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Effect of pyrimethamine in experimental rheumatoid arthritis

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C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: Recent evidence has shown that the antimalarials are useful drugs in the treatment of various rheumatic diseases. The present study was designed to test the therapeutic effect of pyrimethamine (PYR) in collagen-induced arthritis (CIA).

Material/Methods: CIA was induced in Lewis rats. The intraperitoneal administration of PYR and methotrexate (MTX) were started on day 25 post-immunization and continued until final assessment on day 35. During this period, clinical examination was performed intermittently. Anti-C II Ab and nitric oxide (NO) synthesis were measured. The paws and knees were then removed for histopathology and radiography assay. The biocompatibility of PYR and MTX were assessed using a fibrosarcoma cell line.

Results: Data showed that i.p. injection of pyrimethamine to arthritic rats induced a significant reduction in paw edema. This beneficial effect was associated with a significant decrease in anti-CII antibody response compared with untreated rats. Histopathological assessment showed reduced inflammatory cell infiltrate in the joints of treated rats, and tissue edema and bone erosion in the paws were markedly reduced following PYR therapy. Moreover, our radiography results paralleled our histological findings. Cytotoxicity analysis of PYR showed greater tolerability compared with MTX. Treatment with PYR significantly diminished nitric oxide formation in treated rats compared with untreated controls.

Conclusions: Our findings shed light on the therapeutic efficacy of pyrimethamine in experimental rheumatoid arthritis compared with a choice drug (methotrexate), which may recommended it as a second-line drug in the treatment of rheumatoid arthritis.

key words: **pyrimethamine • rheumatoid arthritis • antimalarial drugs • nitric oxide**

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BACKGROUND

Rheumatoid arthritis (RA) is a significant chronic disease characterized by the sequestration of various leukocyte subpopulations within both the developing pannus and synovial space. The chronic nature of this disease results in inflammation of multiple joints, with subsequent destruction of the joint cartilage and erosion of bone. While this disease has a worldwide distribution, its pathogenesis is not clearly understood [1]. Collagen-induced arthritis (CIA) is a model of experimental arthritis which is induced by the injection of type II collagen (CII) [2,3]. The similarities in joint pathology as well as in cellular and humoral immunity in CIA and RA suggest that CIA is a relevant animal model useful in the search for new antiarthritic drugs [4–9]. In humans, joint damage typically occurs before patients are diagnosed, and most of the joint destruction occurs within the first two years of diagnosis [10]. Therapeutic agents such as methotrexate (a disease-modifying anti-rheumatic drug, or DMARD) and immnomodulators only slow the progression of the disease, suggesting that these drugs fail to adequately control the underlying pathophysiology of RA [11,12].

On the other hand, inhibition of folate-dependent enzymes by various drugs, such as MTX and/or non-steroidal anti-inflammatory drugs (NSAIDs), could be due to a significant immunosuppressive property, in which supplementation with folate in excess abolishes the effect of MTX. A structurally similar antifolate, aminopterin, also reduced arthritis [13,14]. Since MTX inhibits the activity of thymidylate synthetase (TS) and dihydrofolate reductase essential for DNA synthesis, it is known that MTX interferes with DNA synthesis and subsequently inhibits cytokine production. Moreover, the antifolate activity of NSAIDs, and hence the cytostatic consequences, are important factors in producing anti-inflammatory activity. Aspirin exerts its anti-inflammatory effects after its conversion into salicylic acid, which possesses greater antifolate activity than its parent compound [15,16].

