The antiinflammatory mechanism of methotrexate. Increased adenosine release at inflamed sites diminishes leukocyte accumulation in an in vivo model of inflammation.

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Research Article

Methotrexate, a folate antagonist, is a potent antiinflammatory agent when used weekly in low concentrations. We examined the hypothesis that the antiphlogistic effects of methotrexate result from its capacity to promote intracellular accumulation of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) that, under conditions of cell injury, increases local adenosine release. We now present the first evidence to establish this mechanism of action in an in vivo model of inflammation, the murine air pouch model. Mice were injected intraperitoneally with either methotrexate or saline for 3-4 wk during induction of air pouches. Pharmacologically relevant doses of methotrexate increased splenocyte AICAR content, raised adenosine concentrations in exudates from carrageenan-inflamed air pouches, and markedly inhibited leukocyte accumulation in inflamed air pouches. The methotrexate-mediated reduction in leukocyte accumulation was partially reversed by injection of adenosine deaminase (ADA) into the air pouch, completely reversed by a specific adenosine A2 receptor antagonist, 3,7-dimethyl-1-propargylxanthine (DMPX), but not affected by an adenosine A1 receptor antagonist, 8-cyclopentyl-dipropylxanthine. Neither ADA nor DMPX affected leukocyte accumulation in the inflamed pouches of animals treated with either saline or the potent antiinflammatory steroid dexamethasone. These results indicate that methotrexate is a nonsteroidal antiinflammatory agent, the antiphlogistic action of which is due to increased adenosine release at inflamed sites.

The Antiinflammatory Mechanism of Methotrexate

Increased Adenosine Release at Inflamed Sites Diminishes Leukocyte Accumulation in an In Vivo Model of Inflammation

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Abstract

Methotrexate, a folate antagonist, is a potent antiinflammatory agent when used weekly in low concentrations. We examined the hypothesis that the antiphlogistic effects of methotrexate result from its capacity to promote intracellular accumulation of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) that, under conditions of cell injury, increases local adenosine release. We now present the first evidence to establish this mechanism of action in an in vivo model of inflammation, the murine air pouch model. Mice were injected intraperitoneally with either methotrexate or saline for 3–4 wk during induction of air pouches. Pharmacologically relevant doses of methotrexate increased splenocyte AICAR content, raised adenosine concentrations in exudates from carrageenan-inflamed air pouches, and markedly inhibited leukocyte accumulation in inflamed air pouches. The methotrexate-mediated reduction in leukocyte accumulation was partially reversed by injection of adenosine deaminase (ADA) into the air pouch, completely reversed by a specific adenosine A_2 receptor antagonist, 3,7-dimethyl-1-propargylxanthine (DMPX), but not affected by an adenosine A_1 receptor antagonist, 8-cyclopentyl-dipropylxanthine. Neither ADA nor DMPX affected leukocyte accumulation in the inflamed pouches of animals treated with either saline or the potent antiinflammatory steroid dexamethasone. These results indicate that methotrexate is a nonsteroidal antiinflammatory agent, the antiphlogistic action of which is due to increased adenosine release at inflamed sites. (*J. Clin. Invest.* 1993. 92:2675–2682.) Key words: leukocyte • adenosine • purine • inflammation • methotrexate

Introduction

Methotrexate is a folate antagonist first developed for the treatment of malignancies and now widely used in the treatment of rheumatoid arthritis (1). Unlike its use in the treatment of malignancies (pulses of 20–250 mg/kg), methotrexate is administered weekly in low doses (0.1–0.3 mg/kg) to treat rheumatoid arthritis and other inflammatory diseases (1). Although the original rationale for the use of methotrexate in the treatment of rheumatoid arthritis was "immunosuppression," the molecular mechanism by which methotrexate suppresses inflammation is not well understood. It is unlikely that, in the doses given, methotrexate diminishes proliferation of immune cells by inhibiting de novo purine and pyrimidine synthesis since leukopenia and mucosal ulcerations, phenomena best explained by the antiproliferative effects of methotrexate, are considered evidence of drug toxicity and indications to decrease or stop therapy. Other proposed mechanisms include a decrease in neutrophil (but not macrophage) leukotriene synthesis (2) and inhibition of transmethylation reactions by inhibiting the formation of *S*-adenosyl-methionine, a methyl donor required for protein and lipid methylation (3).

We have recently proposed an alternative biochemical mechanism of action of methotrexate; methotrexate promotes the release of the antiinflammatory autocoid adenosine at inflamed sites (4). Previous studies have suggested that methotrexate accumulates within cells and, both directly and indirectly, inhibits 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR)[1] transformylase, resulting in the intracellular accumulation of AICAR (Fig. 1; references 5–9). Increased intracellular concentrations of AICAR promote, by a complex mechanism, the increased release of the potent antiinflammatory autocoid adenosine (10, 11). Results of in vitro studies support this hypothesis (4); low concentrations ($\leq$ 10 nM) of methotrexate or higher concentrations of the cell-soluble, nonphosphorylated precursor of AICAR, AICARibonucleoside (acadesine), promote adenosine release from fibroblasts and endothelial cells. The increase in extracellular adenosine concentration diminished, via occupancy of specific cell surface receptors, the capacity of stimulated neutrophils to adhere to the methotrexate-treated endothelial cells and fibroblasts, in an in vitro model of an inflammatory interaction. Asako et al. (12) have confirmed that methotrexate suppresses inflammation by increasing adenosine release using the hamster cheek pouch model of acute inflammation but high concentrations of topically applied methotrexate (1 μM) were used in their study.

