ARTHRITIS & RHEUMATISM
Vol. 43, No. 3, March 2000, pp 656–663

REVERSAL OF THE ANTIINFLAMMATORY EFFECTS OF METHOTREXATE BY THE NONSELECTIVE ADENOSINE RECEPTOR ANTAGONISTS THEOPHYLLINE AND CAFFEINE

Evidence that the Antiinflammatory Effects of Methotrexate are Mediated Via Multiple Adenosine Receptors in Rat Adjuvant Arthritis

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Objective. **Weekly low-dose methotrexate (MTX) remains the mainstay of second-line therapy for rheumatoid arthritis (RA). We have previously reported that adenosine, acting at specific receptors on inflammatory cells, mediates the antiinflammatory effects of MTX in both in vitro and in vivo models of acute inflammation, but the mechanism by which MTX suppresses the chronic inflammation of arthritis remains controversial. The present study was undertaken to further investigate the means by which adenosine mediates the antiinflammatory effects of MTX.**

Methods. **The effects of 2 nonselective adenosine receptor antagonists, theophylline and caffeine, were examined, using the rat adjuvant arthritis model of RA. These agents were given alone and in conjunction with MTX, and arthritis severity was assessed clinically, radiologically, and histologically. Since rodent adenosine A_3 receptors are not blocked by theophylline, selective A_1, A_{2A}, and A_{2B} receptor antagonists were tested as well.**

Results. **Control animals developed severe arthritis, which was markedly attenuated by weekly treatment with MTX (0.75 mg/kg/week). Neither theophylline alone nor caffeine alone (each at 10 mg/kg/day) significantly affected the severity of the arthritis, but both agents markedly reversed the effect of MTX as measured by a severity index, hindpaw swelling, and hindpaw ankylosis. Radiographic and histologic analyses confirmed these observations. Neither A_1, A_{2A}, nor A_{2B} receptor antagonists affected the capacity of MTX to ameliorate inflammation in adjuvant arthritis.**

Conclusion. **These results provide strong evidence that adenosine mediates the antiinflammatory effects of MTX in this model of RA. Moreover, the findings suggest that abstinence from caffeine, a ubiquitous food additive and medication, may enhance the therapeutic effects of MTX in RA.**

Low-dose, intermittently administered methotrexate (MTX) is among the most widely used forms of therapy for inflammatory arthritis (particularly rheumatoid arthritis [RA]), psoriasis, and inflammatory bowel disease. MTX was introduced for the treatment of inflammatory diseases, with very little understanding of its mechanism of action. We, and subsequently others, have reported that the antiinflammatory actions of MTX are mediated by its capacity to increase extracellular adenosine concentrations (1–4). However, the studies reported to date have demonstrated that adenosine is responsible for the antiinflammatory actions of MTX only in acute inflammation; the mechanism of action of MTX in the treatment of chronic inflammation has not been fully explored.

Supported by grants from the NIH (AR-41911, GM-56268, and HL-1972), by the General Clinical Research Center (M01-RR-00096), and by the Kaplan Cancer Center (CA-160877). Dr. Montesinos was the recipient of a fellowship from the S.L.E. Foundation, Inc. Ms Posadas was the recipient of a fellowship from the Generalitat Valenciana.

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Submitted for publication July 26, 1999; accepted in revised form December 1, 1999.

It has been known since the work of Sattin and Rall (5) that adenosine modulates cellular behavior by interacting with specific receptors on the cell surface. It was subsequently recognized, using pharmacologic methods, that there were 2 distinct adenosine receptor subtypes, (6,7) and, more recently, cloning techniques have revealed the existence of at least 4 subtypes (A_1, A_{2A}, A_{2B}, and A_3) (for review, see ref. 8). Most of the known antagonists at adenosine receptors are methylxanthines, as documented in receptor binding and other pharmacologic experiments (9,10), and it is now generally accepted that the pharmacologic effects of theophylline and caffeine, 2 methylxanthines that are commonly encountered in medications and in foods and beverages, are mediated by antagonism of adenosine at its receptors (11). Pharmacologic studies with the murine air pouch model of acute inflammatory disease demonstrate that MTX-mediated increases in exudate adenosine inhibit inflammation via interaction with an A_2 (probably A_{2A}) receptor. Other pharmacologic studies have indicated that adenosine may also act at A_1 or A_3 receptors to inhibit inflammation (12–16).

