LY309887, antifolate via the folate receptor suppresses murine type II collagen-induced arthritis

R. Nagayoshi¹, M. Nakamura², K. Ijiri³, H. Yoshida⁴, S. Komiya¹, T. Matsuyama²

¹Department of Orthopaedic Surgery, ²Department of Immunology, ³Department of Physiotherapy, and ⁴First Department of Pathology, School of Medicine, Kagoshima University, Sakuragaoka, Kagoshima City, Japan.

Abstract
Objective
To examine the effect of LY309887, an inhibitor of glycaminamide ribonucleotide formyltransferase in de novo purine biosynthesis on murine type collagen-induced arthritis (CIA).

Methods
CIA was induced by immunization with bovine type II collagen in adjuvant. The expression of folate receptors was examined in dissected synovial tissues and bone marrow cells from arthritic and non-arthritic mice by the semi-quantitative reverse transcription-polymerase chain reaction. LY309887 was administered to CIA mice after the onset of arthritis. Mice were monitored for arthritis index for 21 days. Levels of IgG₁ and IgG₂a antibodies against bovine type II collagen were measured in sera from CIA mice with or without LY309887 treatment by the enzyme-linked immunosorbent assay. Histologic analyses were also performed in synovial tissues from arthritic joints with or without LY309887 treatment.

Results
Levels of mRNA of folate receptor beta (FR-β) were elevated in arthritic joints from CIA mice, compared with those in nonarthritic joints. The expression of mRNA of FR-β was dominant in bone marrow cells of CIA mice. The administration of LY309887 suppressed the disease progression of CIA mice as defined by the lower arthritis index, and decreased production of serum IgG₁ and IgG₂a anti-type II collagen antibody, and the damage to cartilage or bone.

Conclusion
The administration of LY309887 was effective on CIA mice. It was suggested that LY309887 might be useful in the treatment of rheumatoid arthritis.

Key words
Antifolate, folate receptor, collagen type II, arthritis.
LY309887 suppresses collagen-induced arthritis / R. Nagayoshi et al.

Ryusaku Nagayoshi, MD; Setsuro Komiya, MD, PhD; Motoyuki Nakamura, PhD; Takami Matsuyama, MD, PhD; Kosei Ijiri, MD, PhD; Hiroki Yoshida, MD, PhD.

This work was supported by grants from the Japanese Ministry of Culture, Education, and Science.

Please address correspondence and reprint requests to: Dr. T. Matsuyama, Department of Immunology, School of Medicine, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima City, Japan 890-8520. E-mail: matuyama@m.kufm.kagoshima-u.ac.jp.

Received on March 31, 2003; accepted in revised form on July 18, 2003. © Copyright CLINICAL AND EXPERIMENTAL RHEUMATOLOGY 2003.

Introduction

It has been known that oxidized folates such as folic acid are transported via folate receptors (FRs), while reduced folates such as 5-methyl tetrahydrofolates and methotrexate are transported via reduced folate carrier (RFC) (1-3). At present, three folate receptors alpha (FR-α), beta (FR-β), gamma (FR-γ) are functionally identified in humans (4). FR-γ may not be functional as the membrane protein because it has an incomplete glycosylphosphatidyl inositol anchor portion. These receptors are expressed at low or undetectable levels in normal tissues and levels of their expression were elevated in epithelial and hematopoietic tumors. Thus, it is considered that folate derivatives with a high affinity for these receptors may become efficient drugs or be useful as drug delivery agents for the treatment of tumors (5, 6).

In rheumatoid arthritis (RA), the dominant cell type in the joints is the macrophage. Several reports showed a correlation between the number of macrophages present in the joint and severe cartilage destruction (7). In fact, selective removal of synovial macrophages after the onset of experimental arthritis downregulated synovial inflammation (8, 9). Previously, we showed that the expression of FR-β was selectively elevated in synovial macrophages from RA (10). Likewise, Turk et al. showed that folic acid was transported via the FRs, probably FR-β on activated macrophages in arthritic joints of mycoplasma-induced arthritic rat (11). These findings suggested to us that drugs with a high affinity for FR-β may selectively downregulate synovial inflammation mediated by synovial macrophages.

Recently, several reports showed the effectiveness of folate derivatives with a high affinity for FR-α on tumors in experimental models (12, 13). Among these drugs, LY309887 is a potent inhibitor of glycaminide ribonucleotide formyltransferase (GARFT) involved in de novo purine biosynthesis and it has a high affinity for FR-α and a significant affinity for FR-β. Most recently, it was reported that a phase I trial using LY309887 in humans was successfully performed (14).