We report here the first evidence from in vivo studies that demonstrates that low-dose weekly methotrexate treatment causes intracellular accumulation of AICAR, increased adenosine release at sites of inflammation, and adenosine-dependent inhibition of inflammation. Moreover, we have confirmed that in methotrexate-treated mice adenosine diminishes inflammation via occupancy of adenosine A_2 receptors. These data provide the first in vivo demonstration of a novel biochemical mechanism of action for methotrexate.

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1. *Abbreviations used in this paper:* AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; DMPX, 3,7-dimethyl-1-propargylxanthine.

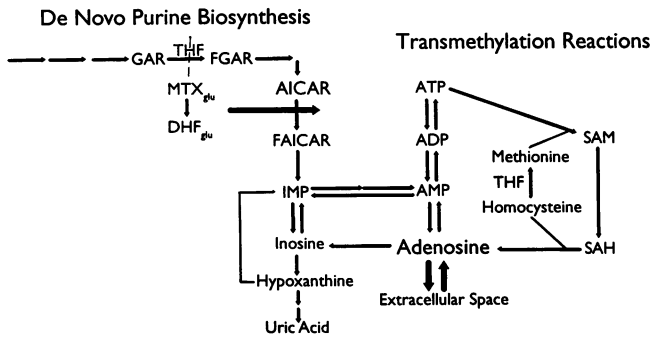

Figure 1. Proposed molecular mechanism of action of methotrexate. Shown here are the major steps in purine synthesis and degradation. Abbreviations: GAR, β-glycinamide ribonucleotide; FGAR, α-*N*-formylglycinamide ribonucleotide; MTX_{glu}, methotrexate polyglutamate; DHF_{glu}, dihydrofolate polyglutamate; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; FAICAR, formyl-AICAR; IMP, inosinic acid; THF, tetrahydrofolate (reduced); SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine.

Methods

Materials. 3,7-Dimethyl-1-propargylxanthine and 8-cyclopentyl-dipropylxanthine were obtained from Res. Biochem. Inc. (Natick, MA). Injectable methotrexate (US Pharmacopeia) was purchased from Lederle Laboratories Division of American Cyanamid (Wayne, NJ). Adenosine deaminase (Type IV, calf intestinal), carrageenan, and dexamethasone were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were the highest grade that could be obtained.

Induction of air pouches and carrageenan-induced inflammation. To induce an air pouch, mice (6-wk-old, female Balb/c mice, Taconic Farms Inc., Germantown, NY) were injected subcutaneously with 3 cc of air (on the back) three times per wk. After 3–4 wk, the air pouch was injected with 1 ml of a 2% (wt/vol) suspension of carrageenan and mice were returned to their cages, where they were allowed to run free for 4 h. At the end of 4 h, the animals were killed, 2 cm^3 of normal saline was injected into the pouch and the contents of the pouch were aspirated, diluted 1:2 with normal saline, and samples were taken for cell count. Smears of the undiluted fluid were prepared and an aliquot was stained (Wright-Giemsa), revealing that > 95% of the exudate cells were PMNs. In some experiments the air pouch was dissected from the subcutaneous tissue, fixed in formalin, processed for histologic sections, and stained (hematoxylin and eosin and Giemsa stains) using standard methods (13). These studies were reviewed and approved by the Institutional Animal Care and Use Committee of New York University Medical Center.

Treatment of mice with methotrexate. Mice were treated with weekly intraperitoneal injections (1 ml) of methotrexate (U.S.P., 0.05–0.5 mg/kg) or pyrogen-free (U.S.P.) normal saline (0.9%) for 3 or 4 wk. There were no apparent adverse effects of either treatment that could be detected by visual inspection of the animals and there were no apparent differences between the animals treated with saline and those treated with methotrexate.

Preparation of adenosine deaminase. Adenosine deaminase (50 μl, 4000 U/ml) was dialyzed against PBS overnight (4°C) before dilution and injection into the air pouch. For some experiments, dialyzed adenosine deaminase was incubated with deoxycoformycin (1 μM) for 30 min at room temperature before dilution (1:2600) in PBS containing carrageenan (14).

Injection of adenosine deaminase and adenosine receptor antagonists. In some experiments adenosine deaminase, previously inactivated adenosine deaminase (see above), and adenosine receptor antagonists were added to the carrageenan suspension to an appropriate final concentration. The final volume of the carrageenan suspension (with inhibitors) did not differ from that in control mice (1 ml).

Histologic analysis of sections of air pouches. Slides of stained (Giemsa) sections of mouse air pouch were examined microscopically using a Leitz research microscope to which was attached a high-resolution video camera. Video images were projected directly onto a screen for analysis by use of JAVA software (Jandel Sci., Corte Madera, CA) run on a Zenith 386 computer. All images were digitized directly and enhanced for contrast and brightness using PHOTOSTYLER software (Aldus, Inc., Seattle, WA).