To better understand the mechanism of action of MTX in the treatment of RA, we investigated whether the nonselective adenosine receptor antagonists theophylline (an agent that nonselectively blocks A_1, A_{2A}, and A_{2B}, but not A_3, adenosine receptors in the rat [17]) and caffeine (which blocks all receptors [9,10]), or more selective adenosine receptor antagonists reverse the antiinflammatory actions of MTX in the adjuvant arthritis model. We found that MTX inhibited the development of adjuvant arthritis and that blockade of A_1, A_{2A}, and A_{2B} receptors, but not the individual receptors alone, reversed the antiinflammatory effects of MTX.

MATERIALS AND METHODS

Materials. Heat-killed *Mycobacterium butyricum* was purchased from Difco (Detroit, MI), and Freund's complete adjuvant (CFA) was mixed as a 1% (weight/volume) suspension of the heat-killed bacteria in heavy mineral oil (Sigma, St. Louis, MO). MTX was purchased from Immunex (San Juan, PR), and methylprednisolone was purchased from Upjohn (Kalamazoo, MI). Theophylline, enprofylline, and caffeine were obtained from Sigma. 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) was obtained from Research Biochemicals (Wayland, MA), and ZM241385 was from Tocris-Cookson (Ballwin, MO). All other reagents used were the highest quality that could be obtained.

Animals. Female 8–12-week-old Lewis rats (Charles River, Wilmington, MA) weighing 130–190 gm were studied. The rats were housed in the New York University (NYU) animal facility, fed regular rat chow, and given access to drinking water ad libitum.

Induction of adjuvant arthritis. Arthritis was induced on day 0, by injection of 0.1 ml of CFA into the base of the tail. Synovitis developed 7–10 days postimmunization in 100% of the rats that did not receive any other treatment (18–20).

Treatment regimens. Animals were treated with a single weekly intraperitoneal injection of MTX (0.75 mg/kg/week in 1 ml of phosphate buffered saline) or a similar volume of saline, starting on the day of the injection of CFA (day 0) and continuing for the full 4 weeks of the experiment. Adenosine receptor antagonists were mixed into the drinking water of groups of animals to achieve a dosage of 10 mg/kg/day (adjusted daily to account for the weight and water intake of the animals); this dosage was higher than those previously reported to achieve effective levels in rats (21–24). All of these treatments were reviewed and approved by the Institutional Animal Care and Use Committee of NYU Medical Center and carried out under the supervision of the facility veterinary staff.

In each experiment, groups of 4–6 animals were treated as described, and each drug or combination was tested on at least 2 separate occasions. The control and MTX-treated groups were pooled from all of the experiments performed and consisted of 30 rats and 20 rats, respectively.

Arthritis assessments. The progress of arthritis was monitored by determining the ankle joint width, global arthritis severity index for swelling and erythema in 60 joints (scored on a scale of 0–3, with 0 representing no change and 3 representing most severe changes; maximum score of 180), and percentage of animals developing ankle joint ankylosis (assessed by the ability to extend/flex the joint). All measurements were performed on day 0 and biweekly for the duration of the study. Body weight was measured on day 0 and then weekly (18–20).

