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

R. Nagayoshi[1], M. Nakamura[2], K. Ijiri[3], H. Yoshida[4], S. Komiya[1], T. Matsuyama[2]

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

Abstract

Objective

To examine the effect of LY309887, an inhibitor of glycinamide 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 CIAmice after the onset of arthritis. Mice were monitored for arthritis index for 21 days. Levels of IgG_1 and IgG_{2a} 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 analyse 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 IgG_1 and IgG_{2a} 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.

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.

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 hematopioetic 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 glycinamide 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 *Myco - bacterium 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 RNAwas isolated using TRIzol LS reagent (Life Technologies, Gaithersburg, MD). The first strand cDNA kit (SuperScript Preamplication 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 CAGGAAGCACATAAGGACATT (upstream primer), TCAAACCACATCTGAATGCAG (downstream primer); FR-β CTCTTG CTTTTGGTCTACATG (upstream primer), GAAGTAGTACTCAAACGTGTG (downstream primer); RFC ACTAACGAGATCATTCCGATG (upstream primer), GATCTAGTTTCCTGGTTTCTG (downstream primer); GAPDH GGAGCCAAACGGGTCATCATCTC (upstream primer), ATGCCTGCTTCACCACCTTCTTG (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 plateu 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 IgG_1 or IgG_{2a} anti-C II.

The plates were then incubated with biotinylated rabbit anti-mouse IgG_1 (Zymed, San Francisco, CA) or IgG_{2a} (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 IgG_1 and IgG_{2a} 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 IgG_1 and IgG_{2a} 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 metatarsophalangeal 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. $P \leq 0.05$ 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 CIAmice

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 mRNAof FR-β in murine bone marrow cells, we examined levels of mRNA of FRs in the bone marrow of CIAmice. The expression of mRNAof 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 LY-309887 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, CIAmice 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 CIAmice compared with the control group. In contrast, low-dose treatment with LY-309887 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 IgG_1 and IgG_{2a} anti-C II in CIA mice following the administration of LY309887

We next examined antibody titers of IgG_1 and IgG_{2a} anti-C II in serum samples from treated and non-treated mice on day 21 after the onset of arthritis. As