Pyrimethamine (Tindurin, Chloridin, Daraprim, Malocide) has been used for many years against malaria, initially on its own, but subsequently in combination with other drugs. Pyrimethamine has also been used recently against toxoplasmic encephalitis relapses and *Pneumocystis carinii* pneumonia in HIV-infected patients [17]. Pyrimethamine inhibits dihydrofolate reductase-thymidylate synthase (DHFR-TS) in the folate biosynthetic pathway and resistance to it arises from mutation in the *dhfr* domain of the *dhfr-ts* gene [18]. In humans, DHFR and TS are separate gene products, DHFR being monomeric and TS dimeric, so the effect of this medicinal agent is specific [18,19]. The purpose of the present study was to determine whether the dihydrofolate reductase (DHFR) inhibitor could suppress the development of arthritis caused by injection of CII in an experimental model.

MATERIAL AND METHODS

Material

Reagents were obtained from Sigma-Aldrich (Milan, Italy). All other chemical reagents were of the highest commercial grade available. All stock solutions were prepared in non-pyrogenic saline (0.9% NaCl; Baxter Healthcare, Thetford, UK).

Animals

Male Lewis rats weighing 160–180 g (Charles River, Milan, Italy) were used for these studies. The animals were housed in a controlled environment and provided with standard rodent chow and water. The rats were divided at random into four groups: N: normal group (n=8), C: control group (n=9), T1: treated with pyrimethamine at 1 mg/kg/day (n=8), and T2: treated with methotrexate at 0.3 mg/kg three times weekly (n=8). Animal care was in compliance with Italian regulations on the protection of animals used for experimental and other scientific purposes (document DM 116192) as well as with the European Economic Community regulations (document OJ of EC L 358/1, ratified Dec. 18, 1986).

Induction of CIA and treatment protocol

Bovine CII was dissolved in 0.1 M acetic acid at a concentration of 2 mg/ml by stirring overnight at 4°C. Dissolved CII was frozen at –70°C until used. Freund's complete adjuvant (CFA) was prepared by adding *Mycobacterium tuberculosis* H37Ra at a concentration of 5 mg/ml. Before injection, CII was emulsified with an equal volume of CFA. CIA was induced as previously described [20]. Briefly, on day 1, rats were injected intradermally at the base of the tail with 100 µl of emulsion (containing 100 µg of CII). On day 21, a second injection of CII in CFA was administered. The intraperitoneal administration of pyrimethamine and methotrexate (MTX) were started on day 25 post-immunization (p.i.) and continued until final assessment on day 35. During this period, clinical examination was performed intermittently. The paws and knees were then removed for histopathological assay.

Clinical assessment of CIA

The rats were evaluated daily for arthritis according to a macroscopic scoring system using a scale from 0 to 4 for each paw: 0 = no signs of arthritis, 1 = swelling and/or redness of the paw or 1 digit, 2 = involvement of 2 joints, 3 = involvement of >2 joints, and 4 = severe arthritis of the entire paw and digits [21].

Histological processing and assessment of arthritis damage

On day 35, the animals were anesthetized with sodium pentobarbital (45 mg/kg intraperitoneally) and killed. Blood was collected by intracardiac puncture and the paws and knees were removed, trimmed, and fixed in 10% buffered formalin, decalcified, and then embedded in paraffin, sectioned to 5 µm, and stained with hematoxylin and eosin for histological examination. Joint damage was assessed based on synovial hypertrophy, pannus formation, inflammatory cell infiltration, and cartilage and subchondral bone destruction [22]. Scoring was carried out in a blinded manner. Joint erosion was graded on a scale of 0–4 for each limb, according to the severity of damage.

Radiographic evaluation

Radiological scoring was determined by an investigator blinded to the treatment protocol on day 35. A score was assigned to each joint on the basis of the degree of soft tissue swell-

ing, joint space narrowing, periosteal new bone formation, and bone destruction. Scores were 0–3 per joint (0: normal, 3: maximum joint destruction) [23,24].