At the end of day 28, the rats were killed by Co_2 administration and, in some experiments, total-body radiographs were obtained (anteroposterior and lateral views), using a General Electric portable x-ray machine with a 3-second exposure (60-cm film-to-source distance). Radiographic scoring (18–20) was done based on the degree of soft tissue swelling, extent of bone erosion/destruction, bone mineralization, and joint space narrowing at both ankle joints. Radiographs were scored on a scale of 0–3 (0 = normal, 3 = maximum joint destruction) for each limb, by an observer who was blinded to the treatment group. The radiographic joint index score was then determined; this score represents the mean of the scores for both hind limbs from each rat, with a maximum possible score of 3 per rat.

Histopathologic analysis. Immediately after radiography, the hind limbs were removed just distal to the knee and placed in 10% buffered formalin. The fixed tissues were then decalcified and slides of sagittal slices through the hindpaw, stained with hematoxylin and eosin, were prepared using standard techniques. Slides were reviewed for soft tissue swelling, bone demineralization, pannus formation, cartilage erosions, and joint space narrowing.

Statistical analysis. The data were analyzed by analysis of variance, followed by analysis of differences between groups using Tukey's highest significant difference test performed with SigmaStat software (SPSS, Chicago, IL). All values are

reported as the mean ± SEM with the exception of ankylosis, which is reported as a simple percentage of the number of ankles.

RESULTS

Arthritis developed between 7 days and 10 days after injection and, similar to the findings in prior studies, disease in the control animals was characterized by increasing activity until day 20 and persistent joint inflammation through at least day 28 (at which time the experiment was terminated). The animals that were not injected with CFA did not develop arthritis (Figure 1). MTX treatment markedly attenuated the arthritis ($P < 0.00001$) (Figure 1A). Treatment with theophylline alone, a methylxanthine that blocks A_1, A_{2A}, and A_{2B}, but not A_3, adenosine receptors (17), appeared to diminish the activity of the arthritis at the early time points, although this difference did not reach statistical significance. More interestingly, theophylline markedly reversed the effect of MTX ($P < 0.003$) (Figure 1A). Injection of depot methylprednisolone completely abrogated the development of arthritis, and theophylline did not reverse the effect of methylprednisolone treatment (mean ± SEM severity index 0 ± 0 in rats treated with either methylprednisolone or methylprednisolone + theophylline, on any day of measurement).

As a separate indicator of arthritis activity, we measured hindpaw width. Injection of CFA caused a marked increase in the width of the hindpaw (Figures 1B and 2). Again, MTX significantly attenuated the hindpaw swelling in the CFA-treated rats ($P < 0.0001$). Theophylline did not diminish the increase in hindpaw width in these animals, but it partially reversed the antiinflammatory effect of MTX on CFA-induced hindpaw swelling, although this difference did not reach statistical significance. The greater severity of arthritis in the theophylline + MTX–treated rats, described above, appeared more marked than the difference in hindpaw swelling because it reflects, in addition to hindpaw swelling, involvement of a greater number of joints with more pain and ankylosis.

Ankylosis of the ankle joints was also assessed as an indicator of joint inflammation and destruction. Ankylosis was observed by the end of the study period in 94% of the ankles of the animals treated with either CFA alone or CFA + theophylline (Figure 1C). MTX diminished the percentage of animals that developed ankylosis, to 25%. The antiinflammatory effect of MTX was almost completely reversed by theophylline (63% of

