# MTX Affects Inflammation and Tissue Destruction Differently in the Rat AA Model

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***ABSTRACT.*** ***Objective.*** To investigate the dose response relationships of methotrexate (MTX) therapy in rat adjuvant arthritis (AA), an animal model of rheumatoid arthritis (RA).

***Methods.*** Female Lewis rats were fed a defined diet and were treated with 0, 0.3, 1, 2, 3, 5, and 10 mg MTX per week beginning 3 days after adjuvant injection and lasting 6 weeks. The presence or absence of arthritis, and its degree were measured by hindpaw edema scores, ankle widths, and radiographic and histopathologic scores.

***Results.*** The 2, 3, 5, and 10 mg MTX per week doses resulted in deaths before the end of the protocol and suppressed normal body weight gain. Tissue destruction, measured by radiographic and histopathologic scores, was reduced in a dose dependent manner with increasing MTX dose. Suppression of inflammation, measured by ankle widths and radiographic and histopathologic scores, reached a maximum at the 1 mg MTX dose and declined at higher doses.

***Conclusions.*** Suppression of tissue destruction and inflammation in rat AA does not occur in a concerted fashion as the dose of MTX increases. The implications of these findings to human disease remain to be determined. (J Rheumatol 2001;28:1476–81)

*Key Indexing Terms:*
ADJUVANT ARTHRITIS METHOTREXATE RHEUMATOID ARTHRITIS

Rat adjuvant arthritis (AA) is a reproducible animal model of rheumatoid arthritis (RA)[1]. Methotrexate (MTX), used in low doses, is efficacious in the treatment of both RA[2,3] and rat AA[4-10]. The dose dependent efficacy of the lower range of MTX doses in RA is fairly linear[11]. High dose MTX therapy (i.e., 500 mg/m$^2$) has produced more variable results in inducing joint improvement in patients with RA[12-14] and it is possible that similar results would be obtained in the rat AA model.

We investigated the dose response to MTX in rat AA fed a defined diet. Body weights and mortality were used to evaluate toxicity whereas ankle widths, foot edema scores and histopathologic scores and radiographic scores were used to evaluate the severity of inflammation and tissue destruction. We hypothesized that variables such as edema, ankle widths, radiographic, and histopathologic scores would indicate a similar presence and severity of the AA and that there would be a normal dose response curve, i.e., uniformly increasing efficacy, as the dose of MTX was increased.

## MATERIALS AND METHODS

*Animals*. The Animal Use Review Board at the University of Alabama at Birmingham approved this protocol. Inbred female Lewis rats, 105-115 g at 35 days of age were purchased from Jackson Laboratories (Bar Harbour, ME, USA). AIN-93M laboratory chow pellets (Dyets, Bethlehem, PA, USA) and water were freely available. As soon as the arthritis was clinically evident, acetaminophen at a concentration of 3 g/l was added to all drinking water. Animals were housed in cages with woodchip bedding with a 12 h sleep/light cycle. Long sipper straws and food in the bottom of cages were provided to animals with arthritis. The rats were randomized to treatment groups on the basis of weights to form groups with the same mean weight.

*Adjuvant*. To produce an adjuvant mixture, dried heat killed *Mycobacterium butyricum* (DIFCO, Detroit MI, USA) was ground in light mineral oil (Humco Laboratory, Texarkana, TX, USA) at a concentration of 6 mg/ml. Each rat was immunized with 0.1 ml of the suspension at the base of the tail under light ether anesthesia at Day 38[15].

*MTX therapy.* MTX (Sigma, St. Louis, MO, USA) dissolved in PBS at doses of 0, 0.3, 1, 2, 3, 5, and 10 mg/kg/week (designated 0 MTX = positive controls, 0.3 MTX, 1 MTX, 2 MTX, 3 MTX, 5 MTX, and 10 MTX) were given intraperitoneally (ip) twice per week (Tuesday and Friday) starting 3 days after adjuvant injection. The dosing continued for 6 weeks. Clinical severity of the disease reaches a maximum in the positive controls at 6 weeks. Non-adjuvant injected animals served as negative controls. The

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trial was done in 3 blocks over a one year period, using the same lot of MTX. Because of excessive mortality in the 3 MTX, 5 MTX, and 10 MTX groups, these doses were discontinued after the first block of animals. Due to deaths, some of the higher dose groups were combined for data analyses (e.g., 2 MTX + 3 MTX).