In this study, we examined the expression of FRs in arthritic joints because there are no information about the expression of FRs in experimental arthritic joints. Furthermore, the effect of LY309887 on murine type II collagen-induced arthritis (CIA) was examined by means of clinical scores, serum levels of anti-type II collagen antibody, and histological analysis.

Materials and methods

Induction of arthritis

Seven-week-old male DBA/1J mice were purchased from Charles River Japan (Yokohama, Japan). The mice were housed in filter-topped cages, and water and a low-folate diet (0.02 mg%) (Dyets, Bethlehem, PA) were provided ad libitum. This study was carried out after permission was received from the Committee of Animal Experimentation, Faculty of Medicine, Kagoshima University.

Bovine type II collagen (C II) was prepared according to the method of Miller and Rhodes (15). Briefly, C II was diluted with 0.1 M acetic acid to a concentration of 4 mg/ml and was emulsified in an equal volume of Freund's incomplete adjuvant (IFA; Difco, Detroit, MI) containing H37Ra Mycobacterium tuberculosis (Difco). At the age of 9 weeks, the mice fed for at least two weeks with a low-folate diet were immunized intradermally at the base of the tail with 100 µl emulsion (200 µg collagen).

LY309887, a gift from Lilly Research Laboratories, Indianapolis, IN, was diluted to 2.5 µg/ml or 7.5 µg/ml in phosphate-buffered saline (PBS).

Treatment and evaluation of arthritis

Mice were randomly assigned to 1 of 3 groups after the onset of arthritis: the low dose or high dose treatments with LY309887, or control. In LY309887 treatment, mice were treated with an injection of 200 µl solution containing 0.5 µg or 1.5 µg of LY309887 intraperitoneally on days 1, 3, 5, 7, and 9.

Mouse paws were scored for arthritis as previously described (16). Briefly, a 4-point scale was used, where 0 = normal, 1 = slight swelling and erythema, 2 = pronounced edema, and 3 = joint rigidi-
ty. The cumulative score of all 4 paws of each mouse was used as the “arthritis index” (maximum score of 12 per mouse) to represent the overall disease severity and progression in an animal.

Analysis of tissue samples

Arthritic and non-arthritic mice were killed on day 21 after the onset of arthritis. The arthritic joints and the clinically non-arthritic joints were dissected and used for the preparation of total RNA. Additionally, the bone marrow from arthritic and non-arthritic mice was dissected and used for preparation of total RNA.

Analysis was carried using the reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated using TRIzol LS reagent (Life Technologies, Gaithersburg, MD). The first strand cDNA kit (SuperScript Preamplification System; Life Technologies) was used to make cDNA from 5 µg of each RNA. Amplification of each cDNA was performed with a AccuPower PCR Premix kit (Bioneer, Seoul, Korea). Polymerase chain reaction (PCR) was performed according to a standard protocol. Primers used were as follows: FR-α CAGGAAGCA-CATAAAGGACATT (upstream primer), TCACACCACATCTGTAATGCAG (downstream primer); FR-β CTCTTGT CTTTTGGTCACAG (upstream primer), GAAGTAGATCTCAACGGC-TGTG (downstream primer); RFC ACTAACAAGATCATTCCGATG (upstream primer), GATCTAGTTTGC TGGTTTCTG (downstream primer); GAPDH GGAGCCCAAACGGGTCAT CATC (upstream primer), ATGCTGCTCACCACCTCTTG (downstream primer). The message for CD163 and IL-1β was amplified using the primers described elsewhere (17, 18). The amplified PCR products for FR-α, FR-β, IL-1β, RFC, CD163, and GAPDH were 430 basepairs (bp), 501 bp, 166 bp, 535 bp, and 151 bp, and 454 bp, respectively. In preliminary experiments, we examined linearity of PCR amplification of FR-α, FR-β, IL-1β, RFC, CD163, and GAPDH cDNA by increasing the number of PCR cycles from 18 to 35. Data indicated that PCR amplification of FR-β cDNA reached a plateau by 33 cycles. Therefore, we used 30 cycles to determine the relative abundance of FR-β mRNA. In the same way, we used 26 cycles, 28 cycles, 27 cycles, 26 cycles, and 21 cycles to determine those of FR-α, IL-1β, RFC, CD163, and GAPDH, respectively.

We then measured anti-collagen type II antibody (anti-C II). Individual serum samples were collected at the end of the experiment by retro-orbital puncture. They were divided into aliquots and stored at -20ºC until they were used for the ELISA. Immunoplates (MaxiSorp; Nunc, Roskilde, Denmark) were coated overnight with 2 µg/ml of bovine type II collagen in PBS at 4ºC. Non-specific binding was blocked with 10% fetal bovine serum (FBS) in PBS for 30 minutes at 37ºC. Serum samples (diluted with 10% FBS-0.05% Tween 20 in PBS) were added and incubated for 2 hours at 37ºC. Serum dilutions were chosen after preliminary assays and ranged from 1:400 to 1:40,000 for IgG1 or IgG2 anti-C II.