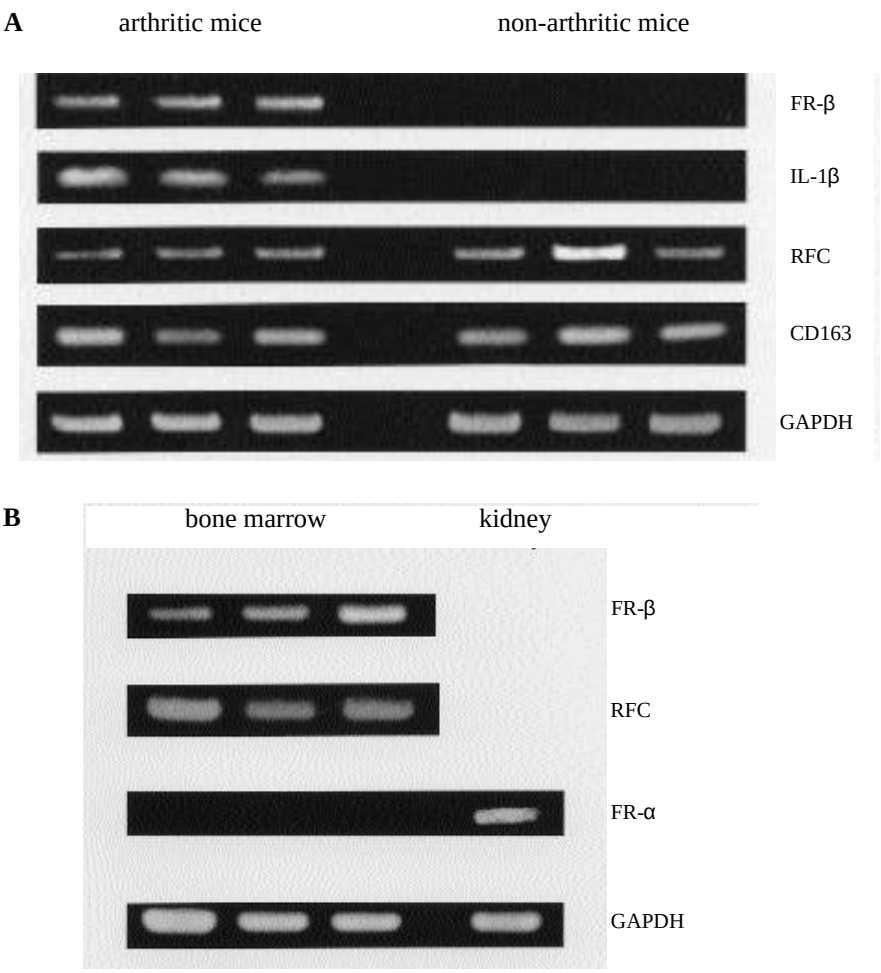

Fig. 1. (**A**) RT-PCR determination of selective FR-β mRNAexpression in the joints of CIAmice. Total RNAwas isolated from arthritic and non-arthritic joints of CIAmice and subjected to RT-PCR analysis for FR-β, IL-1β, RFC, CD163 and GAPDH. mRNAwas collected from the dissected joints of 2 arthritic and 2 non-arthritic mice. (**B**) RT-PCR determination of selective FR-β mRNAexpression in bone marrow cells of arthritic and non-arthritic mice. Total RNA was isolated from bone marrow cells of arthritic mice and subjected to RT-PCR analysis for FR-β, RFC, FR-α and GAPDH. Additionally, total RNA was isolated from the kidney of CIA mice and subjected to RT-PCR analysis for FR-α and GAPDH. Each mRNAwas collected from bone marrow cells obtained from 2 arthritic mice.

shown in Figure 3, antibody titers of both IgG_1 and IgG_{2a} 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-

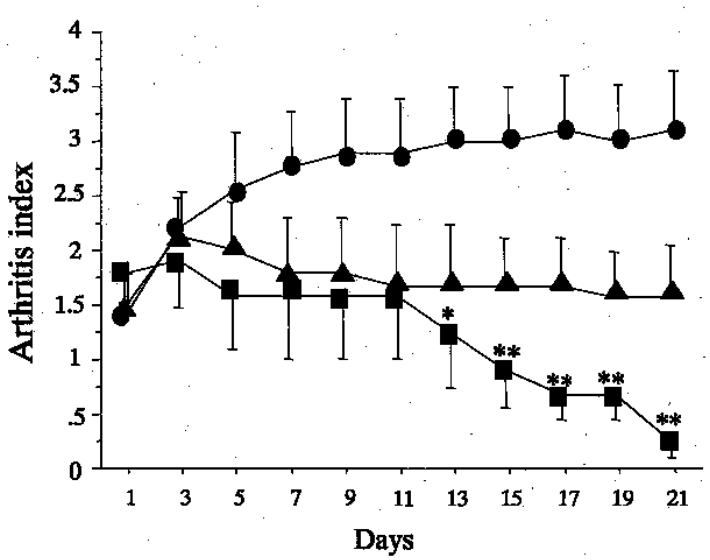

Fig. 2. Suppression of CIAby the administration of LY309887. After the onset of arthritis, mice were treated with intraperitoneal injections of 200 µl solutions containing 0.5 µg or 1.5 µg LY309887 on days 1, 3, 5, 7, and 9. Control mice were treated with PBS alone. ● control, ▲ LY309887, 0.5 µg/dose, ■ LY309887, 1.5 µg/dose. Bars show the mean and SEM.
* P< 0.03 versus the control group. ** P< 0.01 versus the control group. Pvalues were determined by the Mann-Whitney U test. Data are representative of two separate experiments with at least 9 mice per group.