Anti-CII antibody quantification

Individual sera were obtained by intracardiac puncture from bCII/CFA-immunized rats treated with PYR, MTX, or vehicle. Sera were obtained on day 35 p. i. and then stored at –20ºC until the total IgG anti-CII levels were analyzed. ELISA microtiter plates (Dynatech, Plochingen, Germany) were coated with native bCII and incubated overnight at 4ºC. After washing with PBS-Tween (0.05%), sera were added, diluted in 0.1% BSA in PBS-Tween. As a secondary antibody, biotinylated goat anti-rat Ab was added (Serotec, Oxford, UK). After incubation, streptavidin-alkaline phosphatase (Jackson Immuno Research Lab, West Groove, PA, USA) was added and finally the substrate buffer containing phosphatase substrate (Sigma, St Louis, MO, USA) was added. The OD was read at 402 nm using a microplate reader (Labsystems).

Measurment of nitrite/nitrate

Nitrite + nitrate production, an indicator of NO synthesis, was measured in the plasma samples as previously described [25]. Briefly, the nitrate in the plasma was first reduced to nitrite by incubation with nitrate reductase (670 µg/ml) and NADPH (160 µM) at room temperature for 3 h. The nitrite concentration in the samples was then measured by the Griess reaction by adding 100 µl of Griess reagent (0.1% naphtylethylendiamide dihydrochloride in H_2O and 1% sulphanilamide in 5% concentrated H_3PO_4, vol. 1:1) to 100-µl samples. The optical density at 550 nm (OD_{550}) was measured using an ELISA microplate reader (SLT-Labinstruments, Salzburg, Austria). Nitrate concentrations were calculated by comparison with the OD_{550} of a nitrate standard solution.

Cell culture

The fibrosarcoma WEHI-164 cell line was seeded at an initial density of 2×10^4 cells/well on 96-well tissue culture plates. The cells were maintained in RPMI-1640 medium supplemented with 5% fetal calf serum, penicillin at 100 units/ml, and streptomycin at 100 µg/ml, with 5% CO_2 and at 37°C and saturated humidity.

Dose-response analysis

Triplicate, two-fold dilutions of PYR and MTX preparations at concentrations of 0–10 µg/ml were transferred to overnight cultured cells. Untreated cells were used as controls. The cells were cultured overnight and then subjected to colorimetric assay. A sample of the media was used for zymography.

Colorimetric assay

After each experiment, the cells were washed three times with ice-cold phosphate-buffered saline (PBS), followed by fixation in a 5% formaldehyde solution. Fixed cells were washed three times and stained with 1% crystal-violet. Stained cells were washed, lysed, and solubilized with 33.3% acetic acid solution. The density of the developed purple color was read at 580 nm.

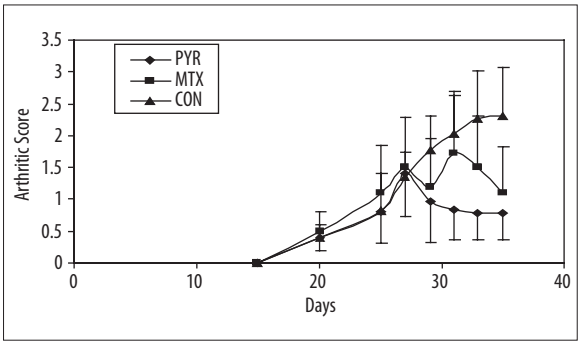

Figure 1. Severity of arthritis score in groups treated with PYR (pyrimethamine) or MTX (methotrexate) compared with untreated control rats (CON). The number of rats in each group was eight. Total i.p. injections was10, and PYR and MTX were administered at 1 mg/kg/day and 0.3 mg/kg three times weekly, respectively. The peak of clinical signs of arthritis in PYR-treated rats occurred on day 27 p. i. The severity of arthritic lesions was then significantly reduced on days 30–35 p. i. in PYR-treated animals compared with control rats. $P<0.05$ was considered statistically significant.

