Roxithromycin Specifically Inhibits Development of Collagen Induced Arthritis and Production of Proinflammatory Cytokines by Human T Cells and Macrophages

YASUYO URASAKI, MAMORU NORI, SATOSHI IWATA, TAKAHIRO SASAKI, OSAMU HOSONO, HIROSHI KAWASAKI, HIROTOSHI TANAKA, NAM H. DANG, EIJI IKEDA, and CHIKAO MORIMOTO

ABSTRACT. ***Objective.*** Roxithromycin (RXM) is a macrolide antibiotic that is effective in treatment of chronic lower respiratory tract diseases including diffuse panbronchiolitis and bronchial asthma. Its mechanism of action apart from its antibacterial action remains unclear. To determine the mechanism of action of RXM, we evaluated the effect of RXM on T cell functions and the inflammatory responses in mice with collagen induced arthritis (CIA).

Methods. T cell proliferation, cytokine production by T cells stimulated through CD28, CD26, or PMA with or without anti-CD3 Mab, cytokine production by macrophages stimulated with lipopolysaccharide, and transendothelial migration of T cells were analyzed in the presence or absence of various concentrations of RXM. We evaluated the effect of RXM treatment in collagen induced arthritis in mice.

Results. RXM did not affect the production of Th1-type and Th2-type cytokines, whereas it specifically inhibited production of proinflammatory cytokines such as tumor necrosis factor-α and interleukin 6 (IL-6) by T cells and macrophages. RXM inhibited T cell migration. We found that RXM treatment of mice with CIA reduced the severity of arthritis and serum level of IL-6, as well as leukocyte migration into the affected joints and destruction of bones and cartilage.

Conclusion. Our findings strongly suggest that RXM may be useful for the therapy of rheumatoid arthritis as well as other inflammatory diseases such as Crohn's disease. (J Rheumatol 2005; 32:1765–74)

Key Indexing Terms:
ROXITHROMYCIN PROINFLAMMATORY CYTOKINE T CELLS COSTIMULATION COLLAGEN INDUCED ARTHRITIS MACROPHAGES RHEUMATOID ARTHRITIS

There is increasing evidence that macrolides have a variety of biologic activities apart from their antibacterial actions[1]. Recently, low dose and longterm erythromycin treatment was shown to be effective in chronic lower respiratory tract disease, including diffuse panbronchiolitis and bronchial asthma[2,3], but the mechanism of action of this drug remains unclear. Erythromycin may have antiinflammatory properties, in addition to its antimicrobial effects. These immunomodulatory effects are reported to be the result of leukocyte activation, such as stimulation of phagocytosis[4,5], natural killer cell activity[5,6], production of superoxide anion[5], and neutrophil chemotaxis[2,7-9]. Roxithromycin (RXM), a new macrolide antibiotic, has a 14-member macrocycline ring structure resembling that of erythromycin[10]. RXM is characterized by rapid and complete absorption after oral administration, resulting in high serum concentrations[11]. *In vitro* investigation revealed that RXM modifies the function of neutrophils[12] and keratinocytes[13]. RXM also affects lymphocyte functions including proliferation induced by mitogens and purified protein derivative[14], as well as proliferation and cytokine secretion induced by mitogens[10,15].

In the initial stage of immune response, a certain antigen would be engaged by the T cell receptors, followed by

From the Division of Clinical Immunology, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo; Department of Lymphoma/Myeloma, M.D. Anderson Cancer Center, Houston, Texas, USA; and Department of Pathology, Keio University School of Medicine, Tokyo, Japan.

Supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labor and Welfare of Japan.

Y. Urasaki, MS; M. Nori, MD; S. Iwata, MD; T. Sasaki, MD; O. Hosono, MD; H. Kawasaki, MD; H. Tanaka, MD, Division of Clinical Immunology, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo; N.H. Dang, MD, M.D. Anderson Cancer Center; E. Ikeda, MD, Department of Pathology, Keio University School of Medicine; C. Morimoto, MD, Division of Clinical Immunology, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo.

Address reprint requests to Dr. C. Morimoto, Division of Clinical Immunology, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo, 108-8639 Japan. E-mail: morimoto@ims.u-tokyo.ac.jp

Accepted for publication April 1, 2005.

release of various cytokines. However, this process alone is not sufficient for induction of all events that accompany T cell activation. Accumulating evidence suggests the presence of so-called costimulatory signals that occur through additional T cell surface molecules, which are independent of the CD3/T cell receptors[16]. These costimulatory signals are indispensable for full activation of T cells, which is characterized by T cell proliferation and cytokine production. Triggering of costimulatory signals therefore plays an important role in the generation of hypersensitive immune reaction. These costimulatory signals can be provided by a number of accessory molecules such as CD28/CTLA-4[17,18]. In addition, we identified CD26 as a novel costimulatory molecule that is preferentially expressed on CD4+ memory T cells[19-21] and that is speculated to be involved in the functions of effector T cells that migrate to inflammatory sites in immune mediated diseases[22].

In our study, we employed the costimulatory system of peripheral T cells *in vitro* to elucidate the immunomodulatory effect of RXM. We evaluated the therapeutic effect of RXM *in vivo* using the mouse model of rheumatoid arthritis (RA).

MATERIALS AND METHODS

Cells and reagents. Human peripheral blood mononuclear cells were isolated from healthy volunteer donors by Ficoll-Hypaque (Pharmacia Biotech, Piscataway, NJ, USA) density gradient centrifugation[19]. Unfractionated mononuclear cells were separated into an E rosette-positive (E+) population and were used as resting T cells. Monocytes were depleted by adherence to plastic plates for 24 h at 37°C followed by incubation with 5 mM L-leucine methyl ester HCl (Sigma Chemical Co., St. Louis, MO, USA) for 1 h. The monoclonal antibody (Mab) OKT3 was obtained from the American Tissue Culture Collection (ATCC; Rockville, MD, USA). Anti-CD26 (1F7) and the anti-CD28 Mab 4B10 were developed in our laboratory as described[18,19]. RXM (generously supplied by Eisai Ltd., Tokyo, Japan) was dissolved in DMSO and further diluted in the culture media consisting of RPMI-1640 and 10% fetal calf serum (FCS).

T cell proliferation assays. One hundred microliters of phosphate buffered saline containing 0.05 μg/ml of OKT3 in the presence or absence of 5 μg/ml of 1F7 (anti-CD26), or 5 μg/ml of 4B10 (anti-CD28) Mab, and incubated overnight at 4°C, as described[20]. Highly purified T cells were resuspended at 1×10^5 cells in 200 μl of RPMI-1640 medium containing 10% FCS, along with 4 different concentrations of RXM (0, 1.4, 14, and 28 μM). To assess PMA stimulation, the cell suspension supplemented with 5 ng/ml of PMA was applied into OKT3-coated wells. Cells were incubated at 37°C in a 5% CO_2 humidified atmosphere for 3 days. Cells were pulsed with 1 μCi/well of ^{3}H-thymidine (ICN Radiochemicals, Irvine, CA, USA) 8 h prior to harvest onto a glass-fiber filter (Wallac, Turku, Finland), and the incorporated radioactivity was quantified by a liquid scintillation counter (Wallac).