*Evaluation*. Animals were weighed and ankle widths were evaluated twice per week (Tuesdays and Fridays) using a Fowler Ultra-cal III digital caliper (Lux, Tucson, AZ, USA). To eliminate bias, the caliper was held with the digital display facing away from the investigator, so that the width value was not seen until after the measurement was made. Fore and hindpaws were graded biweekly for swelling by an investigator blinded to drug treatment. Paw swelling was graded as: 0 = no edema, 1 = slight edema of the small digital joints, 2 = edema of the digital joints and foot pad, 3 = gross edema of the entire foot pad below the ankle or elbow, 4 = gross edema of the entire foot pad including the ankle or elbow joint[15].

At 77 days the animals were sacrificed using $CO_2$ asphyxiation. The hindpaws were removed and immediately fixed in neutral buffered formalin. The hindpaws were placed on radiographic magnification cassettes and radiographs using the following settings: 28 kV/8 mA seconds for anterior posterior views, 30 kV/8 mA seconds for lateral views using single emulsion film. The radiographs were graded for bone mineralization, erosions, periostitis, cartilage space, soft tissues, alignment, and associated degenerative changes using Clarke's method with a scale of 0 for no involvement to 4 for extensive involvement[16]. Two observers blinded to the treatment assignment (WKB and GSA) evaluated the radiographs. The 2 individuals who evaluated the radiographs have significant experience in reading and rating radiographs for patients with RA. For this study, they underwent an intense training period followed by their independent reading of the radiographs, followed by resolution of disagreements. The study radiographs were not read until there was a less than 10% difference between observers on the set of practice rat radiographs.

After radiographic evaluation, the right hindpaws were submitted for histopathological examination. The bones were decalcified in decalcifying solution (Fisher, Chelating Agent, 0.003 M; hydrochloric acid 1.35 M) and then embedded in paraffin (58ºC, 3 × 45 min) and stained by a hematoxylin and eosin stain. Two sections of each joint were read for synovial cell proliferation, cartilage erosion, bone erosion, fibroproliferative pannus, diffuse inflammatory synovitis, and synovial vasculitis (0 = normal, 1 = mild, 2 = moderate, and 3 = severe)[17]. The sections were examined by one investigator (REG) who was blinded to the treatment assignment.

The presence of AA was defined as any positive clinical, histopathological, or radiographic score. The severity of inflammation in clinical terms was defined as the edema score and the ankle width. Mean ankle widths were reported at 77 days of age. In our experience with this model, the ankle and footpad are the most severely affected anatomical sites. The severity of inflammation in radiographic terms was defined as the sum of soft tissue swelling plus mineralization plus periostitis scores for a hind paw (maximum possible score: 12). The severity of inflammation in histopathologic terms was defined as the sum of synovial cell proliferation plus fibroproliferative pannus plus diffuse inflammatory synovitis plus synovial vasculitis score for a hind paw (maximum possible score: 12).

The severity of tissue destruction in radiographic terms was defined as the sum of the alignment plus cartilage space plus secondary degenerative changes plus erosion scores for a hind paw (maximum possible score: 16). The severity of tissue destruction in histopathologic terms was defined as the sum of bone erosion plus cartilage erosion scores for a hind paw (maximum possible score: 6).

The toxicity of MTX was defined as death of the animal before the end of the protocol or a statistically significant suppression of body weight gain during the protocol. An efficacious or toxic response was defined as a statistically significant difference in the means ($p < 0.05$) of the above measurements or scores when compared to the means of the appropriate controls.

*Statistical analyses*. The summary statistics are reported in the form of proportions and mean ± standard deviation (SD) for discrete and continuous data, respectively. The Kruskal-Wallis test was used to compare the histopathologic and radiographic data across the dose groups. Subsequently, pairwise comparisons were done using Wilcoxon tests to identify the subgroups with significantly different responses.

Changes in body weight and differences in ankle width were log transformed and $\sqrt{+1}$ transformed, respectively, in order to create normally distributed data. Analyses of variance followed by the least significant difference test were used to compare means. Intent to treat analysis is not used for dose-finding laboratory animal experiments.

