF-α and IL-6 is derived from T cells in AIA.

It is known that IL-1ß is a potent mediator of inflammatory pain, hyperalgesia[29,30]. In a previous report by this group, it was shown that MTX significantly reduced joint hyperalgesia[13], although it did not affect paw swelling in therapeutic treatment of AIA. The reduction of hyperalgesia by therapeutic treatment with MTX may be ascribed to selective suppression of IL-1ß level in arthritic paws. Interestingly, therapeutic treatment with MTX increased the plasma (systemic) level of IL-6 by approximately 3-fold. Although the mechanism of increase of IL-6 levels in plasma is unknown, treatment with MTX may enhance IL-6 mediated responses, such as autoantibody production, induction of acute-phase proteins, and osteoclast activation[5]. Indeed, erythrocyte sedimentation rate (ESR) was elevated by therapeutic treatment with MTX in AIA (data not shown). It has been reported that MTX increased osteoclast-like cell formation in rats[31] and also induced osteopathy in rheumatic disease[32].

TNF-α has been identified as a crucial cytokine in the pathogenesis of RA, based on marked clinical efficacy from anti-TNF therapies[2,3]. However, anti-IL-1 and anti-IL-6 therapy have also been shown to be effective in RA[4,5]. A recent preclinical study shows that combination therapy with IL-1 receptor antagonist and soluble TNF receptor is more effective than either alone in the treatment of AIA or collagen-induced arthritis[17,33]. We found that FK506, but not MTX, suppressed production of all cytokines measured (TNF-α, IL-1ß, and IL-6) in both prophylactic and therapeutic treatments. Thus, orally active agents that inhibit T cell activation and inflammatory cytokine production, such as FK506, may therefore provide significant benefits comparable to combination of anticytokine therapies using protein agents in the treatment of RA, if AIA is predictive of the response in patients with RA.

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