Cytokine production assays. Antibody-coated plates and purified T cells were prepared in the manner described above, with the exception of OKT3 concentration being 0.5 μg/ml. Cytokine production by T cells was assayed in triplicates in 96-well, flat-bottomplates as described above. After 24 h incubation, culture supernatants were subjected to ELISA [interleukin 2 (IL-2) and IL-4: Biosource International, Camarillo, CA, USA; interferon-γ (IFN-γ) and IL-5: R&D Systems, Minneapolis, MN, USA] to measure the levels of IL-2, IFN-γ, IL-4, and IL-5 as well as tumor necrosis factor-α (TNF-α) and IL-6. For TNF-α and IL-6 production by macrophages, macrophages were enriched from E-rosette-negative cells by adherence to plastic plates. Macrophages (1×10^6/ml) were suspended in 10% FCS-RPMI-1640 and stimulated with 1 μg/ml lipopolysaccharide (LPS; Sigma). After 8 h culture, supernatants were harvested and were subjected to ELISA (TNF-α and IL-6; R&D Systems). The serum levels of IL-6, TNF-α, IFN-γ, and IL-4 were also detected using an ELISA kit as described.

Assessment of cell viability. The trypan blue dye exclusion test was used to assess cell viability. In all experiments, the viability was found to be > 95% at each point measured (data not shown).

Transendothelial migration assay. Transendothelial migration activity was assessed using a kind of Boyden-chamber assay as described[23] with modifications. Human umbilical vein endothelial cells (HUVEC) obtained from ATCC were precultured to make a monolayer sheet on Transwell cell culture inserts with 3.0 μm pore size (Corning Costar, Cambridge, MA, USA) for 48 h. RXM was first dissolved in DMSO and further diluted in the assay medium consisting of RPMI-1640 and 0.6% bovine serum albumin, then added to culture plates in a final volume of 600 μl (the lower chamber) just before the migration assay. PHA-activated T cells (1×10^6 cells/well) were added to each insert in a volume of 200 μl simultaneously with the same concentration of RXM as in the corresponding culture wells (the upper chamber). Spontaneous migration (chemokinesis) assay was performed at 37°C for 8 h in the presence or absence of RXM, then harvested and counted by flow cytometry (FACS Calibur, Nippon Becton-Dickinson, Tokyo, Japan) for 1 min.

Induction of CIA. Male DBA/1J mice were purchased from Japan Charles River Breeding Laboratories (Tokyo, Japan). Bovine type II collagen (Collagen Research Center, Tokyo, Japan) was dissolved at 4 mg/ml in 0.05 M acetic acid and then emulsified with an equal volume of complete Freund's adjuvant (Difco). For the primary immunization, 100 μl of the immunogen were injected intradermally into 8-week-old mice at the tail base. After 3 weeks, the mice received the same dose of immunogen subcutaneously. Arthritis developed within 10 days of the second immunization. These mice were kept under specific pathogen-free conditions in a clean room at the Animal Research Center, Institute of Medical Science, University of Tokyo.

Assessment of CIA disease severity. After physical examination, legs were scored as follows: 0, normal; 1, erythema and mild swelling confined to the ankle joint or toes; 2, erythema and mild swelling extending from the ankle to the midfoot; 3, erythema and severe swelling extending from ankle to the metatarsal joints; 4, ankylosing deformation with joint swelling[24]. The disease score for each mouse was calculated as the sum of the scores for the 2 hind legs.

Oral roxithromycin for CIA mice. RXM was dissolved in 5% arabic gum in 0.9% NaCl, and different doses of RXM (100, 200, 400, and 800 μg) were orally given to 5 different groups comprising 8 mice in each group. The 5% arabic gum, 0.9% NaCl combination alone was also given to the control mouse group orally. RXM or 5% arabic gum in 0.9% NaCl was given orally to mice every day up to Day 14 after second immunization of type II collagen.

ELISA of cytokines and type II collagen antibody levels in CIA mice. Serum samples from CIA mice were collected on the day of the first and second immunizations and Days 7, 14, and 21 after the second immunization. IL-6, TNF-α, IL-4, and IFN-γ levels were assayed by ELISA. Type II collagen antibody levels were assayed by ELISA (Chondrex, Redmond, WA, USA). Comparison of antibody levels was performed at 490 nm optical density.

Histology. Mice were euthanized by CO_2 asphyxiation and hind paws taken from CIA mice 3 weeks after the second immunization were fixed in 10% phosphate-buffered formalin (pH 7.4), decalcified in 10% EDTA, and embedded in paraffin. Sections (4 μm) were stained with hematoxylin and eosin.

Statistical analysis. Statistical analysis was performed by the 2-tailed Student's t test for all the assays (^{3}H-thymidine incorporation assay, ELISA, and transendothelial migration assay). Statistical differences of ankle width and paw width of CIA mice were assessed by Student t test, and the disease score was evaluated by Mann-Whitney U test.

RESULTS

Effect of roxithromycin on T cell proliferation through different costimulatory pathways. As shown in Figure 1, CD3 stimulation alone resulted in the induction of low levels of T cell proliferation. Marked T cell proliferation was observed with CD3 stimulation in combination with an additional second signal, such as anti-CD26 Mab, anti-CD28 Mab, or PMA. Under these conditions, RXM did not inhibit T cell proliferation from different donors at virtually any concentration tested (1.4 to 28 μM). It should be noted that at higher concentration (28 μM), only slight inhibition of T cell proliferation was observed in certain donors.

Effect of roxithromycin on Th1-type and Th2-type cytokine production through different costimulatory pathways. As shown in Figure 2A, RXM, even at 28 μM, did not inhibit IL-2 production under all costimulatory conditions. In addition to IL-2, RXM did not show any apparent effect on the production of IFN-γ at any concentrations tested (1.4 to 28 μM; Figure 2A).

Since Th2-type CD4+ T cells may play a role in allergic disorders such as asthma[25,26], we next examined the effect of RXM on Th2-type cytokine production. As shown in Figure 2A, RXM did not inhibit IL-4 and IL-5 production under each costimulatory condition at any tested doses (1.4 to 28 μM). Our results therefore indicated that RXM did not inhibit Th1-type and Th2-type cytokine production in our experimental systems.

Effect of roxithromycin on proinflammatory cytokine production through different costimulatory pathways. We next examined the effect of RXM on the production of proinflammatory cytokines. As shown in Figure 2B, production of the proinflammatory cytokines IL-6 and TNF-α was significantly inhibited by RXM in a dose-dependent manner under each costimulatory condition. Therefore RXM inhibited proinflammatory cytokine productions by T cells stimulated by our costimulatory conditions.