Zymography

This technique was used for determining gelatinase (collagenase type IV or matrix metalloproteinase type 2, MMP-2) and MMP-9 activity, in conditioned media according to the modified Heussen and Dowdle method [26]. Briefly, aliquots of conditioned media were subjected to electrophoresis in (2 mg/ml) gelatin containing polyacrylamide gels in the presence of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions. The gels underwent electrophoresis for 3 hours at a constant voltage of 80 volts. After electrophoresis, the gels were washed and gently shaken in three consecutive washings in 2.5% Triton X100 solution to remove SDS. The gel slabs were then incubated at 37ºC overnight in 0.1 M Tris HCl gelatinase activation buffer (pH 7.4) containing 10 mM $CaCl_2$ and were subsequently stained with 0.5% Coomassie Blue. After intensive destaining, proteolysis areas appeared as clear bands against a blue background. Quantitative evaluation of both the surface and the intensity of lysis bands, on the basis of gray levels, were compared relative to untreated control wells and expressed as the "relative expression" of gelatinolytic activity.

Statistical analysis

All data are expressed as the mean ± SEM. The significance of differences in arthritic scores and histopathological and radiological assessment were evaluated with the Mann-Whitney test. The anti-CII antibody titers as well as nitric oxide assay were compared using the student's *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

Effect of pyrimethamine in CIA

As shown in Figure 1, CIA developed in rats immunized with CII, and clinical signs (periarticular erythema and edema) of the disease first appeared in the hind paws between days 25 and 27 after CII challenge, with a 100% incidence of CIA

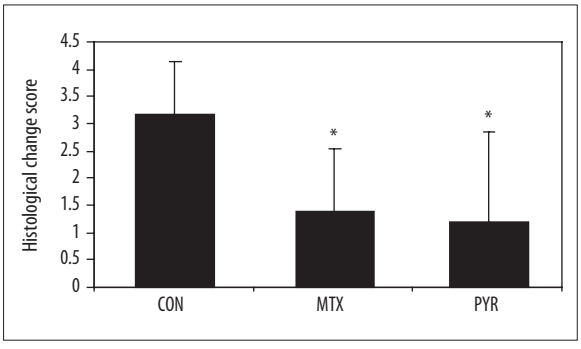

Figure 2. Semi-quantitative scoring of histological changes of the involved joints in groups PYR (treated with pyrimethamine) and MTX (treated with methotrexate) versus CON (controls) were significant. Bars show the mean ±SEM. *$P<0.05$ was considered statistically significant.

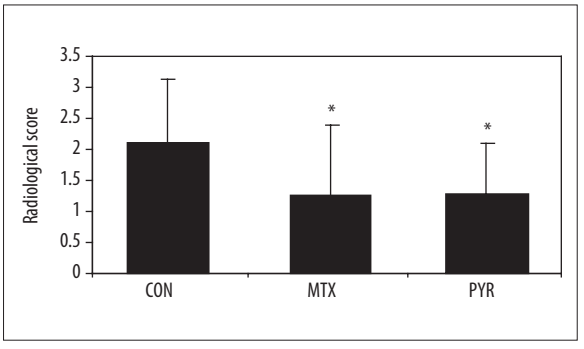

Figure 4. Semi-quantitative scoring of radiological examination of the involved joints in the groups PYR (treated with pyrimethamine) and MTX (treated with methotrexate) versus CON (controls) were significant. Bars show the mean ±SEM. *$P<0.05$ was considered statistically significant.

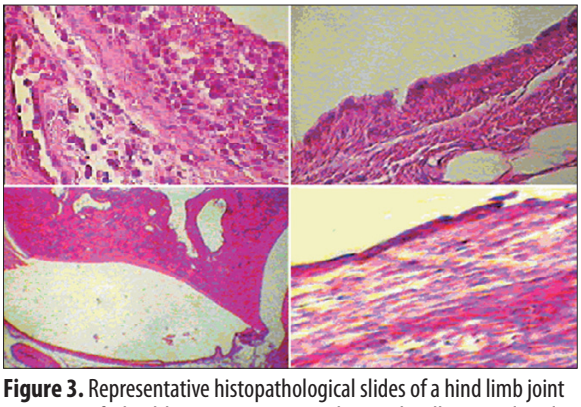

Figure 3. Representative histopathological slides of a hind limb joint of a healthy Lewis rat, a control rat with collagen-induced arthritis, an arthritic animal treated with pyrimethamine (PYR), and an arthritic animal treated with methotrexate (MTX). The joint of the rat treated with pyrimethamine shows significantly fewer signs of joint destruction (hematoxyline and eosin, original magnification ×40).