Quantitation of AICAR. In some experiments the spleens were harvested and the cells were isolated by scraping through gauze. The cells were washed and resuspended at 100×10^6/ml in PBS. The cells were then lysed and the proteins were denatured by addition of 1 vol of trichloroacetic acid (10% vol/vol). The trichloroacetic acid was extracted with freon-octylamine and the supernatants were collected and stored at −80°C before analysis. Nucleotides were quantitated by HPLC by a modification of the method of Chen et al. (15). Briefly, nucleotides were injected onto a Partisil-10 SAX column (Whatman Inc., Clifton, NJ), isocratic elution with 0.007 M $NH_4H_2PO_4$, pH 4.0, followed by a linear gradient to 0.25 M $NH_4H_2PO_4$, pH 5, formed over 30 min at a flow rate of 2 ml/min. Absorbance was monitored at 250 and 260 nm and concentration was calculated by comparison to standards. Preliminary studies showed that 85% of added AICARibotide was recovered using this technique.

Adenosine determination. Aliquots of pouch exudates were added to a similar volume of trichloroacetic acid (10% vol/vol), followed by extraction of the organic acid, as described above. The adenosine concentration of the supernatants was determined by reverse-phase HPLC, as we have previously described (14). Briefly, samples were applied to a C18μBondapack column (Waters Chromatography Div., Milford, MA) and eluted with a 0–40%-linear gradient (formed over 60 min) of 0.01 M ammonium phosphate (pH 5.5) and methanol, with a 1.5 ml/min flow rate. Adenosine was identified by retention time and the characteristic UV ratio of absorbance at 250/260, and the concentration was calculated by comparison to standards. In some experiments the adenosine peak was digested by treatment with adenosine deaminase (0.15 IU/ml, 30 min at 37°C) to confirm that the peak so identified contained only adenosine (16). Preliminary studies demonstrated that 90% of added adenosine was recovered using this technique.

Digitization of chromatograms. Chromatograms were digitized using a Hewlett-Packard (Palo Alto, CA) Scanjet apparatus and the resulting images were enhanced for contrast and brightness using PHOTOSTYLER software run on a Zenith 386 personal computer.

Statistical analysis. The data were analyzed by the appropriate level of ANOVA performed by EXCEL 4.0 software (Microsoft, Inc., Bothell, WA).

Results

Low-dose weekly methotrexate markedly inhibits leukocyte accumulation in the air pouch in response to carrageenan. Low-dose weekly methotrexate is a potent form of antiinflammatory therapy in patients suffering from rheumatoid arthritis. To confirm that the murine air pouch model of inflammation was a reasonable model in which to study the antiinflammatory effects of methotrexate, we determined the effect of various doses of low-dose weekly methotrexate (administered intraperitoneally) on accumulation of leukocytes in the murine air pouch after injection of carrageenan. Methotrexate diminished, in a dose-dependent manner, the number of leukocytes that accumulated in carrageenan-treated air pouches by as much as 60% (IC_{50} = 0.08 mg/kg per wk, $P < 0.0002$ vs. saline-treated animals; Fig. 2). Moreover, the doses of methotrexate required to achieve a maximal antiinflammatory effect in this animal model are similar to those required for the treatment of rheumatoid arthritis (the equivalent of 10–15 mg/wk in a 70-kg individual). In other experiments we observed that metho-

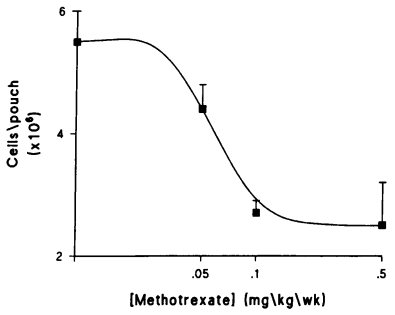

Figure 2. Weekly injection of low-dose methotrexate is antiinflammatory in the air pouch model. Methotrexate was given to the mice by intraperitoneal injection at the indicated doses for 3 to 4 wk during induction of the air pouch. The air pouch was injected with carrageenan (2% wt/vol), exudates were harvested 4 h later, and the cells were counted. Each point represents the mean (±SEM) of cell counts from three mice. Analysis of variance demonstrates that the exudate cell count varied significantly with the dose of methotrexate ($P < 0.0002$).

trexate-mediated inhibition of leukocyte accumulation was similar even when inflammation was induced up to 6 d after the last dose of methotrexate (data not shown).

Low-dose weekly methotrexate treatment increases intracellular concentrations of AICAR. We have proposed that the antiinflammatory actions of methotrexate result, both directly and indirectly, from the inhibition of AICAR transformylase (4). If this mechanism is correct, specific inhibition of AICAR transformylase should result in higher intracellular concentrations of AICAR. We directly tested the validity of this hypothesis by examining AICAR concentrations in splenocytes from saline- and methotrexate-treated mice (0.5 mg/kg by weekly intraperitoneal injection for 4 wk) by HPLC. We found that splenocytes from mice treated with methotrexate contained significantly more AICAR than those treated with saline (Table I, Fig. 3). These results are consistent with the hypothesis that low-dose methotrexate treatment leads to functional inhibition of AICAR transformylase.