Effect of RXM on proinflammatory cytokine production by macrophages. Since macrophages play a role in host defense against infection and in the local modulation of immune and inflammatory responses[27], we also examined the effect of RXM on the production of proinflammatory cytokines by macrophages. The preliminary time-course

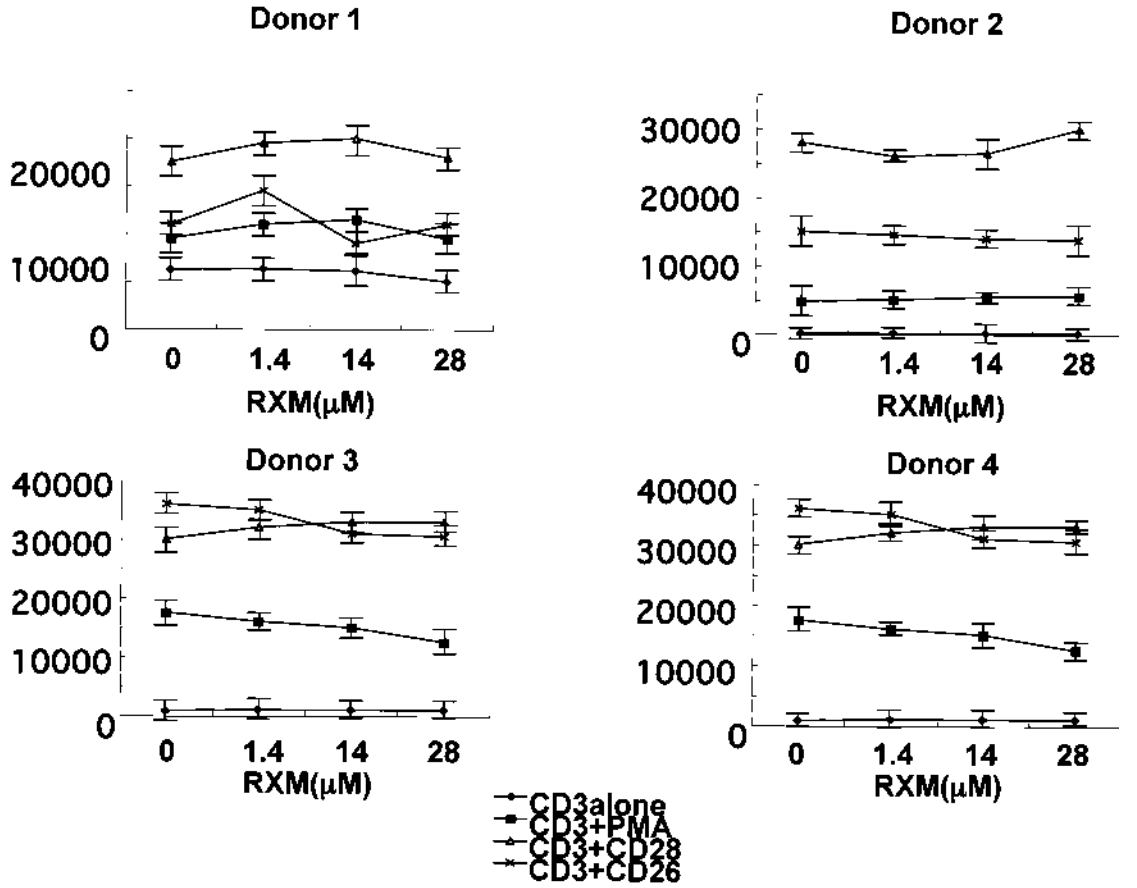

Figure 1. Effect of RXM on the proliferative response of peripheral T cells stimulated with anti-CD3 Mab alone, anti-CD3 Mab plus PMA, anti-CD3 plus anti-CD28 (4B10) Mab, and anti-CD3 plus anti-CD26 (1F7) Mab. Counts per minute (cpm) value in the case of nonstimulated T cells was near background level (data not shown). Mean cpm values ± SD from triplicate samples from 4 different donors are shown. RXM did not significantly inhibit proliferative response of T cells induced by the stimuli described above.

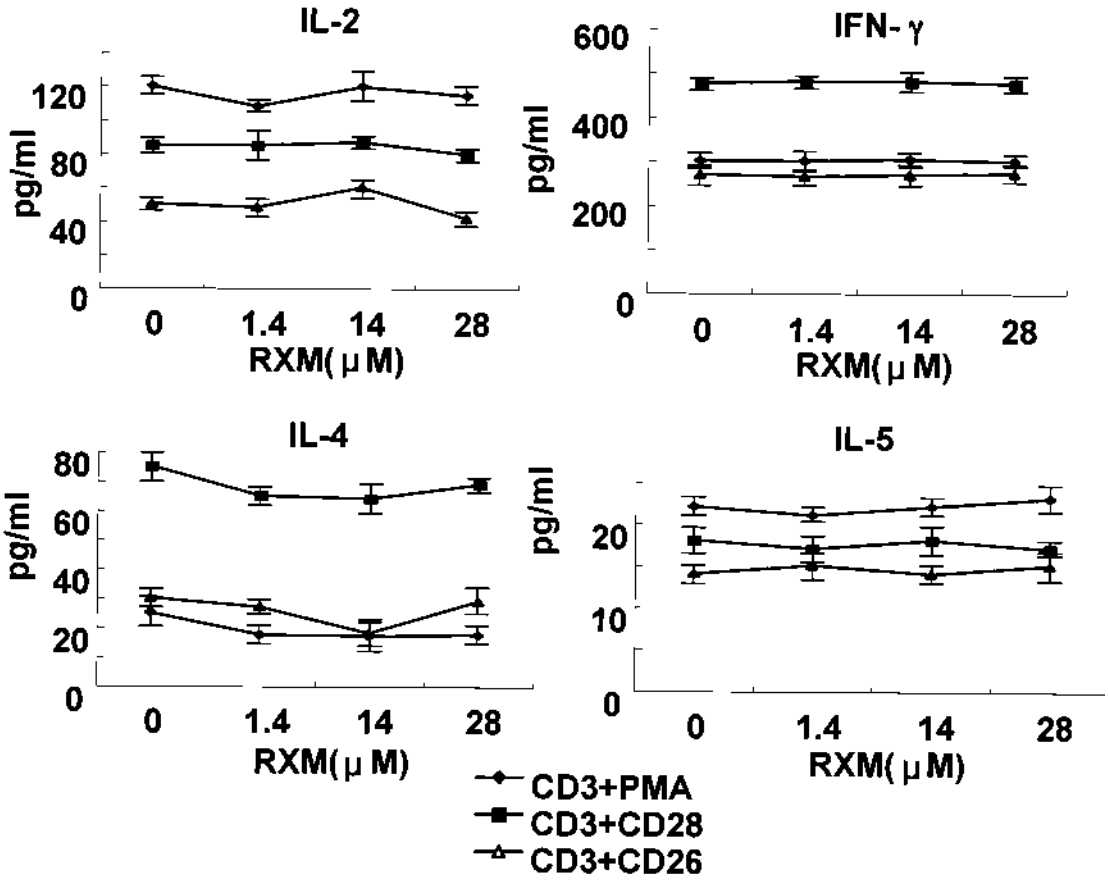

Figure 2A. Effect of RXM on peripheral T cell production of IL-2, IFN-γ, IL-4, and IL-5. T cells were stimulated with anti-CD3 plus PMA, CD3 plus CD28, and anti-CD3 plus anti-CD26 for 24 h, and culture supernatants were assayed by ELISA for cytokine levels shown here. Cytokine levels of T cells stimulated with anti-CD3 alone were always the background level (data not shown). Mean values ± SD from triplicate samples are shown; data are representative of 3 independent experiments.

experiment showed that 8 hours after stimulation was optimal for LPS-stimulated proinflammatory cytokine production by macrophages. As shown in Figure 2C, RXM inhibited both IL-6 and TNF-α production by macrophages in a dose-dependent manner.