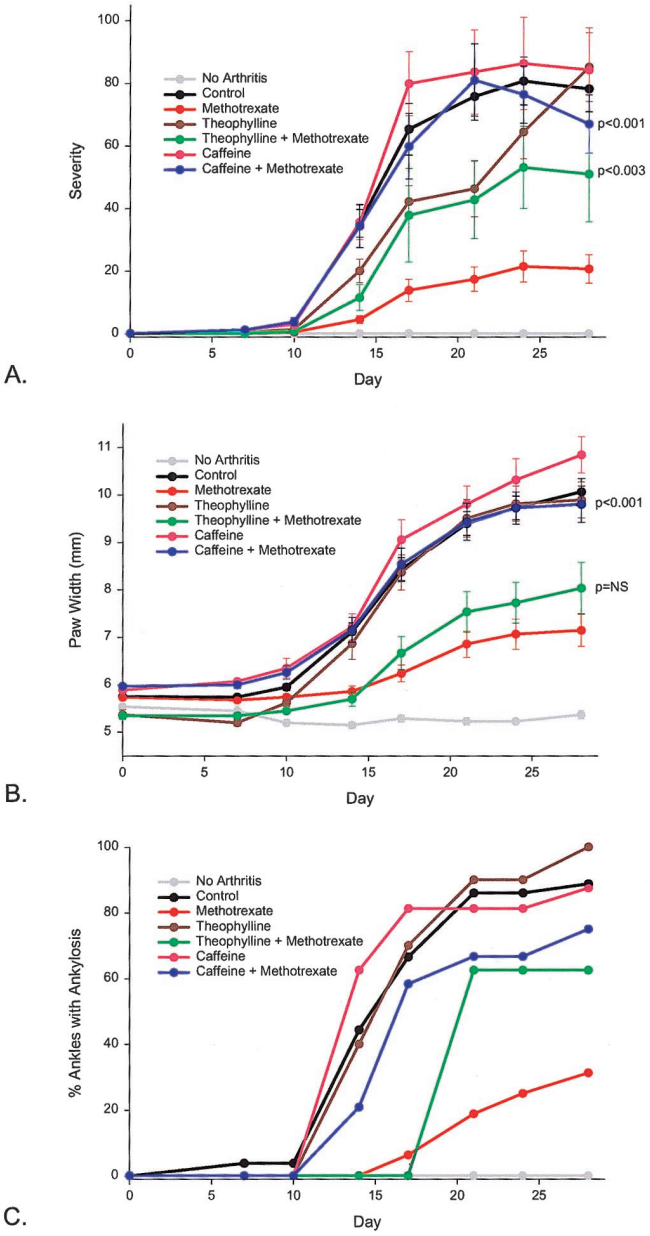

Figure 1. Effects of theophylline and caffeine on methotrexate (MTX) inhibition of the development of adjuvant arthritis. Rats were injected with Freund's complete adjuvant on day 0 and then, starting on day 0, were given either a weekly intraperitoneal injection of MTX (0.75 mg/kg/week) or an equal volume of saline. Severity index (**A**), hindpaw width (**B**), and ankylosis (**C**) were assessed twice weekly, as described in Materials and Methods. Treatment groups were as follows: 30 control rats, 20 MTX-treated rats, 8 theophylline-treated rats, 9 theophylline + MTX–treated rats, 12 caffeine-treated rats, and 12 caffeine + MTX-treated rats. In **A** and **B,** values are the mean ± SEM; in **C,** values are the mean. *P* values are versus the MTX-treated group. NS = not significant.