## RESULTS

*Toxicity: mortality and body weights*. None of the positive control animals (0/20) and negative control animals (0/13) died prior to the completion of the protocol. Therefore, deaths of MTX-treated animals were regarded as due to drug toxicity. All animals treated with 5 MTX and 10 MTX died prior to the completion of the protocol and those doses were not continued further. The mean age at death was 57 (range 55-61) and 55 (range 52-59) days in these 2 groups, respectively. Fifty percent (4/8) of the animals in the 2 MTX and 3 MTX groups died prior to the completion of the protocol or on the day that the protocol was completed. The mean age at death was 68 and 61 days in these 2 groups, respectively. No deaths occurred during the protocol in the 0.3 MTX or 1 MTX groups.

The mean differences in body weight change at 77 days of age are listed in Table 1. Maximum weight gain was seen in the 1 MTX group at 77 days of age. The 1 MTX group showed significantly greater weight gain than the 0.3 MTX group, the 2 MTX + 3 MTX group and the positive controls. The weight gain of the 1 MTX group was not significantly different from the negative controls.

*Presence of arthritis*. The presence of AA in the positive control group was 75% (15/20), 75% (15/20), and 100% (20/20) by clinical, histopathological, and radiographic criteria, respectively. These data as well as the data for the 0.3, 1, and 2 + 3 MTX group are shown in Table 2. There is an apparent lack of consistency in the presence of arthritis as measured by the 3 methods. Arthritis was found more frequently by radiographic examination than by clinical or histopathological examination, especially at the higher MTX doses (e.g., 1 MTX group and the 2 + 3 MTX group) (Table 2).

*Table 1*. Mean body weight changes (in grams) (from 43 to 77 days of age) in MTX-treated animals with AA.

| Group (n) | Mean (± SD)* |
|---|---|
| 0 MTX (Positive Control) (20) | 53 (± 13)[a] |
| 0.3 MTX (12) | 68 (± 15)[b] |
| 1 MTX (12) | 78 (± 7)[c] |
| 2 MTX + 3 MTX (9) | 62 (± 16)[a, b] |
| Negative Control (13) | 76 (± 8)[c] |

*Means with different letter superscripts are different from each other ($p < 0.05$).

*Table 2*. Presence of AA by clinical (edema), histopathologic and radiographic examination in MTX dose groups.

| Group (n) | Edema (%) | Histopathologic (%) | Radiographic (%) |
|---|---|---|---|
| 0 MTX (Positive Control) (20) | 75 | 75 | 100 |
| 0.3 MTX (12) | 50 | 67 | 100 |
| 1 MTX (12) | 0 | 58 | 75 |
| 2 + 3 MTX (9) | 0 | 44 | 89 |
| Negative controls (13) | 0 | 54 | 54 |

*Table 3*. Response to MTX as measured by mean edema scores*.

| Group (n) | 53–56 Days of Age | 60–63 Days of Age | 67–70 Days of Age |
|---|---|---|---|
| 0 MTX (Positive Control) (20) | 6.6 ± 5.3 | 7.1 ± 5.6 | 7.8 ± 5.1 |
| 0.3 MTX (12) | 2.2 ± 4.4 | 2.8 ± 4.7 | 2.6 ± 4.4 |
| 1 MTX (12) | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| 2 + 3 MTX (9) | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| Negative controls (13) | 0 ± 0 | 0 ± 0 | 0 ± 0 |

*The means (± sd) are calculated from an average of 2 edema scores per animal. Maximum edema score is 16.

*Clinical response*. Table 3 shows the mean edema scores during the final weeks of the protocol. Using this measure, the 2 highest doses of MTX completely prevented rat AA. Table 4 displays differences in ankle widths as an indicator of severity of inflammation. Since the animals are both growing and potentially have arthritis, their right and left ankle widths were subtracted from the mean right and left ankle widths of age matched animals not receiving adjuvant (i.e., the negative controls). Thus, a positive difference in ankle widths will indicate relative swelling of that ankle for that age. As expected, differences in ankle widths of the positive controls were greater than those of animals treated with MTX. At 77 days of age, there was a trend in the data which suggested that the 1 MTX dose was better than the higher doses (i.e., 2 and 3 MTX).

*Radiographic response*. Radiographic scores are shown in Table 5. The 1 MTX dose resulted in mean total radiographic scores that were not different from negative controls. All other adjuvant treated animals had mean total radiographic scores greater than the negative controls (Table 5). The 1 MTX dose produced a statistically better result in suppressing severity of inflammation than did higher or lower MTX doses. Both the 1 MTX and the 2 and 3 MTX doses reduced the severity of tissue destruction to about the same extent and neither was statistically different from the negative controls.