Effect of RXM on transendothelial migration of activated T cells. We next examined its effect on transendothelial migration of PHA-stimulated T cells. These preactivated T cells spontaneously migrate from the upper chamber to the lower chamber through the endothelial monolayer, which is therefore regarded as chemokinesis. As shown in Figure 3, T cell migration was significantly inhibited in a range from 14 to 28 μM in a dose-dependent manner when RXM was present during the endothelial migration assay ($p < 0.05$). Compared to proinflammatory cytokine production by T cells and macrophages, RXM at dose of 1.4 μM did not inhibit migration of T cells from 5 different donors; but from 14 to 28 μM RXM, T cell migration was always inhibited. In contrast, when we pretreated HUVEC with various concentrations of RXM for 48 hours, and then HUVEC were washed and exposed to PHA-stimulated T cells, RXM did not affect the migration of T cells through HUVEC even at the highest concentration tested (28 μM; data not shown).

Effect of RXM therapy on the development of CIA. Finally, we investigated whether RXM affects the pathophysiology of CIA. Oral treatment of RXM was started after the second immunization of type II collagen, and daily treatment of RXM was continued up to Day 14. As shown in Figure 4, disease scores were suppressed in a dose-dependent manner after 7 days of treatment. In mice treated with RXM at a dose of 100, 200, 400, and 800 μg and control mice, statistically significant differences in disease score suppression were observed ($p < 0.05$ and $p < 0.01$). It should be noted that in the groups of mice treated with RXM 400 μg/day and 800 μg/day, disease scores were markedly inhibited, but the differences in the disease scores between the 2 groups did not reach statistical significance differences after 14 days of treatment. These results therefore indicate that RXM treatment inhibited the development of CIA.

Effect of RXM treatment on serum levels of IL-6, TNF-α, IL-4, and IFN-γ and type II collagen antibody levels. Since IL-6 and TNF-α appear to be involved in the development of

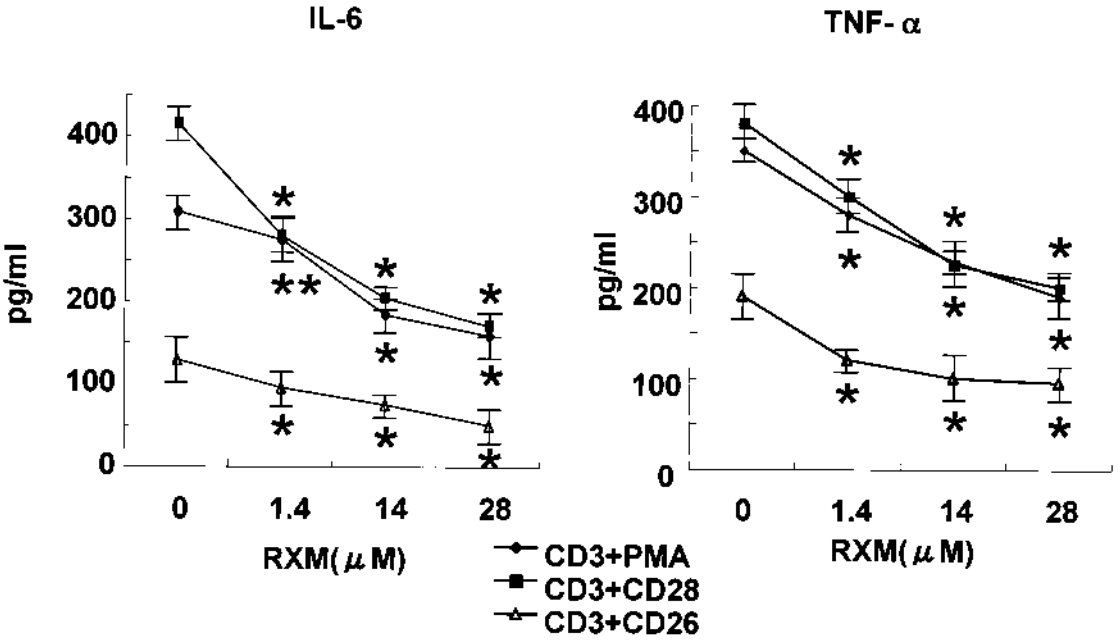

Figure 2B. Effect of RXM on peripheral T cell production of IL-6 and TNF-α. T cells were stimulated by the same conditions as in (A), and culture supernatants were assayed by ELISA for IL-6 and TNF-α. Cytokine levels of T cells stimulated with anti-CD3 alone were always the background level (data not shown). Mean values ± SD from triplicate samples are shown; data are representative of 3 independent experiments. *p <0.01, **p < 0.05 between 0 and 1.4 μM, 0 and 14 μM, 0 and 28 μM RXM; 2-tailed Student t test.

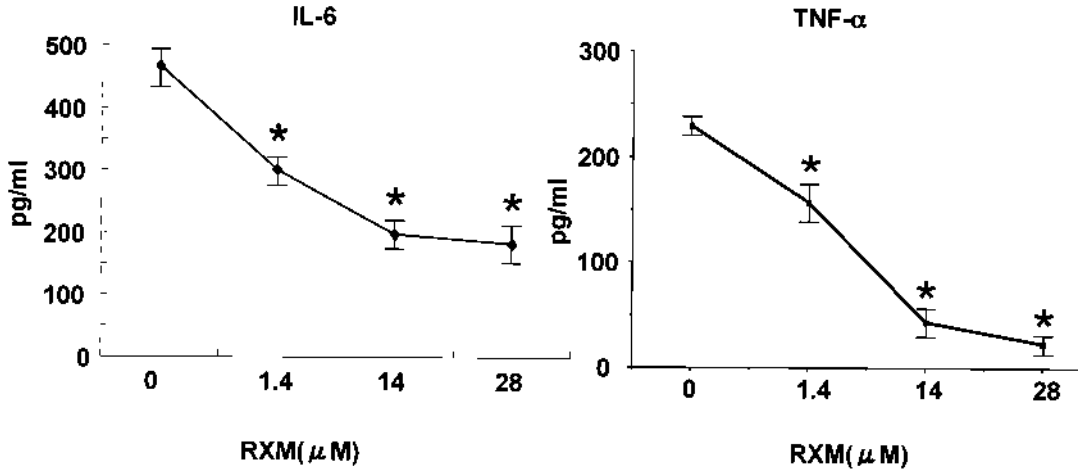

Figure 2C. Effect of RXM on production of IL-6 and TNF-α by macrophages stimulated with LPS (1 μg/ml) for 8 h; culture supernatants were assayed by ELISA for IL-6 and TNF-α. Mean values ± SD from triplicate samples are shown; data are representative of 3 independent experiments. *p <0.01 between 0 and 1.4 μM, 0 and 14 μM, 0 and 28 μM RXM; 2-tailed Student t test.