The plates were then incubated with biotinylated rabbit anti-mouse IgG1 (Zymed, San Francisco, CA) or IgG2 (Santa Cruz Biotechnology, Santa Cruz, CA) for 1.5 hrs at 37ºC. Streptavidin-horseradish peroxidase (HRP; Zymed) was used as substrate, and the optical density (OD) at 405 nm was measured using a microplate reader (model 550; Bio-Rad, Hercules, CA). All incubations were carried out in a volume of 100 µl/well, and between steps the plates were washed 4 times with 200 µl 0.05% Tween 20 in PBS. The relationship of the optical density measured in standard serum diluted serially and arbitrary units showed a good linear correlation in all determinations (data not shown). The concentrations of IgG1 and IgG2 anti-C II in the sera diluted 1:4000 are presented as relative values (arbitrary units) compared with the optical density of the standard sera. Each plate was incubated with a standard curve of positive serum used to define arbitrary units of total IgG1 and IgG2 anti-C II Abs.

Hematology and histopathology

White blood cell counts were performed on samples isolated by orbital vein puncture on day 21. Blood hemoglobin concentrations were measured using a diagnostic kit (Hemoglobin Test Wako, Wako Pure Chemical Company, Ltd., Osaka, Japan). Arthritic hind paws from at least 4 mice from each group were collected at the end of the experiment, fixed with 10% buffered formalin, decalcified in 5% EDTA and then embedded in paraffin. Sagittal serial sections of whole hind paws (5 µm thickness) were cut and stained with hematoxylin and eosin for microscopic evaluation, which was performed in a blinded manner. To determine whether the treatment had an effect on the degree of joint inflammation, the ankle, the metatarsophalan-geal and proximal and distal interphalangeal joints were examined for the presence or absence of synovitis, pannus formation, cartilage loss, bone erosions, and disruption of the joint architecture.

Statistical analysis

The non-parametric Mann-Whitney U test was used to test for differences in clinical parameters among mice across the experimental groups. P ≤ 0.05 was accepted as significant. Data were analyzed using Stat-View 4.5 statistical software for Windows (SAS Institute, Cary, NC).

Results

Elevated levels of mRNA of FR-β in arthritic joints from CIA mice

We examined levels of mRNA of FR-α, FR-β, RFC, IL-1β and the macrophage-specific marker CD163 in the arthritic and non-arthritic joints of CIA mice by semi-quantitative RT-PCR analysis. As shown in Figure 1A, levels of mRNA of FR-β were elevated in arthritic joints compared to those in non-arthritic joints and paralleled those of IL-1β in both joints. In contrast the levels of mRNA of RFC and CD163 detected were similar between arthritic and non-arthritic joints. Levels of mRNA of FR-α were not detectable in either joints (data not shown). In human tissues, it was reported that the expression of mRNA of FR-β was observed in myeloid lineage cells of...
the bone marrow (19, 20). To determine the expression of mRNA of FR-β in murine bone marrow cells, we examined levels of mRNA of FRs in the bone marrow of CIA mice. The expression of mRNA of FR-β and RFC but no expression of mRNA of FR-α were observed in the bone marrow of CIA mice (Fig. 1B).

Suppression of clinical symptoms in CIA mice by administration of LY309887
Mice were fed a low-folate diet during the experiments to obtain low-folate concentrations similar to those of humans. Previously, it was shown that low-folate serum concentrations enhanced the effects of LY309887 (21). In the pharmacokinetics of LY309887 in humans, it was reported that LY309887 concentrations declined rapidly for 24 to 36 hours. Since the clearance of LY309887 in mice has been described to be close to that of humans, CIA mice were treated every other day. Preliminary experiments using our treatment protocol showed that the administration of 5 doses (1.5 µg/dose) every other day was safe and effective. CIA mice were treated with low (0.5 µg/dose) or high doses (1.5 µg/dose) of LY309887 and with PBS alone. As shown in Figure 2, on day 13 the high-dose treatment with LY309887 had significantly suppressed the development of clinical symptoms in CIA mice compared with the control group. In contrast, low-dose treatment with LY309887 resulted in only a modest effect on the clinical symptoms of CIA mice. Concerning the white blood cell count, serum hemoglobin concentration, and body weight, no significant differences were found among these groups (data not shown). Additionally, no side effects (for example, diarrhea or hematochezia) were observed in either group during this experimental period.