Low-dose weekly methotrexate treatment increases adenosine concentrations in inflammatory exudates. We have previously shown that treatment of cells in culture with either methotrexate or AICARibonucleoside (acadesine), a nonphosphorylated, cell-soluble precursor of AICAR, promotes release of adenosine into the supernate and that adenosine release was

Table I. Methotrexate (0.5 mg/kg per wk) Treatment Increases Intracellular AICAR and Extracellular Adenosine

Condition	AICAR concentration (pmol/10^6 splenocytes±SEM)	Exudate adenosine concentration (μM, ±SEM)
	n = 6	*n* = 16
Control	26.5±10	0.57±0.09
Methotrexate (0.5 mg/kg per wk)	72.4±16*	1.11±0.19‡

Mice were treated with a weekly intraperitoneal injection of sterile saline or methotrexate for 4 wk during which time an air pouch was induced on the backs of these mice, as described. After 4 wk the air pouches were injected with carrageenan (2%wt/vol), the splenocyte lysates and inflammatory exudates were collected and analyzed by HPLC, as described. * $P < 0.02$ vs. control, Student's *t* test. ‡ $P < 0.008$ vs. control, Student's *t* test.

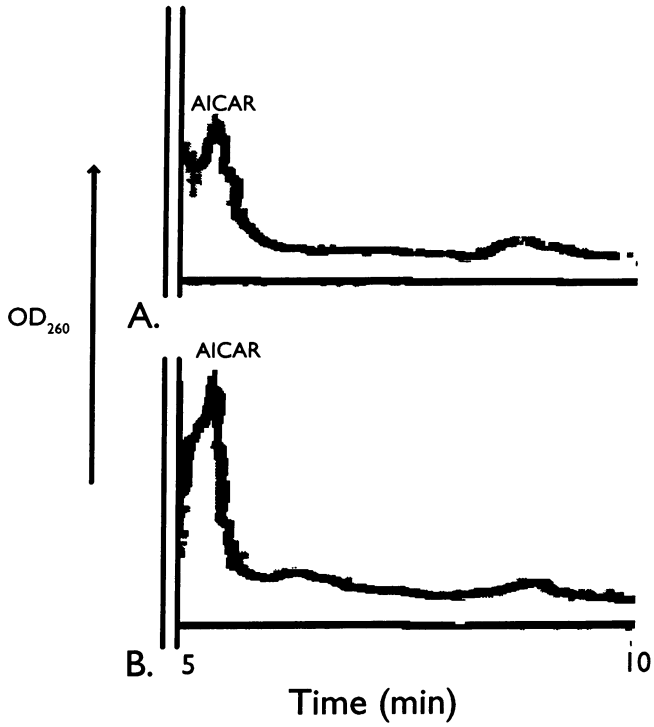

Figure 3. Intracellular concentration of AICAR is higher in splenocytes from animals treated with methotrexate (0.5 mg/kg per wk). Mice were treated with methotrexate for 4 wk during induction of the air pouch. After the animals were killed, and the air pouch exudate was harvested, the spleens were collected, and the cells were collected. The cell number was adjusted, the cells were lysed, and the AICAR-concentration analyzed by reverse-phase, ion exchange HPLC and detected at OD_{260}. Shown is a representative chromatogram of six of splenocyte AICAR from (*A*) a control (22.6 pmol/10^6 splenocytes) and (*B*) a methotrexate-treated mouse (87.3) pmol/10^6 splenocytes).

enhanced under conditions of "stress" (4). To determine whether low-dose weekly methotrexate treatment also promotes adenosine release in vivo we quantitated the adenosine concentration in inflammatory exudates taken from air pouches in saline- and methotrexate-treated (0.5 mg/kg per wk) mice. We found that methotrexate treatment led to a significantly higher adenosine concentration in the pouch exudate (Table I, Fig. 4). Thus, low-dose, intermittent methotrexate therapy promotes adenosine release at an inflamed site.

Adenosine mediates the antiinflammatory effect of methotrexate in the air pouch. To determine whether the methotrexate-induced increase in adenosine concentration observed in pouch fluid exudates was related to the antiinflammatory effects of methotrexate, we studied the effect of adenosine deaminase on leukocyte accumulation in methotrexate-treated mice. Adenosine deaminase irreversibly deaminates extracellular adenosine to its inactive metabolite, inosine, and thereby renders it inactive at adenosine receptors. Adenosine deaminase (0.15 IU/ml) did not significantly affect the number of leukocytes recovered from pouches of saline-treated animals, but partially reversed the antiinflammatory effect of methotrexate treatment (Fig. 5). Histologic examination of the air pouch tissue revealed that, similar to its effects on leukocyte counts in the exudate, methotrexate diminished leukocyte in-