CIA[28], we examined the serum levels of those proinflammatory cytokines as well as IFN-γ and IL-4 in CIA mice on Days 0, 7, 14, and 21 after RXM treatment. While treatment of RXM did not affect the serum level of IFN-γ (Figure 5A), serum IL-6 levels increased in control CIA mice on Day 7, and then decreased to an undetectable level by Day 14. In contrast, in RXM-treated CIA mice, serum IL-6 levels were reduced on Day 7 in a dose-dependent manner. Particularly in CIA mice treated with 400 μg and 800 μg RXM, serum IL-6 levels were significantly reduced on Day 7 ($p < 0.05$; Figure 5B).

Serum IL-4 and TNF-α levels could not be detected in these groups of CIA mice due to the low degree of sensitivity of the available detection kits. Regarding type II collagen antibody levels (Figure 5C), a type II collagen antibody was detected after Day 7 of the second immunization of collagen, but RXM treatment did not affect the serum titer of this antibody. Thus we concluded that RXM treatment, particu-

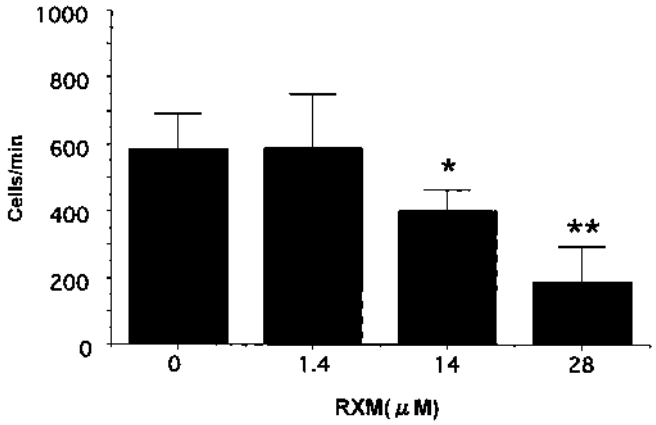

Figure 3. Inhibitory effect of RXM on transendothelial migration (chemokinesis) of PHA-activated T cells, as described in Materials and Methods. Bars show mean values ± SD of triplicate cultures. *p < 0.05 between 0 and 14 μM RXM, **p < 0.01 between 0 and 28 μM RXM; 2-tailed Student t test. Data are representative of samples from 5 different donors.

larly at the higher dose levels 400 μg and 800 μg, significantly inhibited the production of IL-6 in the serum of CIA mice.

RXM inhibition of leukocyte migration and bone destruction in affected joints of CIA mice. Histological analysis of inflamed joints and synovial tissue from those mice revealed large numbers of cells of leukocyte origin, synovial membrane proliferation, and pannus formation as well as destruction of bone and cartilage in control CIA mice not treated with RXM (Figure 6). On the other hand, in CIA mice treated with 200 and 800 μg RXM per day, the infiltration of leukocytes, cartilage and bone destruction, and pannus formation were strongly suppressed in a dose-dependent manner (Figure 6). Especially in 800 μg RXM-treated CIA mice, no cartilage and bone destruction, pannus formation, and synovial membrane proliferation was observed. These results indicated that RXM inhibited leukocyte migration as well as cartilage and bone destruction.

DISCUSSION

We demonstrated that RXM clearly inhibited the production of the proinflammatory cytokines TNF-α and IL-6 by activated T cells and macrophages. In addition, RXM inhibited T cell migration. Most importantly, RXM treatment of CIA mice inhibited the development of CIA, serum IL-6 levels, the migration of leukocytes into affected joints, and the destruction of bone and cartilage.

Previous studies showed that RXM could not inhibit concanavalin A (ConA)-induced T cell proliferation, but could inhibit ConA-induced IL-2 and IL-4 production by T cells[15]. Moreover, the same investigators reported that RXM inhibited production of Th2-type cytokine IL-4 and IL-5 but not Th1-type cytokine IL-2 and IFN-γ by T cells stimulated with the same costimulatory stimuli used in our study[29]. Although the precise reasons for the discrepancy between our findings and their data are unclear, it may be due to differences in the methods used for stimulation. Moreover, other researchers reported that other macrolides such as midecamycin, clarithromycin, and josamycin inhibited production of both Th1-type and Th2-type cytokines, such as IL-2, IL-4, and IL-5, by ConA-stimulated T cells[30]. Meanwhile, erythromycin and RXM inhibited TNF-α pro-

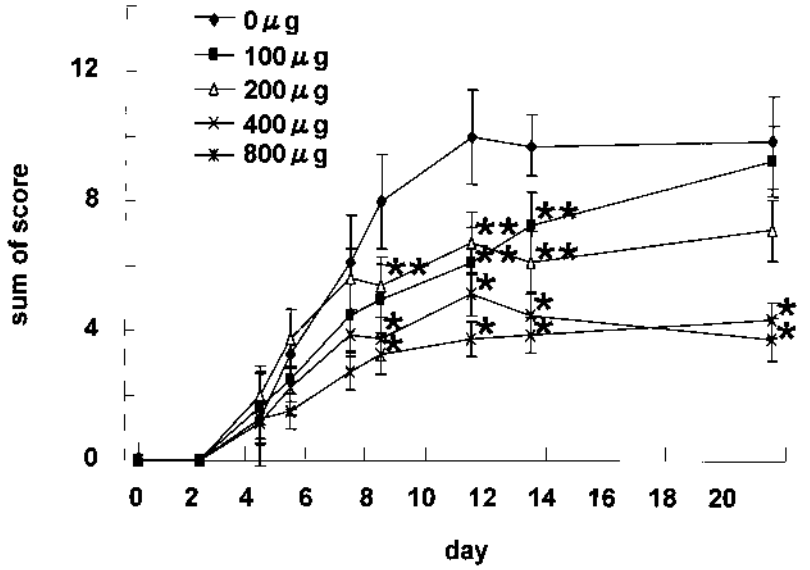

Figure 4. Effect of RXM therapy on development of CIA. Disease scores of CIA mice measured from Day 0 to Day 21 after the second immunization. Sums of scores are shown for 4 different doses of RXM (100, 200, 400, 800 μg; n = 8 for each group) and control group (n = 8). Mean values ± SD from 8 mice are plotted. *p < 0.01, **p < 0.05 between 0 and 100, 200, 400, 800 μg/day; 2-tailed Student t test.