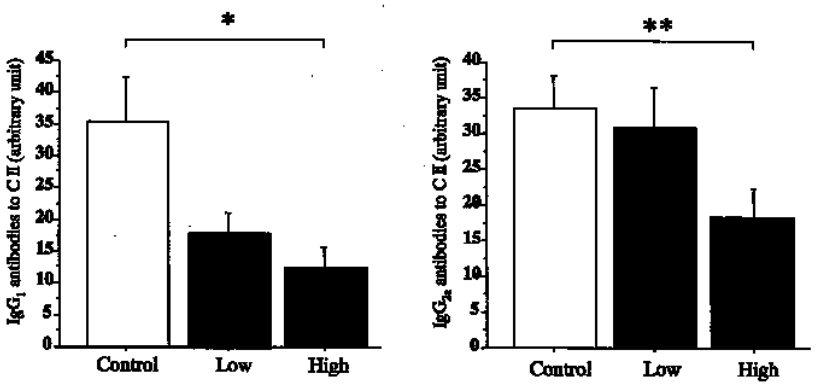

Fig. 3. Decreased production of IgG_1 and IgG_{2a} anti-C II in CIA mice by the administration of LY309887. Levels of IgG_1 (**A**) and IgG_{2a} (**B**) anti-C II were measured in sera from mice treated with PBS alone (controls, open bars), low dose LY309887 (gray bars) and high dose LY309887 (solid bars). After the onset of arthritis, mice were treated with 200 µl injections containing 0.5 µg or 1.5 µg of LY309887 intraperitoneally on days 1, 3, 5, 7, and 9. Control mice were treated with PBS alone. Bars show the mean and SEM. * P< 0.01 versus control group. ** P< 0.02 versus the control group. Pvalues were determined by the Mann-Whitney U test.
Data are representative of two separate experiments with at least 9 mice per group.

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 LY-309887 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-

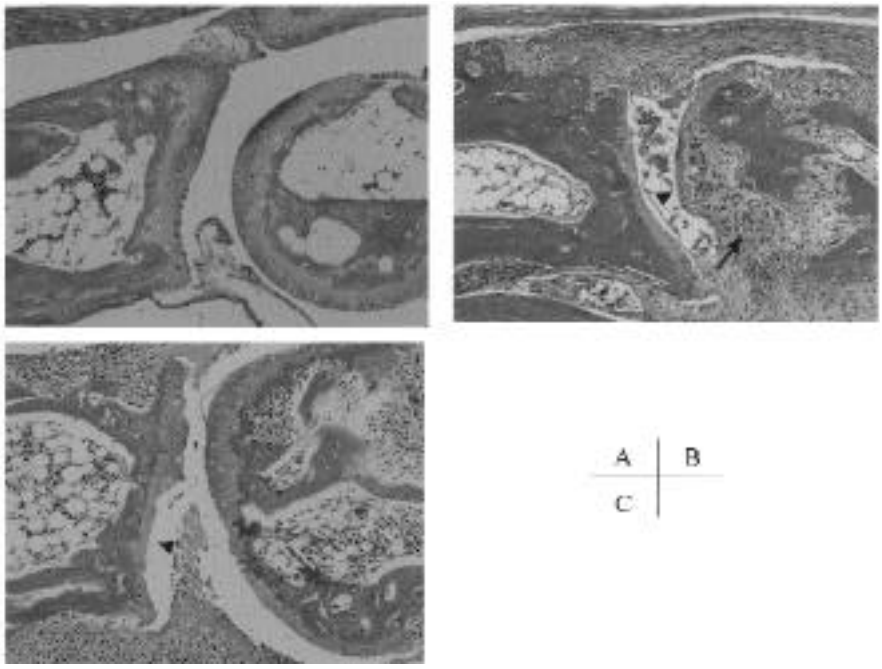

Fig. 4. Histopathology of collagen-induced arthritis (CIA) in PBS and LY309887 treated mice. For a direct comparison between control and LY309887 treated mice, we selected animals whose arthritis index equaled the average arthritis index of their respective group. All specimens were collected on day 21 after the onset of disease. (**A**) Normal interphalangeal (IP) joint. (**B**) Affected IPjoint of a control mouse with an arthritic index of 2, showing severe synovitis with synovial hyperplasia and pannus formation (**arrow**), massive infiltration of polymorphonuclear and mononuclear cells, severe cartilage erosion (**arrowhead**) with loss of chondrocytes resulting in a pitted appearance of the surface, and lateral bone erosion. (**C**) Affected IP joints (as defined by footpad swelling) in LY309887-treated mice with an arthritic index of 2, displaying mild synovitis and less damage to the cartilage or bone (**arrowhead**). (Original magnification x100.)