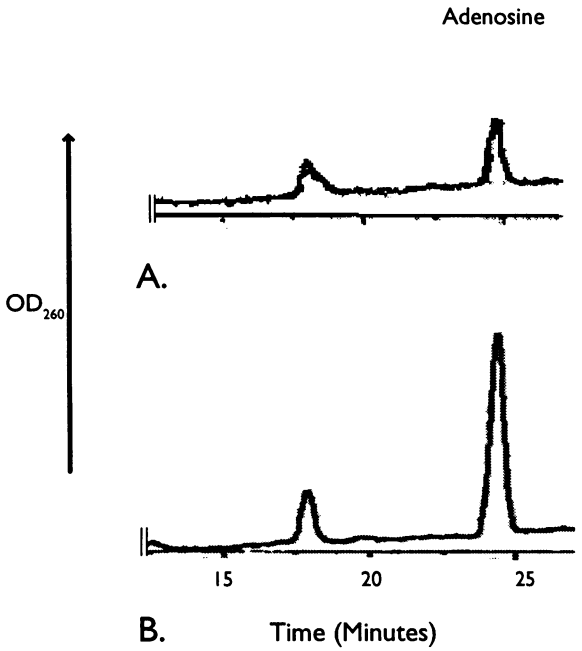

Figure 4. The concentration of adenosine is higher in exudates of mice treated with methotrexate (0.5 mg/kg per wk). Mice were treated with methotrexate for 4 wk during induction of the air pouch. After the animals were killed the air pouch exudate was harvested and soluble adenosine was extracted after treatment of the exudates with 10% trichloroacetic acid. The adenosine concentration of exudate extracts was then analyzed by reverse-phase HPLC, as described, and detected at OD_{260}. Shown is a representative chromatogram of 16 of pouch exudate adenosine from (*A*) a control (0.39 μM) and (*B*) a methotrexate-treated mouse (1.3 μM).

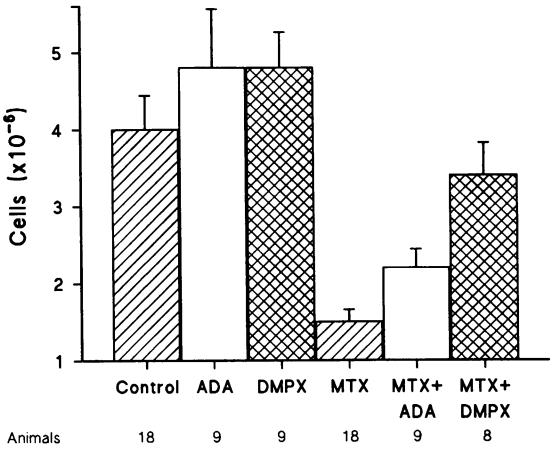

Figure 5. Adenosine deaminase (ADA, 0.15 IU/ml) and DMPX (mg/kg) reverse the antiinflammatory effects of methotrexate treatment (0.5 mg/kg per wk). Mice were treated with saline (control) or methotrexate for 3 to 4 wk before inflammation was induced in the air pouch. Shown are the means (±SEM) of the number of cells that accumulated in the pouch exudates. Methotrexate significantly inhibited the accumulation of leukocytes in the pouch exudate (4.0±0.4 vs. 1.5±0.1 $\times 10^6$ cells/pouch, control vs. methotrexate, $P < 3 \times 10^{-6}$). Neither ADA (4.8±0.5 $\times 10^6$ cells/pouch) nor DMPX (4.8±0.4 $\times 10^6$ cells/pouch) significantly affected the number of cells that accumulated in the control air pouches, but both ADA (2.3±0.8 $\times 10^6$ cells/pouch) and DMPX (3.8±0.5 $\times 10^6$ cells/pouch) significantly reversed the antiinflammatory effect of methotrexate ($P < 0.006$ and $P < 0.001$ vs. methotrexate alone, respectively).

filtration into the pouch tissue (38±2 vs. 106±14 cells/160× field, methotrexate vs. control, $P < 0.01$), and adenosine deaminase reversed the antiinflammatory effect of methotrexate (88±3 cells/160× field, $P < 0.01$ vs. methotrexate alone) without affecting leukocyte infiltration in control mice (91±6 cells/160× field, P = NS vs. control, Fig. 6). Adenosine deaminase-mediated reversal of the antiinflammatory effect of methotrexate treatment was specific since adenosine deaminase did not reverse the antiinflammatory effects of dexamethasone (1.5 mg/kg, injected intraperitoneally 1 h before injection of the pouch with carrageenan, Fig. 7). Moreover, conversion of adenosine to inactive metabolites was responsible for reversal of the antiinflammatory effect since adenosine deaminase which was inactivated by prior incubation with its tight-binding, irreversible inhibitor deoxycoformycin (1 μM), did not affect the antiinflammatory capacity of methotrexate treatment (data not shown). We conclude from these experiments that the increase in extracellular adenosine in the methotrexate-treated animals is responsible, at least in part, for the antiinflammatory effects of methotrexate.

The antiinflammatory effect of adenosine is mediated via adenosine A_2 receptors. There are at least two major subtypes of adenosine receptor, A_1 and A_2, that can be differentiated, in part, on the basis of agonist and antagonist specificity (17, 18). Since extracellular adenosine appeared to mediate the antiinflammatory effects of methotrexate, we sought to determine whether the antiinflammatory actions of adenosine were mediated by occupancy of a specific adenosine receptor. We therefore injected receptor-specific adenosine receptor antagonists into the air pouch with the inflammatory stimulus. The adenosine A_1 receptor antagonist 8-cyclopentyl-dipropylxanthine (0.2 mg/kg) did not affect leukocyte accumulation in the air pouch in either control animals or methotrexate-treated animals (Fig. 8). Because of its poor solubility in aqueous medium, higher concentrations of 8-cyclopentyl-dipropylxanthine could not be utilized for study. In contrast, a specific adenosine A_2 receptor antagonist, 3,7-dimethyl-1-propargylxanthine (DMPX), completely reversed the antiinflammatory effect of methotrexate treatment (IC_{50} = 0.2 mg/kg, $P < 0.01$; Fig. 9) without affecting accumulation of leukocytes in either control animals (Fig. 5) or dexamethasone-treated animals (Fig. 7). We conclude from these experiments that the increased adenosine found at inflamed sites in methotrexate-treated animals mediates the antiinflammatory effects of methotrexate by engaging adenosine A_2 receptors.