*Table 4*. Response to MTX as measured by mean differences (±SD) in ankle widths (mm) in animals with AA by MTX dose.

| Group in mg/kg/week (n) | 77 Days of Age |
|---|---|
| 0 MTX (Positive Control) (20) | $1.56^{a}$ (± 1.74) |
| 0.3 MTX (12) | $0.36^{b}$ (± 1.21) |
| 1 MTX (12) | $-0.11^{c}$ (± 0.25) |
| 2 + 3 MTX (9) | $-0.03^{b,c}$ (± 0.19) |

The data were generated by subtracting the right and left ankle widths of adjuvant-treated animals from the average of the right and left ankle widths of negative control animals, respectively at the 2 time periods. Means with different superscript letters are significantly different from each other, $p < 0.05$.

*Table 5*. Mean (± sd) radiographic scores of hind limbs in animals with AA treated with MTX.

| Group (n) | Score indicating inflammation[3] | Score indicating tissue destruction[3] | Total score |
|---|---|---|---|
| Negative Control (13) | $0.5^{a}$ (± 1.0) | $0.8^{a}$ (± 1.4) | $1.3^{a}$ (± 2.4) |
| 0 MTX (Positive Control) (20) | $8.2^{b}$ (± 6.1) | $10.0^{b}$ (± 9.3) | $17.8^{b}$ (± 15.0) |
| 0.3 MTX (12) | $3.6^{c}$ (± 6.3) | $4.8^{c}$ (± 7.4) | $8.5^{c}$ (± 13.5) |
| 1 MTX (12) | $0.7^{a}$ (± 1.0) | $0.8^{a}$ (± 1.1) | $1.5^{a,d}$ (± 1.9) |
| 2 + 3 MTX (9) | $1.7^{c}$ (± 1.5) | $0.6^{a}$ (± 1.0) | $2.3^{c,d}$ (± 1.8) |

Means with different letter superscripts are significantly different from each other, $p < 0.05$. The right and left hind limb of each animal was examined. All animals compared survived until 77 days of age.

*Table 6.* Mean (± sd) histopathologic scores in animals with AA receiving different doses of MTX.

| Group (n) | Inflammation | Tissue Destruction |
|---|---|---|
| Negative Control (13) | 0.42[a] (± 0.33) | 0.23[a] (± 0.37) |
| 0 MTX (Positive Control) (20) | 3.24[b] (± 2.71) | 2.15[b] (± 1.74) |
| 0.3 MTX (12) | 2.17[b,c] (± 2.46) | 1.50[b,c] (± 1.61) |
| 1 MTX (12) | 0.75[a,c] (± 0.38) | 0.54[a,c] (± 0.55) |
| 2 + 3 MTX (9) | 0.83[a,b,c] (± 0.82) | 0.50[a,c] (± 0.82) |

Means with different letter superscripts are significantly different from each other, $p < 0.05$. The right hind limb of each animal was examined. All animals compared survived until 77 days of age.

*Histopathologic response*. As shown in Table 6, the 1 MTX dose group was the only group that had a statistically significant lower mean severity of inflammation score when compared to the positive controls. Mean severity of tissue destruction histopathological scores were approximately the same for the 1, 2, and 3 MTX dose groups.

*MTX dose response relationship*. Figure 1 shows the plot of the log of mean radiographic and histopathologic scores versus the dose of MTX used. The log of the mean scores is utilized since changes that are proportionally similar (but different in absolute amount) will have a similar change on the *y* axis. Thus, the radiographic or histopathologic scores for an unaffected hind limb should be the same as the negative controls and the approach to these values by ever smaller increments produced by increasing drug dose is best displayed using the log of the mean scores. As shown in Figure 1, increasing doses of MTX produce a normal (i.e., upward or plateauing) dose response curve when considering mean radiographic or histopathologic scores for severity of tissue destruction. Mean scores at the highest MTX dose level approach those of animals not given adjuvant (i.e., negative controls) and thus are free of disease. The dose response for the tissue destruction was expected. That, however, cannot be said for inflammation as determined both radiographically and histopathologically which peaks in the 1 MTX group only to diminish at higher and lower doses. However, only mean radiographic scores for inflammation were significantly different between the 1 MTX versus the 2 and 3 MTX groups.