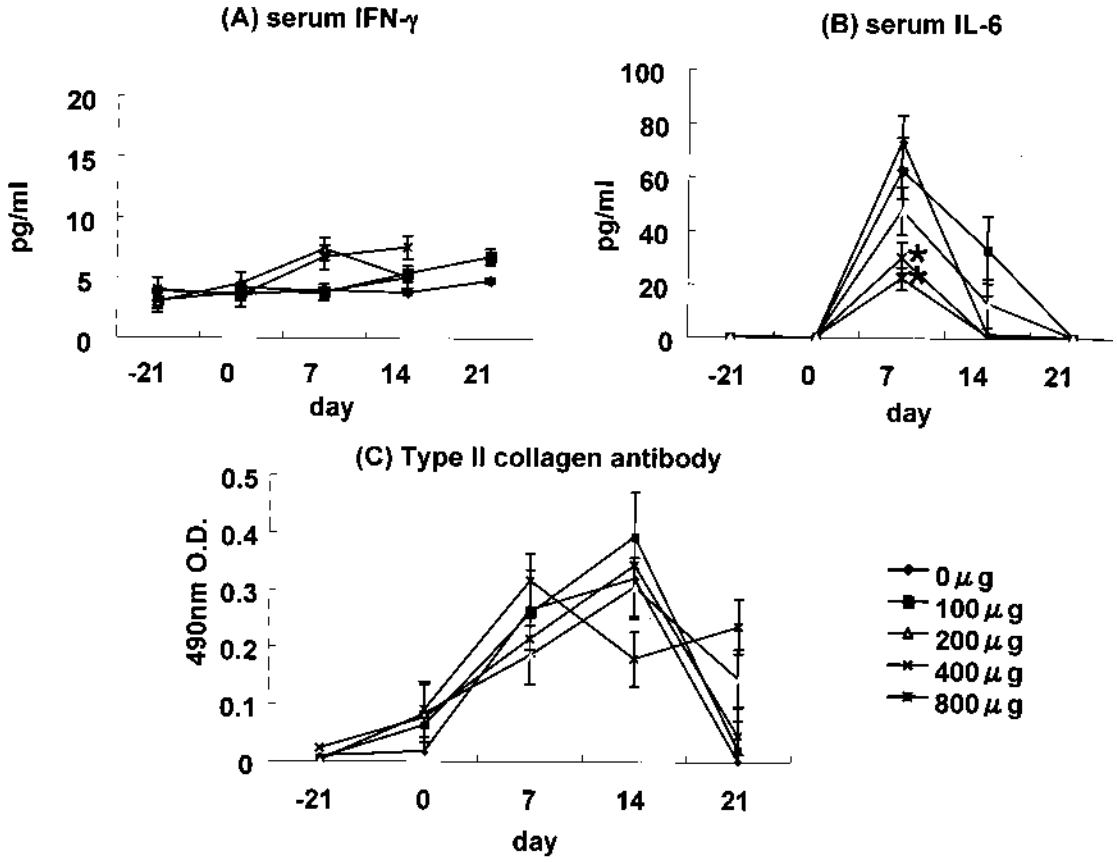

Figure 5. Effect of RXM treatment on serum levels of IFN-γ, IL-6, and type II collagen antibody in CIA mice, as described in Materials and Methods. Serum levels were plotted from 4 different doses of RXM (100, 200, 400, 800 μg; n = 8 for each group) and controls (n = 8). Mean values ± SD from 8 mice were plotted. IL-4 and TNF-α levels were always below background levels (data not shown). *$p < 0.05$, 0 versus 400 and 800 μg/day; 2-tailed Student t test.

duction by macrophages stimulated with LPS[31,32]. Recently, Guchelaar, *et al* reported that erythromycin inhibited TNF-α and IL-6 production induced by heat-killed *Streptococcus pneumoniae* in human whole blood *ex vivo*[33].

Systemic administration of macrolide antibiotics (erythromycin and RXM) has been shown to be effective in the treatment of lower and upper airway inflammatory diseases such as bronchial asthma and diffuse panbronchiolitis[2,32-34]. Although these inflammatory diseases have been reported to be successfully treated with low dose administration of macrolide antibiotics, which cannot be expected to act as antibacterial agents, the precise mechanisms of action to explain the clinical effectiveness of this therapy are not well understood. It was recently reported that bronchial asthma is a T cell-mediated inflammatory disorder, and selective recruitment of CD4+ T cells into sites of inflammation may contribute to the development of different pathological conditions[35,36]. Current studies suggest that TNF-α may potentially be involved in the development of bronchial hyperresponsiveness by directly altering the contractile properties of the airway smooth muscle[37,38]. As a regulator of IgE synthesis, increased levels of IL-6 have been detected in blood and bronchoalveolar lavage after bronchial challenge of patients with asthma, while bronchial biopsies of these patients reveal increased expression of IL-6[39]. Thus, inhibition of TNF-α and IL-6 production by T cells and macrophages as well as inhibition of T cell migration by RXM may also have important therapeutic implications for bronchial asthma.

In both CIA and RA, joint inflammation and cartilage and bone destruction depend on the concentrations of TNF-α and IL-6 in affected joints[28,40]. At the site of inflammation in the affected RA synovium, infiltration of leukocytes, especially T cells from blood vessels, is the initial step necessary for the development of the RA lesion. The *in vivo* effectiveness of RXM in preventing CIA development is likely linked to the ability of RXM to inhibit T cell migration and proinflammatory cytokine production by T cells and macrophages *in vitro*.

Based on the pharmacokinetics data in rats, oral administration of RXM at 5 mg/kg, which corresponds to 100 μg/mouse, resulted in the maximum plasma concentration around 1.9 μg/ml (2.3 μM). The usual dose in humans, 300 mg/adult, resulted in the maximum plasma concentration