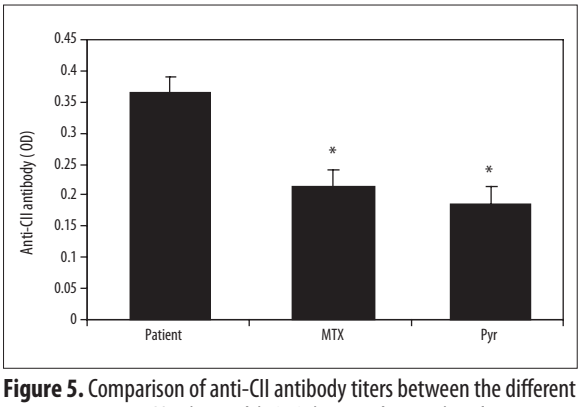

Figure 5. Comparison of anti-CII antibody titers between the different groups CON (controls), PYR (pyrimethamine) and MTX (methotrexate). The sera were collected on day 35 p. i. There were eight rats in each group. Pyrimethamine therapy significantly reduced anti-CII antibody production in PYR versus the CON group. The bars represents mean ±SEM. *$P<0.05$ compared with the CON group.

by day 27. The interaperitoneal injection of pyrimethamine (1 mg/kg/day) to arthritic rats could rapidly reverse paw edema, as did MTX. The difference between the control group and treated rats on days 30–35 of the experiment was significant ($P<0.05$).

Histopathology findings

As shown in Figures 2 and 3, histological evaluation of the paws in the control animals revealed signs of severe arthritis along with inflammatory cell infiltrate. Histopathological assessment showed reduced inflammatory cell infiltrate in the joints of treated rats, as well as the number of osteoclasts present in the subchondral bone; tissue edema and bone erosion in the paws were markedly reduced by both drugs (pyrimethamine and MTX), indicating that the tested drugs were effective in retarding synovial inflammation and preventing destruction of joints.

Radiographical findings

Radiographical analysis of the affected joints in control rats showed soft tissue swelling, joint space narrowing, reduced lucency due to demineralization, and areas of recalcification, indicative of new bone formation. Blinded radiographic scores on day 35 were significantly lower in the pyrimethamine-treated rats than in the untreated animals and paralleled those of MTX (Figure 4). The radiographic appearance of swollen joints was comparable with the results of the arthritic index and histological changes in the joints.

Effect of pyrimethamine on anti-CII antibody production

As is apparent in Figure 5, treatment with 1 mg/kg/day of pyrimethamine significantly reduced the titers of anti-CII antibody on day 35 p. i. compared with those of untreated rats ($P<0.05$). Moreover, the mean anti-CII Ab response was also decreased in the MTX group compared with the untreated group. This difference was statistically significant ($P<0.05$).

Effect of pyrimethamine on nitric oxide formation

The data in Figure 6 demonstrates that the levels of nitric oxide have been significantly ($P<0.05$) increased in plasma