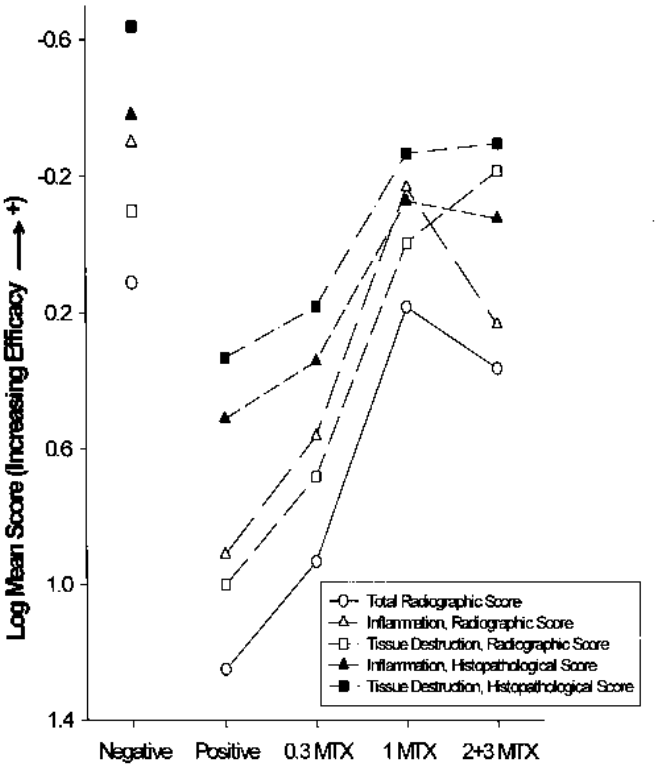


*Figure 1.* Log of mean radiographic and histopathologic scores versus MTX dose. Negative control (no arthritis), 0 MTX (positive control), 0.3 MTX, 1 MTX and 2 + 3 MTX groups are plotted.

## DISCUSSION

Rat AA is a T lymphocyte dependent, chronic, erosive polyarthritis. It is widely used as a reproducible model of RA because of its ease of induction, assessment and clinical features similar to RA[1]. Rat AA was produced in a large percentage of the positive controls; therefore, reductions in the presence and severity of this arthritis are likely to be due to the effect of MTX. In this animal model, MTX is given to the animals prior to the gross appearance of clinical disease because the arthritis rapidly becomes severe and does not respond to MTX if given after the disease has become established[4,6,7,9,18-20]. Because this was a dose finding animal experiment and the animals died in some groups, we did not use intention to treat statistical methods.

Our current trial suggests that 1 mg/kg/week MTX produced optimal efficacy and higher doses were more toxic than the study of Suzuki, *et al* in which 3 mg/kg/week produced optimal suppression of arthritis[10]. Baggott, *et al* found that a 2.7-mg MTX/kg/week dose produced good efficacy in rat AA[20]. These earlier studies used a different diet (i.e., rodent lab blocks *vs* defined diet). The laboratory chow used previously has variable protein and fat sources and possibly micronutrient composition when compared to the defined diets (personal communication, Dr. Merle Stillions, Harlan-Teklad, Madison, WI). The different diets may have produced differences in inducible liver enzymes that metabolize MTX. For example, the metabolism of MTX to 7-hydroxy-MTX substantially reduces its efficacy in rat AA[20]. It may be necessary to feed defined diets to obtain very reproducible results in this animal model.

We cannot conclude that the dose response relationship was the expected one when plotting mean radiographic and histopathologic scores for severity of inflammation or even the total radiographic score. All 3 of these variables indicate more (not less) disease activity when the dose is increased from 1 MTX to either 2 or 3 MTX (see Figure 1). The same

pattern in dose and efficacy is observed with mean differences in ankle widths where maximum suppression in swelling occurs at the 1 MTX, not the higher MTX dose groups (Table 4). However, ankle widths may have been increased in the higher doses of MTX because of protein calorie malnutrition-induced edema. A similar pattern is observed in the frequency of arthritis measured by radiographic data. Table 2 shows that a greater frequency of arthritis is observed in the 2 and 3 MTX dose groups vs the 1 MTX dose group. Although some of these differences were not statistically different, there is a clear trend for reduced (not increased) efficacy at the highest MTX dose.