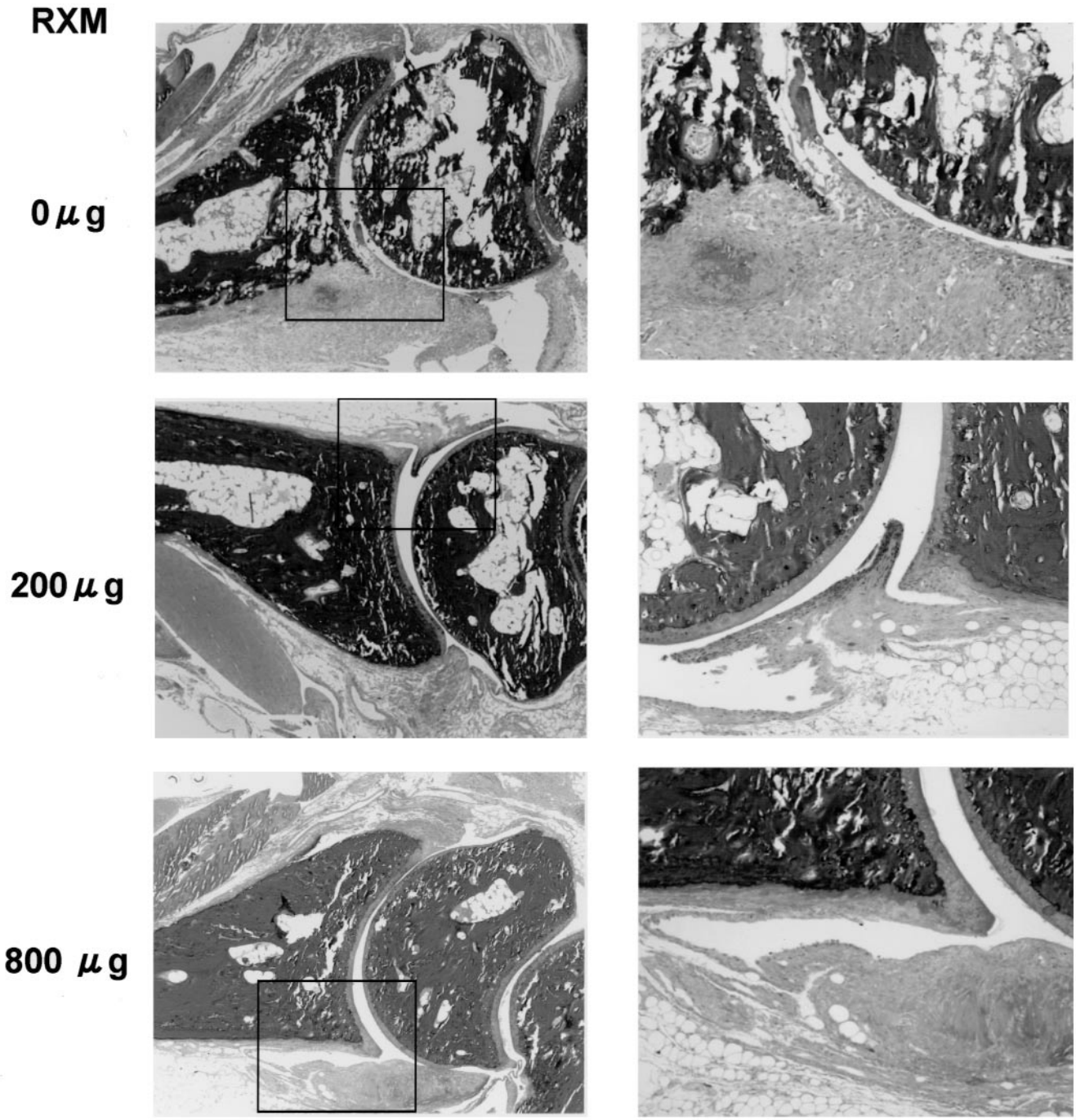

Figure 6. Ankle joints of CIA mice were collected on Day 21 after the second immunization of collagen. Mice had been treated with 0, 200, and 800 μg RXM. H&E stain; original magnification ×40 (left panels) and ×100 (right panels).

around 8.1 μg/ml (9.6 μM). Therefore, the *in vivo* concentration of RXM may correspond to the concentration that inhibits immune/inflammatory phenomena *in vitro*. Further, the doses employed in our *in vitro* and *in vivo* experiments correspond to the human daily dose.

Advances in understanding the pathogenesis of RA based on studies of human tissues and animal models of disease have led to identification of molecular targets for immunotherapeutic intervention. Of these, TNF-α has been validated as an appropriate target for treatment, and to date 2 biological agents that target TNF-α have been licensed for clinical use[40]. These are infliximab, an anti-TNF-α Mab[41], and etanercept, an engineered p75 TNF receptor dimer linked to the Fc portion of human IgG1[42]. Therapies inhibiting TNF-α in patients with active RA result in rapid and sustained improvement in symptoms and signs of disease, improve-

ment in the quality of life, and protection of joints from structural damage[40,43]. Moreover, anti-TNF-α treatment has been reported to be effective in Crohn's disease[44]. IL-6 regulates the production of acute-phase proteins by hepatocytes and activates osteoclasts to absorb bone[45,46]. In a preclinical study, a humanized anti-IL-6R Mab has been used to treat patients with severe RA, and clinical improvements have been reported[47].

Besides bronchial asthma, RA, and Crohn's disease, RXM may also be useful for the treatment of disorders in which TNF-α and IL-6 may play a role in pathophysiology, such as graft versus host disease (GVHD) following allogeneic bone marrow transplant[48], heart failure[49], and Castleman's disease[50].

ACKNOWLEDGMENT

The authors thank Fumiki Nojima for excellent secretarial assistance.

REFERENCES

1. Nelson S, Summer WR, Terry PB, Warr GA, Jakab GJ. Erythromycin-induced suppression of pulmonary antibacterial defenses. A potential mechanism of superinfection. Am Rev Respir Dis 1987;136:1207-12.
2. Kadota J, Sakito O, Kohno S, et al. A mechanism of erythromycin treatment on patients with diffuse panbronchiolitis. Am Rev Respir Dis 1993;147:153-9.
3. Miyatake H, Suzuki K, Taki F, Takagi K, Satake T. Effect of erythromycin on bronchial hyperresponsiveness in patients with bronchial asthma. Arzneimittelforschung 1991;41:552-6.
4. Yokota T, Suzuki E, Arai K. Cefpodoxime proxetil, its in vitro antibacterial activity, affinity to bacterial penicillin-binding proteins, and synergy of bactericidal activity with serum complement and mouse-cultured macrophages. Drugs Exp Clin Res 1988;14:495-500.
5. Fraschini F, Scaglione F, Ferrara F, Marelli O, Braga PC, Teodori F. Evaluation of the immunostimulating activity of erythromycin in man. Chemotherapy 1986;32:286-90.
6. Mikasa K, Sawaki M, Konishi M, et al. The effect of erythromycin treatment on natural killer cell activity in patients with chronic lower respiratory tract infections. Kansenshogaku Zasshi 1989;63:811-5.
7. Ras GJ, Anderson R, Eftychis HA, et al. Chemoprophylaxis with erythromycin stearate or amoxycillin in patients with chronic bronchitis — effects on cellular and humoral immune functions. S Afr Med J 1984;66:955-8.
8. Aho P, Mannisto PT. Effects of two erythromycins, doxycycline and phenoxymethylpenicillin, on human leucocyte chemotaxis in vitro. J Antimicrob Chemother 1988;21:29-32.
9. Anderson R. Erythromycin and roxithromycin potentiate human neutrophil locomotion in vitro by inhibition of leukoattractant-activated superoxide generation and autooxidation. J Infect Dis 1989;159:966-73.
10. Yoshimura T, Kurita C, Yamazaki F, et al. Effect of roxithromycin on proliferation of peripheral blood mononuclear cells and production of lipopolysaccharide-induced cytokines. Biol Pharm Bull 1995;18:876-81.
11. Young RA, Gonzalez JP, Sorkin EM. Roxithromycin. A review of its antibacterial activity, pharmacokinetic properties and clinical efficacy. Drugs 1989;37:8-41.
12. Noma T, Hayashi M, Yoshizawa I, Aoki K, Shikishima Y, Kawano Y. A comparative investigation of the restorative effects of roxithromycin on neutrophil activities. Int J Immunopharmacol 1998;20:615-24.
13. Wakita H, Tokura Y, Furukawa F, Takigawa M. The macrolide antibiotic roxithromycin suppresses IFN-gamma-mediated immunological functions of cultured normal human keratinocytes. Biol Pharm Bull 1996;19:224-7.
14. Konno S, Adachi M, Asano K, Okamoto K, Takahashi T. Inhibition of human T-lymphocyte activation by macrolide antibiotic roxithromycin. Life Sci 1992;51:L231-6.
15. Konno S, Asano K, Kurokawa M, Ikeda K, Okamoto K, Adachi M. Antiasthmatic activity of a macrolide antibiotic, roxithromycin: analysis of possible mechanisms in vitro and in vivo. Int Arch Allergy Immunol 1994;105:308-16.
16. Robey E, Allison JP. T-cell activation: integration of signals from the antigen receptor and costimulatory molecules. Immunol Today 1995;16:306-10.
17. Green JM, Noel PJ, Sperling AI, et al. Absence of B-7 dependent responses in CD28-deficient mice. Immunity 1994;1:501-8.
18. Gimmi CD, Freeman GJ, Gribben JG, et al. B-cell surface antigen B7 provides a costimulatory signal that induces T cells to proliferate and secrete interleukin 2. Proc Natl Acad Sci USA 1991;88:6575-9.
19. Morimoto C, Torimoto Y, Levinson G, et al. 1F7, a novel cell surface molecule, involved in helper function of CD4 cells. J Immunol 1989;143:3430-9.
20. Dang NH, Torimoto Y, Deusch K, Schlossman SF, Morimoto C. Comitogenic effect of solid-phase immobilized anti-1F7 on human CD4 T cell activation via CD3 and CD2 pathways. J Immunol 1990;144:4092-100.
21. Tanaka T, Kameoka J, Yaron A, Schlossman SF, Morimoto C. The costimulatory activity of the CD26 antigen requires dipeptidyl peptidase IV enzymatic activity. Proc Natl Acad Sci USA 1993;90:4586-90.
22. Hafler DA, Fox DA, Manning ME, Schlossman SF, Reinherz EL, Weiner HL. In vivo activated T lymphocytes in the peripheral blood and cerebrospinal fluid of patients with multiple sclerosis. N Engl J Med 1985;312:1405-11.
23. Iwata S, Yamaguchi N, Munakata Y, et al. CD26/dipeptidyl peptidase IV differentially regulates the chemotaxis of T cells and monocytes toward RANTES: possible mechanism for the switch from innate to acquired immune response. Int Immunol 1999;11:417-26.
24. Rosloniec EF, Cremer M, Kang A, Myers LK. Collagen-induced arthritis. In: Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W, editors. Current protocols in immunology. New York: Wiley; 1996:15.5.1-24.
25. Kapsenberg ML, Wierenga EA, Bos JD, Jansen HM. Functional subsets of allergen-reactive human CD4+ T cells. Immunol Today 1991;12:392-5.
26. Romagnani S. Lymphokine production by human T cells in disease states. Annu Rev Immunol 1994;12:227-57.
27. Johnston RB Jr. Current concepts: immunology. Monocytes and macrophages. N Engl J Med 1988;318:747-52.
28. Marinova-Mutafchieva L, Williams RO, Mason LJ, Mauri C, Feldmann M, Maini RN. Dynamics of proinflammatory cytokine expression in the joints of mice with collagen-induced arthritis. Clin Exp Immunol 1997;107:507-12.
29. Asano K, Kamakazu K, Hisamitsu T, Suzaki H. Modulation of Th2 type cytokine production from human peripheral blood leukocytes by a macrolide antibiotic, roxithromycin, in vitro. Int Immunopharmacol 2001;11:1913-21.
30. Morikawa K, Zhang J, Nonaka M, Morikawa S. Modulatory effect of macrolide antibiotics on the Th1- and Th2-type cytokine production. Int J Antimicrob Agents 2002;19:53-9.
31. Suzaki H, Asano K, Ohki S, Kanai K, Mizutani T, Hisamitsu T.