Discussion

The results of the experiments reported here provide the first in vivo demonstration of a molecular mechanism for the antiphlogistic actions of methotrexate. Methotrexate, either acting directly or by promoting the intracellular accumulation of dihydrofolate polyglutamate, increases intracellular content of AICAR. The increase in intracellular AICAR concentration is associated with (and probably leads to) an increase in extracellular adenosine in inflammatory exudates. The increase in local adenosine concentrations at sites of inflammation suppresses inflammation via occupancy of adenosine A_2 receptors on inflammatory or connective tissue cells.

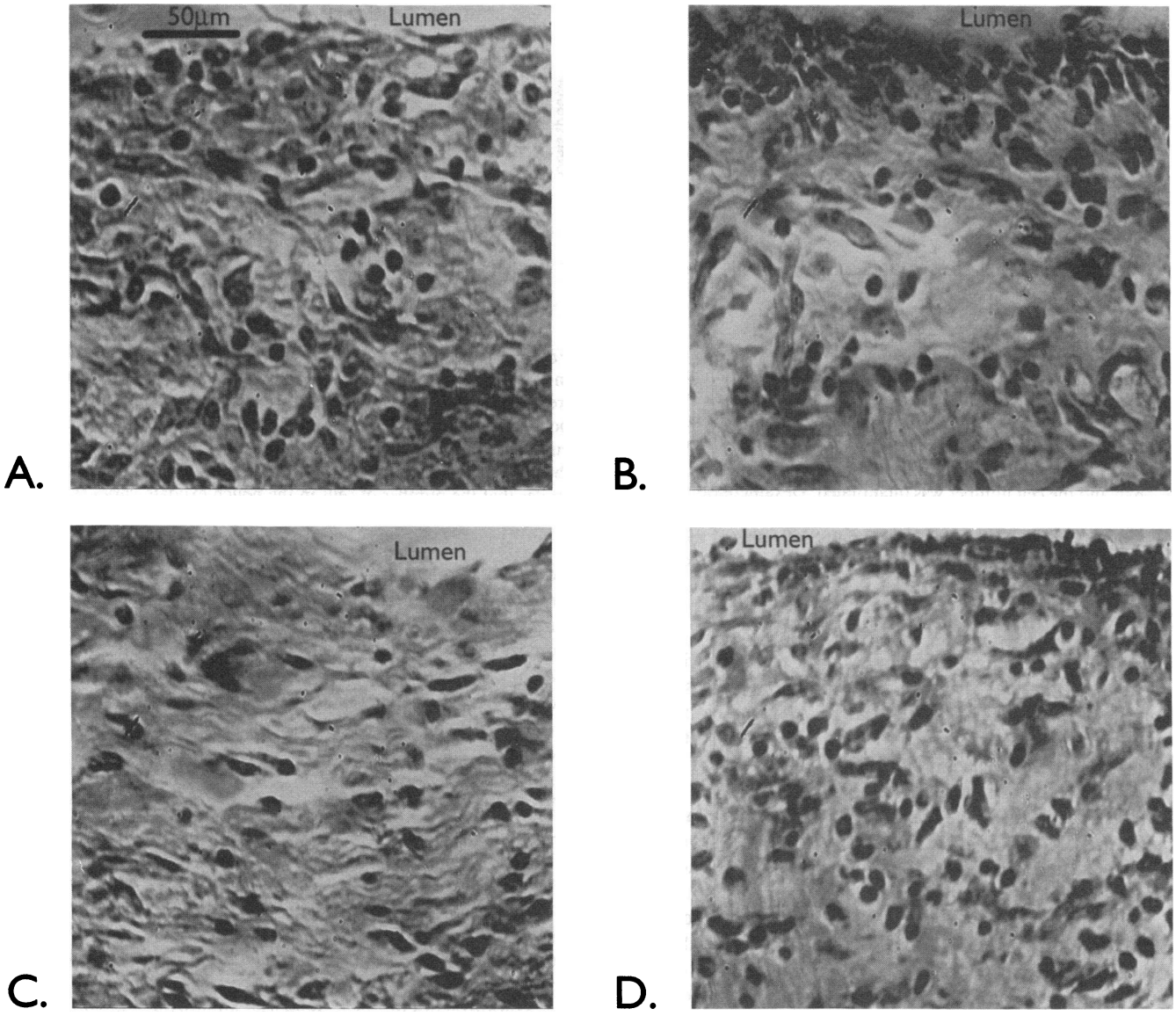

Figure 6. Adenosine deaminase (ADA, 0.15 IU/ml) reverses the antiinflammatory effects of methotrexate treatment (0.5 mg/kg per wk). Mice were treated with saline (control) or methotrexate for 3 wk before inflammation was induced in the air pouch. The air pouches were dissected out of the animals, and fixed and prepared by standard histopathological techniques for photomicroscopy. The photographic images were digitized directly using JAVA software and the images shown were adjusted only for brightness and contrast. Shown are representative fields (of 10 examined) from one section from one of two animals studied under each condition.