eral lines of evidence clearly suggest that antifolates do not act simply as antiproliferative agents for the cells responsible for the joint inflammation in RA and the mechanism of action of low dose anti-folates might be more anti-inflammatory than antiproliferative (27). It has been suggested that LY309887 might inhibit aminoimidazolecarboximide ribonucleotide formyltransferase (AICARFT) activity, in addition to GARFT as a major target (12). In this regard, Cronstein *et al.* reported that MTX downregulates the inflammation of arthritis by the release of adenosine, strong anti-inflammatory metabolite via the inhibition of AICARFT(12, 13, 28). Thus, the effect of LY309887 on CIAmice may be due to the release of adenosine in arthritic joints. Furthermore, the effect of LY309887 might be attributed to the inhibition of mRNA and protein synthesis, which are elevated in inflammatory cells. Consequently, LY309887 may inhibit the production of inflammatory mediators such as proinflammatory cytokines, chemokines and metalloproteinases.

LY309887 suppressed the production of IgG_1 and IgG_{2a} anti-C II. It is well known that CIA is a Th1 cell-mediated disease and that IgG_{2a} anti-C II plays a role in the pathogenesis of arthritis by activating complement levels (29). IL-12 from activated macrophages promotes the development of Th1 cells that help IgG_{2a} production. Thus, suppression of the proliferation or activity of macrophages by LY309887 may cause the decreased production of IgG_{2a} anti-C II. At present, the significance of a marked reduction in IgG_1 anti-C II in the pathogenesis of CIA remains unknown. It was shown that myeloid lineage mast cells promote the development of Th2 cells by producing IL-4 and as a consequence promoting IgG_1 production (30). One possible explanation for the reduced production of IgG_1 anti-C II by LY309887 treatment is that LY309887 might also suppress 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 antifolate 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 RAby 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.
2. RATNAM M, MARQUARDT H, DUHRING JL, FREISHEIM JH: Homologous membrane folate binding proteins in human placenta: Cloning and sequence of a cDNA. *Biochem - istry* 1989; 28: 8249-54.
3. WONG SC, PROEFKE SA, BHUSHAN A, MATHERLY LH: Isolation of human cDNA that restore methotrexate sensitivity and reduced folate carrier activity in methotrexate transport-defective Chinese hamster ovary cells. *J Biol Chem* 1995; 270: 17468-75.
4. SHEN F, ROSS JF, WANG X, RATNAM M: Identification of a novel folate receptor, a truncated receptor, and receptor type beta in hematopoietic cells: cDNA cloning, expression, immunoreactivity, and tissue specificity. *Biochemistry* 1994; 33: 1209-15.
5. WANG S, LOW PS: Folate-mediated targeting of antineoplastic drugs, imaging agents, and nucleic acids to cancer cells. *J Control Release* 1998; 53: 39-48.
6. LEAMON CP, LOW PS: Folate-mediated targeting: from diagnostics to drug and gene delivery. *Drug Discov Today* 2001; 6: 44-51.
7. MULHERIN D, FITZGERALD O, BRESNIHAN B: Synovial tissue macrophage populations and articular damage in rheumatoid arthritis. *Arthritis Rheum* 1996; 39: 115-24.