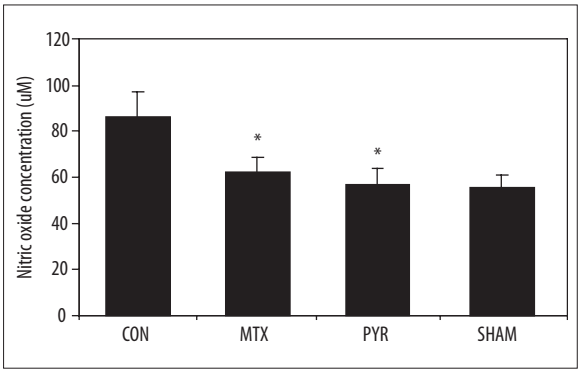

Figure 6. The levels of nitric oxide formation in plasma on day 35 p. i. between the different groups CON (controls), PYR (pyrimethamine), and MTX (methotrexate). The concentration of nitric oxide in the treated CIA rats was significantly increased versus the sham group (*P<0.05). Treatment with pyrimethamine could significantly reduce the level of nitric oxide in plasma compared with control animals (*P<0.05). Values are mean ±SEM with eight rats in each group.

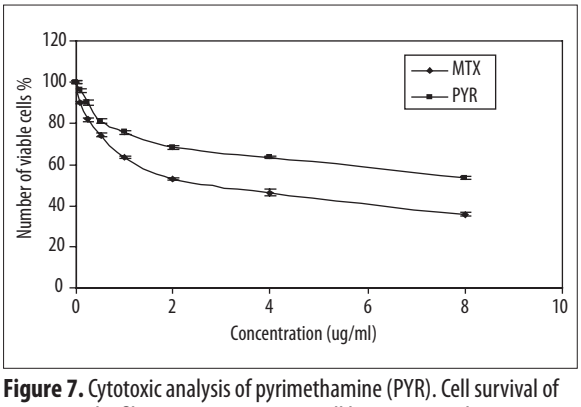

Figure 7. Cytotoxic analysis of pyrimethamine (PYR). Cell survival of the fibrosarcoma WEHI-164 cell line was tested comparing MTX (methotrexate) with PYR. LD_{50} for MTX was 2.5 µg/ml. In contrast, WEHI-164 as a sensitive cell line showed more tolerability to increasing amounts of PYR than of MTX.

obtained from CIA-treated rats (control group). In contrast, the level of NO were significantly lower in the plasma of pyrimethamine-treated as well as MTX-treated rats compared with control groups (P<0.05).

Biocompatibility of pyrimethamine

Figure 7 shows the proliferative response of the fibrosarcoma WEHI-164 cell line to pyrimethamine at different doses (0–8 µg/ml) compared with MTX. The tolerability and biocompatibility of WEHI-164, as a cell line sensitive to increasing amounts of pyrimethamine, was more than to MTX. Pyrimethamine showed less cytotoxic effect compared with methotrexate.

Effect of pyrimethamine on MMP-2

Dose response analysis of pyrimethamine on MMP-2 expression is presented in Figure 8. The inhibitory effect of pyrimethamine at concentrations of 0.05–8 µg/ml was less

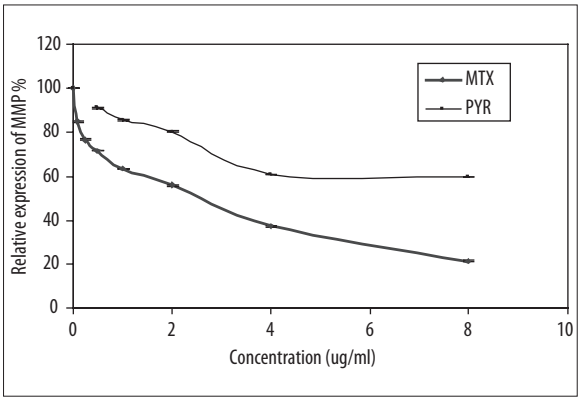

Figure 8. The inhibitory effect of pyrimethamine (PYR) on MMP-2 activity. The fibrosarcoma cell line (2×10^4 cell/well) was incubated overnight with increasing doses of PYR as described in Materials and Methods. Methotrexate (MTX)-treated cells were used as controls. Analyses were performed using the UVI Pro Gel Documentation system (Cambridge, UK). Surface and intensity of lysis bands on the basis of gray level were analyzed. PYR-treated cells were investigated in triplicate. The inhibitory activity of PYR (0.5–8 µg/ml) was less than that of MTX.

than that of MTX.This difference was significant between pyrimethamine and MTX at concentrations of 2–8 µg/ml (P<0.05). It should be noted that because of the similarity of the inhibitory effects of pyrimethamine on MMP-2 and MMP-9, the results for MMP-2 are reported in this study.