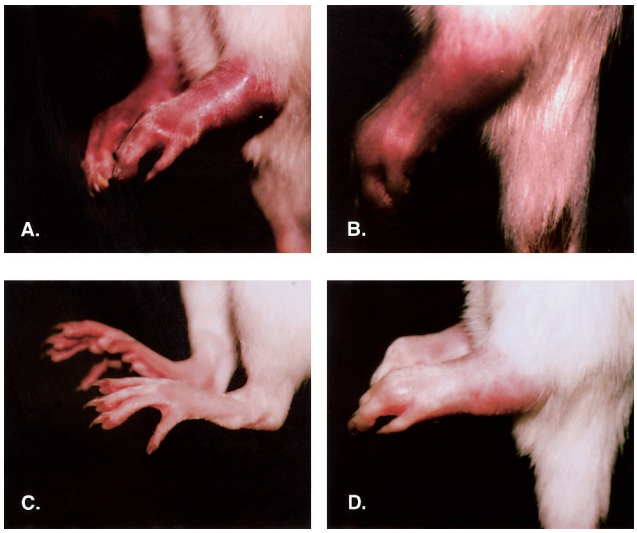

Figure 2. Effects of theophylline on methotrexate (MTX) inhibition of the development of adjuvant arthritis. Rats were injected with Freund's complete adjuvant (CFA) on day 0 and then, starting on day 0, were given either a weekly intraperitoneal injection of MTX (0.75 mg/kg/week) or an equal volume of saline. Shown are the hindpaws of representative rats from each group, photographed on day 21. **A,** Rat treated with CFA alone. **B,** Rat treated with CFA + theophylline (10 mg/kg/day). **C,** Rat treated with CFA + MTX. **D,** Rat treated with CFA + MTX + theophylline.

the animals treated with MTX alone developed joint ankylosis).

Analysis of joint radiographs at the termination of the experiment revealed changes consistent with those observed by physical examination. Both in the animals treated with CFA alone and in those treated with CFA + theophylline, there was complete destruction of the ankle joints (Table 1 and Figure 3). MTX treatment markedly diminished joint destruction. Again, coadministration of theophylline with MTX reversed the antiinflammatory effects of the latter compound ($P < 0.05$) (Table 1).

Histologic analysis (Figure 4) confirmed the clinical and radiologic findings. There was infiltration with inflammatory cells and almost complete loss of the normal joint architecture in the CFA-treated animals, and theophylline did not alter the histologic findings indicating joint destruction. MTX treatment preserved much of the joint architecture but theophylline completely reversed the effect of MTX, as reflected by the histologic changes.

To confirm that the effects of theophylline on MTX-mediated inhibition of inflammation were caused by adenosine receptor blockade in this model, we investigated whether another nonselective methylxanthine adenosine receptor antagonist, caffeine, also reversed the antiinflammatory effects of MTX. Like theophylline, caffeine alone did not significantly affect the onset or severity of arthritis in the rats (Figure 1A). Also like theophylline, caffeine reversed the antiinflammatory

Table 1. Effects of theophylline on methotrexate (MTX)–mediated reduction of radiologic joint damage in adjuvant arthritis*

	Radiographic index, mean ± SEM	
	CFA	CFA + theophylline
No treatment	3.0 ± 0.0	2.7 ± 0.3
MTX, 0.75 mg/kg/week	0.3 ± 0.3†	1.6 ± 0.3‡

* Joint radiographs were obtained in 4 rats from each group and scored on a scale of 0–3 (0 = normal; 3 = complete destruction of the ankle joint), by an observer who was unaware of the treatment (see Materials and Methods). CFA = Freund's complete adjuvant.
† $P < 0.01$ versus no treatment.
‡ $P < 0.05$ versus MTX alone.