8. VAN LENT PL, VAN DEN BERSSELAAR LA, VANDEN HOEKAE, VANDE LOOAA, VANDEN BERGWB: Cationic immune complex arthritis in mice-a new model. Synergistic effect of complement and interleukin-1. *Am J Pathol* 1992; 140: 1451-61.
9. BLOM AB, VAN LENT PL, HOLTHUYSEN AE, VAN DEN BERGWB: Immune complexes, but not streptococcal cell walls or zymosan, cause chronic arthritis in mouse strains susceptible for collagen type II autoimmune arthritis. *Cytokine* 1999; 11: 1046-56.
10. NAKASHIMA-MATSUSHITA N, HOMMA T, YU S *et al*.: Selective expression of folate receptor β and its possible role in methotrexate transport in synovial macrophages from patients with rheumatoid arthritis. *Arthritis Rheum* 1999; 42: 1609-16.
11. TURK MJ, BREUR GJ, WIDMER WR *et al.*: Folate-targeted imaging of activated macrophages in rats with adjuvant-induced arthritis. *Arthritis Rheum* 2002; 46: 1947-55.
12. MENDELSOHN LG, SHIH C, SCHULTZ RM, WORZALLA JF: Biochemistry and pharmacology of glycinamide ribonucleotide formyltransferase inhibitors: LY309887 and lometrxol. *Invest New Drugs* 1996; 14: 287-94.
13. NEWELL DR: Clinical pharmacokinetics of antitumor antifolates. *Semin Oncol* 1999; 26: 74-81.
14. BUDMAN DR, JOHNSON R, BARILE B *et al*.: Phase I and pharmacokinetic study of LY309887: A specific inhibitor of purine biosynthesis. *Cancer Chemother Pharmacol* 2001; 47: 525-31.
15. MILLER EJ, RHODES RK: Preparation and characterization of the different types of collagen. *Methods Enzymol* 1982; 82: 33-65.
16. WILLIAMS RO, FELDMANN M, MAINI RN: Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis. *Proc Natl Acad USA* 1992; 89: 9784-8.
17. SHAER DJ, BORETTI FS, HONGEGGER A *et al.*: Molecular cloning and characterization of mouse CD163 homologue, a highly glucocorticoid-inducible member of the scavenger receptor cysteine-family. *Immunogenetics* 2001; 53: 170-7.
18. GABAY C, MARINOVA-MUTAFCHIEVA L, WILLIAMS RO *et al.*: Increased production of intracellular interleukin-1 receptor antagonist type I in the synovium of mice with collagen-induced arthritis. *Arthritis Rheum* 2001; 44: 451-62.
19. ROSS JF, WANG H, BEHM FG *et al*.: Folate receptor type beta is a neutrophilic lineage marker and is differentially expressed in myeloid leukemia. *Cancer* 1999; 85: 348-357.
20. REDDY JA, HANELINE LS, SROUR EF, ANTONY AC, CLAPP DW, LOW PS: Expression and functional characterization of the beta-isoform of the folate receptor on CD34 (+) cells. *Blood* 1999; 93: 3940-8.
21. ALATI T, WORZALLA JF, SHIH C *et al*.: Augmentation of the therapeutic activity of Lometrexol [(6-R)5,10-Dideazatetrahydrofolate] by oral folic acid. *Cancer Res* 1996; 56: 2331-5.
22. HOLMDAHL R, ANDERSSON M, GOLDSCHMIDT TJ, GUSTAFSSON K, JANSSON L, MO JA: Type II collagen autoimmunity in animals and provocations leading to arthritis. *Immunol Rev* 1990; 118: 193-232.
23. TERATO K, HASTY KA, REIFE RA, CREMER MA, KANG AH, STUART JM: Induction of arthritis with monoclonal antibodies to collagen. *J Immunol* 1992; 148: 2103-8.
24. WESTERHOF GR, SCHORNAGEL JH, KATHMANN I *et al*.: Carrier- and receptor-mediated transport of folate antagonists targeting folate-dependent enzymes: Correlates of molecular-structure and biological activity. *Mol Pharmacol* 1995; 48: 459-71.
25. CHEN VJ, BEWLEY JR, ANDIS SL *et al.*: Preclinical cellular pharmacology of LY231514 (MTA): A comparison with methotrexate, LY309887 and raltitrexed for their effects on intracellular folate and nucleoside triphosphate pools in CCRF-CEM cells. *Br J Cancer* 1998; 78: 27-34.
26. SCHASER K, KINNE RW, BEIL H, KLANDY B, STOSS H: Proliferation of T-cells, macrophages, neutrophilic granulocytes and fibroblast-like cells in the synovial membrane of patients with rheumatoid arthritis. *Verh Dtsch Ges Pathol* 1996; 80: 276-80.
27. CUTOLO M, STRAUB RH: Anti-inflammatory mechanisms of methotrexate in rheumatoid arthritis. *Ann Rheum Dis* 2001; 60: 729-35.
28. CRONSTEIN BN: Molecular therapeutics. *Arthritis Rheum* 1996; 39: 1951-60.
29. WATSON WC, TOWNES AS: Genetic susceptibility to murine collagen II autoimmune arthritis. Proposed relationship to the IgG2 autoantibody subclass response, complement C5, major histocompatibility complex (MHC) and non-MHC loci. *J Exp Med* 1985; 162: 1878-91.
30. FARRAR JD, ASNAGLI H, MURPHY K, MURPHY KM: T helper subset development: Roles of instruction, selection, and transcription. *J Clin Invest* 2002; 109: 431-5.