Decreased production of IgG₁ and IgG₂a anti-C II in CIA mice following the administration of LY309887
We next examined antibody titers of IgG₁ and IgG₂a anti-C II in serum samples from treated and non-treated mice on day 21 after the onset of arthritis. As shown in Figure 3, antibody titers of both IgG₁ and IgG₂a anti-C II in mice treated with high doses of LY309887 were lower than those in mice treated with PBS.

Reduction in CIA histopathology associated with administration of LY309887
Histologic analyses were performed in the hind limbs of mice treated with PBS or LY309887 on day 21 after the onset of arthritis. For a direct comparison between control and LY309887-treated mice, we selected animals whose arthritis index equaled the average arthritis index of their respective groups. The interphalangeal joints of a representative control mouse showed all the signs of severe inflammatory arthritis, including synovitis with massive infiltration of polymorphonuclear and mononuclear cells, pannus formation, cartilage erosion with loss of chondrocytes, and lateral bone resorption. The interphalangeal joints of a representative LY309887-treated mouse showed only mild synovitis and less damage to cartilage and bone (Fig. 4).

Discussion
In this study it was shown that levels of FR-β mRNA were elevated in arthritic joints of hind limbs compared to those in non-arthritic joints, whereas the levels of mRNA of CD163, macrophage-
specific marker detected were similar between these joints. In preliminary experiments involving immunohistochemical analysis of synovium from normal donors and RA patients for anti-FR-β specific antibody, the expression of FR-β protein was observed in synovial-infiltrated macrophages but not in resident synovial macrophage-like cells (synovial A cells). In contrast, similar expression of CD163 protein was observed in both macrophage subsets. Thus, the present findings support the notion that elevated levels of FR-β mRNA seen in the arthritic joints of CIA mice may be attributed to the increased percentage of synovial infiltrated macrophages. In bone marrow cells of arthritic and non-arthritic mice, the expression of FR-β and RFC mRNA was observed, but not FR-α mRNA. These FR-β expressed cells are assumed to be monomyeloid lineage cells from previous findings in human bone marrow cells (19, 20).

Mouse CIA is mediated by synergistic T cells and humoral immune responses specific for type II collagen (22, 23). Additionally, there is accumulating evidence that macrophages and myeloid cells contribute to the severity and chronicity of synovial inflammation and cartilage destruction during experimental arthritis (8, 9). The antiproliferation activity of antifolates depends on their ability to interact with intracellular folate-requiring enzyme targets, on their cellular transport properties and their degree of polyglutamation (13). LY309887 is thought to utilize RFC for uptake at high micromolar concentrations, and FRs at low serum concentrations (24). LY309887 has a high affinity for FR-α, and a 10-fold less affinity for FR-β and its polyglutamated form was shown intracellularly (12). It has been reported that LY309887 is active in a broad spectrum of murine and human xenograft tumors and a phase I trial in humans has already been performed (12,14). The intraperitoneal administration of LY309887 at 1.5 µg per dose every other day for a total of 5 doses was effective in CIA mice, as indicated by the clinical scores and histopathology of arthritic joints, and was well tolerated as reflected in the white blood cell count, serum hemoglobin and body weight. Thus, it is conceivable that LY309887 may act on proliferating monomyeloid lineage cells in bone marrow cells via FR-β. In fact, bone marrow suppression was reported as a side effect of LY309887 in murine tumor models (25). However, in this study we did not find a significant difference in the white blood cell count between treated and untreated mice. Therefore, it seems that LY309887 does not act simply as an antiproliferative agent.

In contrast to myeloid lineage cells in bone marrow, most macrophages in synovitis are believed to be non-proliferating (26). Accordingly, the action of LY309887 on synovial macrophages must be different from the anti-proliferation effect in bone marrow cells. Sev-
The proliferation or activity of myeloid lineage mast cells.

The administration of LY309887 after the onset of arthritis prevented to a large extent the damage to cartilage and bone in arthritis, suggesting the possibility that this drug or reagents with a high affinity for FR-β might serve as disease-modifying drugs against RA. However, the use of folate during anti-folate therapy must be considered on the basis of folate receptor competition in addition to competition with antifolate as a substrate for dihydrofolate reductase.

In conclusion, our findings support the hypothesis that LY309887 may be applicable to the treatment of RA by an alteration of the dosage protocol. In particular, the administration of LY309887 might be useful as a therapeutic agent in MTX-resistant RA patients, in that it acts on a different enzyme target from that of MTX.

Acknowledgments
The authors are would like to express their thanks to T. Kodama for his helpful technical assistance.

References
1. SADASIVAN E, ROTHENBERG SP: The complete amino acid sequence of a human folate binding protein from KB cells determined the cDNA. J Biol Chem 1989; 264: 5806-11.