Discussion

Growing evidence suggests that rheumatoid arthritis should no longer be considered a benign disease. Considerable data suggest that this disease is associated with diminished long-term survival [27]. Clinical trials on different treatment strategies in rheumatoid arthritis have shown that the antimalarials are useful drugs in the treatment of various rheumatic diseases [28–30], so hydroxychloroquine is used as a second-line treatment of RA [31,32]. In this investigation, CIA was induced in Lewis rats to imitate the clinical scenario of RA and the antiarthritic potency of pyrimethamine was subsequently assessed in this experimental model. This drug has been used for many years against malaria and, in combination with other drugs, is also used recently against toxoplasmic encephalitis relapses and *Pneumocystis carinii* pneumonia in HIV-infected patients [33]. It has been reported that pyrimethamine also has antibacterial value [34]. Pyrimethamine inhibits dihydrofolate reductase-thymidylate synthase (DHFR-TS) in the folate biosynthetic pathway, and resistance to it arises from a mutation in the *dhfr* domain of the *dhfr-ts* gene [18]. Here we demonstrate that treatment with pyrimethamine reduces the development of clinical signs in the CIA model and the infiltration of PMNs in the joints (histology) as well as the degree of joint injury (histology, radiography) in rats treated with type II collagen compared with MTX, which is the most widely used DMARD. All of these findings support the view that pyrimethamine attenuates the degree of arthritis and joint damage caused by collagen in the rat.

It has recently been reported that inhibitors of NOS activity reduce the development of arthritis, and these findings sup-

port the role for NO in the pathophysiology associated with inflammatory reactions [35,36]. The results of the present study show that the formation of nitrite and nitrate (metabolites of NO in water) caused by CIA is markedly diminished in pyrimethamine-treated rats. Moreover, pyrimethamine therapy could significantly reduce anti-CII antibody production in the therapeutic group. Since anti-CII antibody could be essential for the development of CIA and its transfer induces arthritis [37], this implies that treatments which affect the antibody response may also affect the disease [38], in concordance with our findings. Conversely, the matrix metalloproteinases (MMPs), especially the gelatinases (MMP-2 and MMP-9), have been implicated in several features of inflammatory arthritis, including angiogenesis and bone erosions [39]. Angiogenesis is a major component of the inflammatory pannus in rheumatoid arthritis, and MMP secretion by microvascular endothelial cells is an essential step in angiogenesis [40].

To investigate the tolerability and MMP-2 activity, we used the fibrosarcoma WEHI-164 cell line, a highly sensitive cell line [41], since the synovial fibroblasts contribute to chronic inflammatory responses in RA as a major part of the invasive pannus [42]. In addition, fibroblast cell lines isolated from RA patients have the potential to produce matrix-degrading enzymes and several cytokines, such as IL-1, IL-8, GM-CSF, and TNF-α [43,44].Thus the inhibitory effect of pyrimethamine in MMP2 could probably diminish the process of angiogenesis progression and cytokine production. Furthermore, our data exhibit greater tolerability of pyrimethamine compared with MTX, which could be an advantage for its therapeutic aims as an antiarthritic drug.

CONCLUSIONS

The current study demonstrates that pyrimethamine could effectively suppress the arthritis in an experimental model and, moreover, reduce anti-CII antibody production, inhibit MMP-2 expression and, based on its tolerability compared with methotrexate, it may be recommended as a second-line drug in the treatment of rheumatoid arthritis.

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