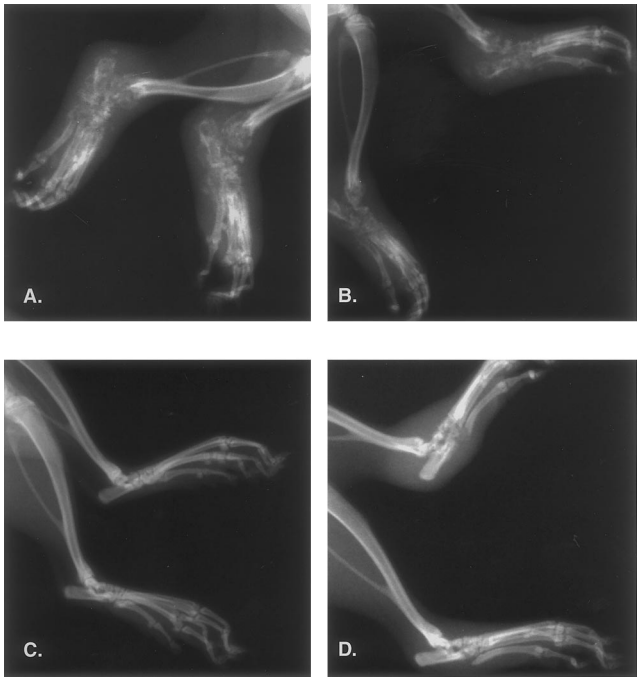

Figure 3. Effects of theophylline on MTX prevention of radiologic joint destruction in adjuvant arthritis. Rats were injected with CFA on day 0 and then, starting on day 0, were given either a weekly intraperitoneal injection of MTX (0.75 mg/kg/week) or an equal volume of saline. Shown are representative radiographs obtained after the rats were killed on day 28. **A,** Rat treated with CFA alone. **B,** Rat treated with CFA + theophylline (10 mg/kg/day). **C,** Rat treated with CFA + MTX. **D,** Rat treated with CFA + MTX + theophylline. See Figure 2 for definitions.

effects of MTX, whether measured as severity ($P < 0.001$), hindpaw width ($P < 0.001$), ankylosis, or radiologic changes ($P < 0.007$) (Figures 1A–C and Table 2). In contrast to either theophylline or caffeine, methylxanthines given in doses that have been administered to animals to selectively antagonize A_1 (DPCPX), A_{2A} (ZM241385), or A_{2B} (enprofylline [25]) receptors did not affect the capacity of MTX to diminish inflammation in this model (data not shown). Combinations of the more selective antagonists were toxic to the animals (causing cachexia, weight loss, and hair loss), and their effect on inflammation could not be evaluated.

During these experiments the animals continued to gain weight until they developed severe arthritis, at which point they began to lose weight, although none of the animals lost more than 20% of their pretreatment weight. Treatment with individual agents did not appear to have a direct effect on the rate of weight gain (26).

DISCUSSION

To date, the mechanism of action of MTX in the treatment of inflammatory arthritis has not been fully established (for review, see ref. 27). We report here that theophylline and caffeine, 2 chemically related, nonselective adenosine receptor antagonists, reverse the antiinflammatory effects of MTX in the adjuvant arthritis model of RA. This is the first direct demonstration in an in vivo model that adenosine mediates the antiinflammatory effects of MTX in chronic inflammatory arthritis. The results of the experiments reported here are consistent with the prior demonstration that adenosine mediates the antiinflammatory effects of MTX in acute inflammation both in vitro and in vivo (1–3). In contrast to prior reports, however, the present results indicate that adenosine must ligate multiple receptors in order to suppress chronic inflammation.

Humans have ingested caffeine in tea, coffee, and chocolate since time immemorial, and theophylline has been used therapeutically for nearly half a century, although its mechanism of action remains in dispute. Currently, theophylline and caffeine are thought to exert their pharmacologic effects primarily by acting as adenosine receptor antagonists (11) or by inhibiting cellular phosphodiesterases (28,29).

Phosphodiesterase inhibition is thought to account for the effects of theophylline in the treatment of asthma, despite the fact that concentrations required to inhibit phosphodiesterase are much greater than those achieved therapeutically. Theophylline and other methylxanthine and non-methylxanthine phosphodiesterase inhibitors raise intracellular cAMP concentrations. Intracellular cAMP in elevated levels suppresses inflammatory cell function and inflammation (28,29), and it has been suggested that this underlies the antiinflammatory effects of theophylline (30–32). Indeed, the use of phosphodiesterase inhibitors (including non-methylxanthine phosphodiesterase inhibitors) has been advocated for the treatment of asthma (28), and phosphodiesterase inhibitors suppress the inflammation of adjuvant arthritis (33) as well. Neither theophylline nor caffeine prevented or augmented the development of adjuvant arthritis in rats that were not exposed to MTX. Moreover, the selective adenosine A_{2B} receptor antag-

Table 2. Effects of caffeine on methotrexate (MTX)–mediated reduction of radiologic joint damage in adjuvant arthritis*

	Radiographic index, mean ± SEM	
	CFA	CFA + caffeine
No treatment	2.4 ± 0.2	2.0 ± 0.5
MTX, 0.75 mg/kg/week	0.7 ± 0.2†	2.1 ± 0.3‡

* Joint radiographs were obtained in 4 rats from each group and scored on a scale of 0–3 (0 = normal; 3 = normal to complete destruction of the ankle joint), by an observer who was unaware of the treatment (see Materials and Methods). CFA = Freund's complete adjuvant.
† $P < 0.001$ versus no treatment.
‡ $P < 0.007$ versus MTX alone.