Results similar to our dose response pattern have previously been reported. Galivan reported an unexpected dose response to therapy in streptococcal cell wall arthritis[4]. An intermediate dose of MTX 15 $\mu$g twice a week was much better in suppressing the arthritis than the higher dose of 120 $\mu$g per week. In a study of similar design to ours, Kawai, *et al*[7] was able to demonstrate MTX efficacy in rat AA at 0.1 and 0.2 mg/kg/week. Although these low doses of MTX did not normalize radiographic findings, bone erosions seemed to be more consistently reduced in a dose dependent manner than did periostitis. Thus, these researchers may have also detected a difference in inflammation and tissue destructive response to the MTX dose. Using the MRL mouse model of arthritis, Baggott, *et al* found that an intermediate MTX dose was better in suppressing histopathologic features of arthritis than was the highest MTX dose[19]. Thus, histopathologic scores for diffuse inflammatory synovitis and synovial vasculitis actually increased in the MRL/*lpr* model as the MTX dose was increased from a moderate to the highest level[19]. Since these features are measurements of inflammation, the MRL/*lpr* model gave results similar to those we report for the rat AA model. On the other hand, several other research groups have apparently observed an expected dose efficacy curve for MTX treatment of rat AA[6,8-10,20].

In RA, an expected dose efficacy relationship has been observed[11] with doses of 5 mg/m$^2$, 10 mg/m$^2$, and 20 mg/m$^2$. There is a report of MTX efficacy at a dose of 500 mg/m$^2$ in patients failing low dose MTX therapy[14]. Shiroky, *et al* documented 50% or greater clinical improvements in 5 out of 8 clinical variables[12,13]. One may have expected higher doses of MTX to produce much better efficacy.

There is a disagreement in the literature whether or not MTX inhibits disease progression as measured progression of radiographic changes including bone erosion and joint narrowing in RA. Rau, *et al*[21] evaluated 31 patients (who had been unresponsive to gold salts) after 24 months of MTX therapy. He determined that radiographic progression of disease was slower during MTX treatment than during gold therapy. However, Nordstrom, *et al*[22] found that pulse MTX for an average of 30 weeks did not significantly slow the rate the joint deterioration. In the Cooperative Systematic Studies of the Rheumatic Diseases program, which compared auranofin and MTX alone and their combination, there was a worsening in the joint erosion score and joint narrowing in all 3 treatment groups[23]. However, the patients receiving MTX progressed at slower rates than those who received auranofin. Studies evaluating azathioprine, MTX, and their combination showed a trend towards decreased radiographic progression in patients treated with MTX[24,25]. Maravic, *et al* found mild radiographic progression in patients with early RA treated with MTX for one year[26,27]. Rich, *et al* determined that patients who start MTX without erosions have slower disease progression than patients who start the drug with erosions already present[27,28]. These discrepancies probably relate to which point in the course of disease MTX was used with most studies showing benefit including either patients with relatively early disease or those in whom MTX is used as the first disease modifying antirheumatic drug[29-32]. Our data in rat AA tend to support the contention that MTX can slow the progression of radiographic disease in RA. Whether or not MTX has an antiinflammatory effect in RA is also controversial[33]. In our animal model, the antiinflammatory effects of MTX reached a maximum at moderate doses and decreased at lower and higher doses. This unexpected dose response could account for the variability in antiinflammatory effects in other animal models. Kirwan and Bresnihan[33,34] discuss the possibility of tissue destruction in RA with and without inflammation. It has been shown in the severe combined immunodeficiency mouse model that human synovial fibroblasts, in the absence of human inflammatory cells, continue to invade and destroy human cartilage[35]. The same observation was made in a patient with acquired immunodeficiency syndrome and RA[36]. These results support the view that tissue destruction in RA may continue in the absence of inflammation. Our dose response results in rat AA also support an alternative model of RA in which synovial inflammation (i.e., pain and swelling) are separate phenomenona from synovial hyperplasia with joint destruction. Inflammation and tissue destruction may not necessarily parallel each other; these findings are in agreement with those of other investigators[34,35].

Our data demonstrate that 1 mg/kg/week is the optimal dose of MTX in our rat AA model. This dose effectively reduced swelling, ankle widths, radiographic and histopathological scores with no toxicity. However, the reduction of severity of tissue destruction and inflammation did not move in concert as the dose of MTX increased. If the rat AA data can be translated to the human condition, then increasing the MTX dose in a patient with RA may not necessarily increase its antiinflammatory effect; it may actually decrease it. Thus, the variable effect of MTX on inflammation in RA may be due to dose levels used that are either lower or higher than the optimal one. Finally, there are likely to be metabolic, biochemical, or pharmacologic reasons for this unexpected finding and further studies are underway.

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