The observation that low-dose weekly methotrexate therapy promotes the intracellular accumulation of AICAR in splenocytes indicates that the "folate antagonism" of low-dose weekly methotrexate is highly specific. Via inhibition of dihydrofolate reductase, high concentrations of methotrexate diminish the cellular content of the methyl donors required for synthesis of purines and pyrimidines (6). In addition to the synthesis of formyl-AICAR from AICAR (Fig. 1), reduced folate is required for the synthesis of α-*N*-formylglycinamide ribonucleotide from β-glycinamide ribonucleotide, precursors of AICAR. Thus, under the conditions studied, if methotrexate inhibited folate-dependent reactions nonspecifically, then we would have expected either no change or a decrease in cellular AICAR content. We found the opposite, a net increase in cellular AICAR content, an observation that indicates that treatment with low concentrations of methotrexate leads to selective inhibition of AICAR transformylase without inhibiting the enzymatic steps required for the production of AICAR. The selective effect of low concentrations of methotrexate on purine biosynthesis most likely follows from the metabolism of methotrexate to its polyglutamated derivatives (for review see reference 6). Polyglutamated methotrexate directly inhibits several steps in the synthesis and metabolism of purines and pyrimidines (5, 7–9). In particular, polyglutamated methotrexate is a potent direct inhibitor of AICAR transformylase (7). Moreover, inhibition of dihydrofolate reductase by methotrexate (and methotrexate polyglutamate) leads to the intracellular accumulation of dihydrofolate polyglutamate, a known and potent inhibitor of AICAR transformylase (7–9). Since relatively high concentrations of methotrexate polygluta-

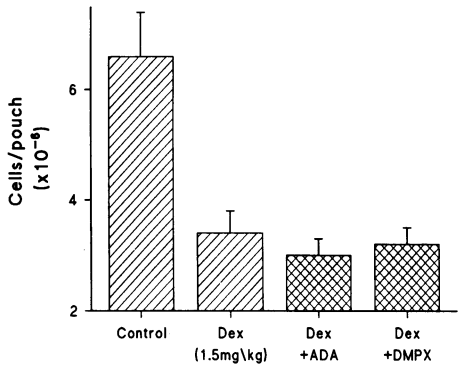

Figure 7. Neither adenosine deaminase (ADA, 0.15 IU/ml) nor DMPX (mg/kg) reverse the antiinflammatory effects of dexamethasone treatment (1.5 mg/kg). Air pouches were induced on mice for 3 wk. 1 h before injection of carrageenan into the air pouch, the mice received an intraperitoneal injection of dexamethasone (1.5 mg/kg) or saline. The exudates were harvested 4 h after injection of carrageenan and the cell number was quantitated. Dexamethasone significantly diminished the number of cells that accumulated in the air pouch and neither ADA nor DMPX significantly altered the number of cells that accumulated in the air pouch of animals treated with dexamethasone.

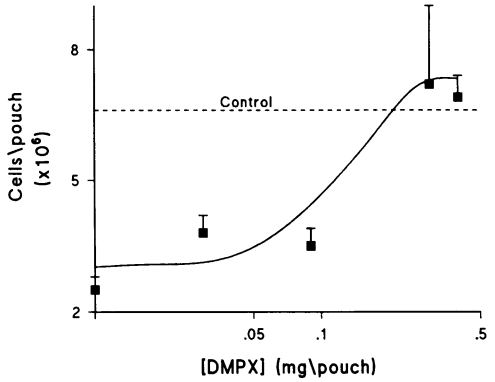

Figure 9. DMPX (mg/kg) reverses the antiinflammatory effect of methotrexate (0.5 mg/kg per wk). Mice were treated with methotrexate for 3 to 4 wk before inflammation was induced in the air pouch. Shown are the means (±SEM) of the number of cells that accumulated in the pouch exudates from three mice in the presence of the indicated concentrations of DMPX. Analysis of variance demonstrates that the number of cells in the pouch exudate varies significantly with the dose of DMPX ($P < 0.01$).

mates (7) are required to inhibit AICAR transformylase, it is more likely that dihydrofolate polyglutamates are responsible for the intracellular accumulation of AICAR. Nonetheless, treatment with methotrexate may lead to inhibition of AICAR transformylase (and accumulation of AICAR) by two different but complementary mechanisms.

Previous studies have demonstrated that intracellular accumulation of AICAR increases adenosine release from some, but not all, cell types (11). Barankiewicz et al. have shown that treatment of B-lymphoblasts with high concentrations of AICARibonucleoside diminishes adenosine uptake and utilization, resulting in increased release of adenosine into the extracellular space, particularly under conditions of ATP degradation (11, 19). In contrast to T lymphoblasts, which release little adenosine under any condition, B lymphoblasts possess increased AMP-5′-nucleotidase activity (adenosine formation) and relatively little adenosine kinase or adenosine deaminase activity (adenosine utilization [11]). Thus, Barankiewicz et al. postulated that, since AICARibonucleoside does not affect adenosine production or transport, intracellular accumulations of AICAR must inhibit adenosine kinase or adenosine deaminase activity in order to promote the increase in extracellular adenosine observed (11, 19). AICARibonucleoside may also lead to an increase in adenosine release at sites of "stress," such as reperfusion after ischemic insult to the heart, and the increased extracellular adenosine that accumulates in ischemic tissue protects the affected tissue from leukocyte-mediated injury (10). Our data suggest that treatment in vivo with low-dose methotrexate similarly increases intracellular AICAR content and, more importantly, promotes adenosine release at inflamed sites.