Suppressive activity of a macrolide antibiotic, roxithromycin, on pro-inflammatory cytokine production in vitro and in vivo. Mediators Inflamm 1999;8:199-204.
32. Iino Y, Toriyama M, Kudo K, Natori Y, Yuo A. Erythromycin inhibition of lipopolysaccharide-stimulated tumor necrosis factor alpha production by human monocytes in vitro. Ann Otol Rhinol Laryngol Suppl 1992;157:16-20.
33. Guchelaar HJ, Schultz MJ, van der Poll T, Koopmans RP. Pharmacokinetic-pharmacodynamic modeling of the inhibitory effect of erythromycin on tumour necrosis factor-alpha and interleukin-6 production. Fundam Clin Pharmacol 2002;15:419-24.
34. Itkin IH, Menzel ML. Pharmacokinetic-pharmacodynamic modeling of the inhibitory effect of erythromycin on tumour necrosis factor-alpha and interleukin-6 production. J Allergy 1970;45:146-62.
35. Robinson DS, Hamid Q, Ying S, et al. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. N Engl J Med 1992;326:298-304.
36. Ying S, Humbert M, Barkans J, et al. Expression of IL-4 and IL-5 mRNA and protein product by CD4+ and CD8+ T cells, eosinophils, and mast cells in bronchial biopsies obtained from atopic and nonatopic (intrinsic) asthmatics. J Immunol 1997;158:3539-44.
37. Baeuerle PA, Henkel T. Function and activation of NF-kappa B in the immune system. Annu Rev Immunol 1994;12:141-79.
38. Amrani Y, Chen H, Panettieri RA Jr. Activation of tumor necrosis factor receptor 1 in airway smooth muscle: a potential pathway that modulates bronchial hyper-responsiveness in asthma? Respir Res 2000;1:49-53.
39. Hirano T. Interleukin 6 and its receptor: ten years later. Int Rev Immunol 1998;16:249-84.
40. Harris ED Jr. Rheumatoid arthritis. Pathophysiology and implications for therapy. N Engl J Med 1990;322:1277-89.
41. Knight DM, Trinh H, Le J, et al. Construction and initial characterization of a mouse-human chimeric anti-TNF antibody. Mol Immunol 1993;30:1443-53.
42. Weinblatt ME, Kremer JM, Bankhurst AD, et al. A trial of etanercept, a recombinant tumor necrosis factor receptor:Fc fusion protein, in patients with rheumatoid arthritis receiving methotrexate. N Engl J Med 1999;340:253-9.
43. Lipsky PE, van der Heijde DM, St. Clair EW, et al. Infliximab and methotrexate in the treatment of rheumatoid arthritis. Anti-Tumor Necrosis Factor Trial in Rheumatoid Arthritis with Concomitant Therapy Study Group. N Engl J Med 2000;343:1594-602.
44. Kam LY, Targan SR. TNF-alpha antagonists for the treatment of Crohn's disease. Expert Opin Pharmacother 2000;1:615-22.
45. Castell JV, Gomez-Lechon MJ, David M, Hirano T, Kishimoto T, Heinrich PC. Recombinant human interleukin-6 (IL-6/BSF-2/HSF) regulates the synthesis of acute phase proteins in human hepatocytes. FEBS Lett 1988;232:347-50.
46. Tamura T, Udagawa N, Takahashi N, et al. Soluble interleukin-6 receptor triggers osteoclast formation by interleukin 6. Proc Natl Acad Sci USA 1993;90:11924-8.
47. Yoshizaki K, Nishimoto N, Mihara M, Kishimoto T. Therapy of rheumatoid arthritis by blocking IL-6 signal transduction with a humanized anti-IL-6 receptor antibody. Springer Semin Immunopathol 1998;20:247-59.
48. Jacobsohn DA. Novel therapeutics for the treatment of graft-versus-host disease. Expert Opin Investig Drugs 2002;11:1271-80.
49. Negrusz-Kawecka, M. The role of TNF-alpha in the etiopathogenesis of heart failure. Pol Merkuriusz Lek 2002;12:69-72.
50. Katsume A, Saito H, Yamada Y, et al. Anti-interleukin 6 (IL-6) receptor antibody suppress Castleman's disease-like symptoms emerged in IL-6 transgenic mice. Cytokine 2002;21:304-11.