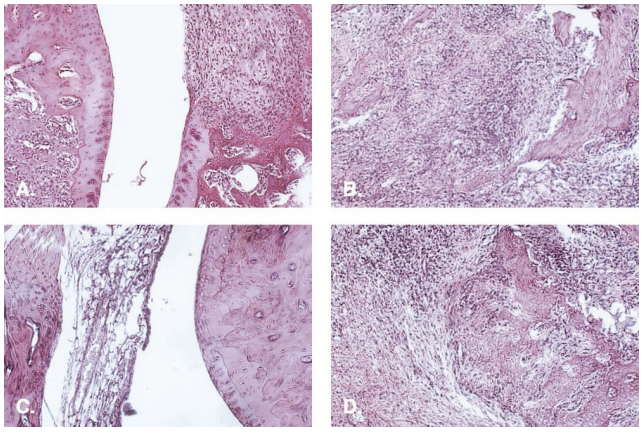

Figure 4. Effects of theophylline on MTX prevention of histologic features of joint destruction in adjuvant arthritis. Rats were injected with CFA on day 0 and then, starting on day 0, were given either a weekly intraperitoneal injection of MTX (0.75 mg/kg/week) or an equal volume of saline. Shown are representative histologic sections obtained after the rats were killed on day 28. **A,** Rat treated with CFA alone. **B,** Rat treated with CFA + theophylline (10 mg/kg/day). **C,** Rat treated with CFA + MTX. **D,** Rat treated with CFA + MTX + theophylline. See Figure 2 for definitions.

onist enprofylline, which is also a methylxanthine and is a more potent inhibitor of phosphodiesterase than is theophylline (34), neither diminished arthritis alone nor affected the capacity of MTX to diminish inflammation in this model. Thus, it is unlikely that phosphodiesterase inhibition accounts for the capacity of theophylline and caffeine to alter the effect of MTX observed in the model of inflammatory arthritis reported here.

Unlike more recently developed methylxanthine derivatives, theophylline and caffeine are nonselective adenosine receptor antagonists (10). We found that neither theophylline nor caffeine alone significantly altered the course of adjuvant arthritis in MTX-treated rats, an observation that suggests that endogenous adenosine levels in the inflamed joints are insufficient to diminish inflammation in this model. None of the agents interfered with the capacity of methylprednisolone to suppress the development of adjuvant arthritis, indicating that the reversal of the antiinflammatory effects of MTX by theophylline and caffeine is specific and limited to MTX. In light of the previous demonstration that adenosine mediates the antiinflammatory effects of MTX in acute inflammation (2,3), the results reported here are most consistent with the hypothesis that the dominant pharmacologic effect of theophylline and caffeine in this model of RA results from adenosine receptor antagonism.

The antiinflammatory effects, as well as other physiologic and pharmacologic effects, of adenosine are clearly mediated via adenosine receptors, and all 4 adenosine receptors appear to act, when occupied, as antiinflammatory receptors. We and others have demonstrated that the inhibitory adenosine receptors on neutrophils, the inflammatory cells involved in acute inflammation, are A_2 (most likely A_{2A}) receptors (for review, see ref. 35). This finding has been confirmed by our subsequent demonstration that an adenosine A_2 receptor antagonist reverses the antiinflammatory effects of MTX, and by inference adenosine, in the murine air pouch model of inflammation (2). Adenosine A_1 receptor agonists have been reported to be the most potent antiinflammatory adenosine receptor agonists in other in vivo models of acute inflammation (12,36), although this finding may be accounted for by the effects of adenosine, via A_1 receptors, on the central nervous system (15).

MTX treatment has been shown to inhibit expression of collagenase by synoviocytes in biopsy specimens from patients with RA, and this specific inhibition of collagenase expression is most likely mediated by adenosine A_{2B} receptors (37,38). Several groups have reported that adenosine A_3 receptors, when occupied, diminish synthesis and release of cytokines, such as tumor necrosis factor α, that are thought to play a central role in the pathogenesis of RA (13–16,39). Because A_3 receptors in rodents are insensitive to theophylline (17), our results are most consistent with the surprising finding that blockade of A_3 adenosine receptors does not contribute to the antiinflammatory effects of MTX in this model of arthritis.

The biochemical mechanism by which MTX promotes adenosine release is not fully established. MTX is taken up by cells and polyglutamated; the polyglutamates of MTX remain metabolically active (40,41). It was originally suggested that MTX polyglutamates potently inhibit an intermediate enzyme in de novo purine biosynthesis, i.e., phosphoribosylaminoimidazolecarboxamide (AICAR) transformylase (42,43), leading to intracellular accumulation of AICAR. Even the low doses of MTX used to treat inflammation in the mouse promote accumulation of AICAR in tissues (2), and recent studies confirm that long-term administration of MTX to rats with adjuvant arthritis promotes the accumulation of AICAR and its metabolites (44). Moreover, excretion of AICAR metabolites is increased in patients taking low-dose MTX for the treatment of psoriasis (45). The intracellular accumulation of AICAR has been associated with enhanced adenosine release (46), most likely as a result of AICAR-mediated inhibition of AMP deaminase (with extracellular accumulation of AMP). Indeed, the excess adenosine found in the supernates of MTX-treated cells or in the inflammatory exudates of MTX-treated mice is derived entirely from extracellular adenine nucleotides by the action of ecto–5′-nucleotidase, and the antiinflammatory effects of MTX in the murine air pouch model are completely blocked by ecto–5′-nucleotidase inhibitors (47). Alternatively, AICA-ribonucleoside inhibits adenosine deaminase, and this may also lead to adenosine accumulation (43,48–51). Whatever the mechanism, blood and urine adenosine concentrations are increased in patients who are taking MTX (45,52).

Although MTX is probably the most commonly used second-line agent for the treatment of RA, not all patients derive benefit from this drug, and treatment response is often less than complete (53). Caffeine is present in high concentrations in coffee, tea, chocolate, and soft drinks and as an ingredient of over-the-counter pain medications. The observation that caffeine completely reverses the antiinflammatory effects of MTX in this model of inflammatory arthritis suggests that avoidance of caffeine ingestion may enhance the efficacy of

MTX in the treatment of inflammatory diseases. However, before it can be recommended that patients taking MTX for the treatment of inflammatory arthritis avoid caffeine in their diets, further studies in humans should be undertaken.

The efficacy of low-dose MTX in the treatment of asthma is controversial (54–57). Our results suggest one explanation for inconsistency in the results of clinical trials of MTX in the treatment of this disease. Theophylline has long been used in the treatment of asthma, and theophylline usage by patients in these trials may have reversed or prevented any beneficial effects of MTX, thereby confounding the study results. Future studies of MTX for the treatment of asthma should control for theophylline use.

In conclusion, our findings further confirm the hypothesis that adenosine, generated endogenously, mediates the antiinflammatory effects of MTX, one of the most commonly used second-line drugs in the treatment of RA. These results indicate that other agents that promote adenosine release at sites of inflammation might also be useful for the treatment of RA and other forms of inflammatory arthritis and also suggest that avoidance of caffeine may enhance the efficacy of MTX in the treatment of inflammatory arthritis.

ACKNOWLEDGMENT

The authors wish to thank Dr. K. Haines for reviewing the manuscript.

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