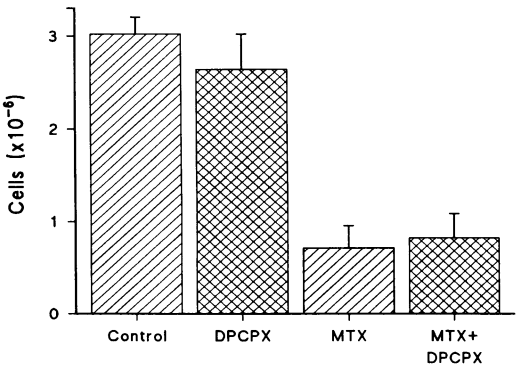

Figure 8. 8-Cyclopentyl-dipropylxanthine (DPCPX, 0.2 mg/kg) does not reverse the antiinflammatory effect of methotrexate (0.5 mg/kg per wk). Mice were treated with methotrexate for 3 to 4 wk before inflammation was induced in the air pouch. Shown are the means (±SEM) of the number of cells that accumulated in the pouch exudates from six mice in the presence of the indicated concentrations of DPCPX.

Methotrexate induced increased adenosine concentrations in inflammatory exudates and was a potent antiinflammatory agent in the air pouch model. To prove that the effects of methotrexate on purine metabolism and inflammation were causally related, we used two different approaches: elimination of extracellular adenosine by adenosine deaminase and antagonism of adenosine at its receptors with a specific antagonist (DMPX). Both of these experimental maneuvers reversed the antiinflammatory effect of methotrexate but did not reverse the antiinflammatory effect of dexamethasone in this same model. Dexamethasone is a potent agonist at glucocorticoid receptors that diminishes leukocyte accumulation at inflammatory sites by a mechanism that is not related to purine metabolism (for review see reference 20). Thus, our observation that both specific elimination and antagonism of adenosine reverse the antiinflammatory effects of methotrexate is strong evidence that adenosine mediates the antiphlogistic effect of methotrexate.

We have previously observed that methotrexate treatment, in vitro, promotes an increase in adenosine release at the expense of hypoxanthine and inosine release (4). In this study we were unable to detect inosine in most samples and the HPLC technique we used does not resolve hypoxanthine from many

other compounds present in these complex biologic fluids. Nevertheless, the adenosine concentration present in inflammatory exudates of methotrexate-treated animals (1.11 μM) is more than sufficient to account for the diminished inflammation observed; maximal inhibition of stimulated neutrophil adhesion and generation of superoxide anion and H_2O_2 is achieved with adenosine concentrations greater than or equal to 1 μM (14, 21). Indeed, the concentration of adenosine found in exudates from control animals was less than the concentration of adenosine found in transudates from "stressed" isolated rabbit hearts (during hypoxia, 1225±300 nM; reference 22). Although the adenosine concentration measured in the inflammatory exudate probably reflects the metabolic changes in methotrexate-treated animals and is sufficient to inhibit the production of toxic oxygen metabolites by the cells present in the inflammatory exudate, it is likely that the increase in extracellular adenosine responsible for diminished inflammation is that which occurs in the surrounding tissues, a less readily accessible site for sampling.

There are at least two major subclasses of adenosine receptor that can be distinguished on pharmacologic grounds, A_1 and A_2 (17, 18). Adenosine A_1 receptors are relatively high-affinity receptors that are linked to pertussis toxin-inhibited G proteins (23–33). Adenosine A_1 receptors have been demonstrated on neutrophils and macrophages (but not peripheral blood mononuclear cells) where they mediate, when occupied, enhanced chemotaxis and phagocytosis of immunoglobulin-coated particles (34–38). Adenosine A_2 receptors are low-affinity receptors linked to Gαs signal transduction proteins in many cell types. Adenosine A_2 receptors are present on neutrophils, monocytes, lymphocytes, and basophils and, when occupied, generally suppress the inflammatory or immune functions of these cells (for review see references 39–41). Using relatively selective antagonists we found that the antiinflammatory effects of adenosine in methotrexate-treated animals were mediated by occupancy of adenosine A_2 receptors, results that were identical to those obtained by Asako et al. (12). In contrast, Schrier et al. (42) observed, utilizing receptor-specific agonists, that occupancy of adenosine A_1 receptors rather than A_2 receptors is antiinflammatory in a rat model of inflammation. The discrepancy may be due to species differences in agonist sensitivity or adenosine receptor expression. Alternatively, the apparent difference in receptor specificity for the antiinflammatory effects of adenosine results from a difference in the distribution, lipid solubility, or other pharmacologic properties of the adenosine receptor-specific agonists studied.

We first suggested that adenosine might be an endogenous antiinflammatory agent when we observed that adenosine inhibits the generation of toxic oxygen metabolites by stimulated neutrophils (14). In subsequent studies we have shown that adenosine, both added exogenously or released endogenously, diminishes endothelial cell injury mediated by stimulated neutrophils (43). The cytoprotective effects of adenosine result from inhibition of the generation of toxic oxygen metabolites and inhibition of the stimulated adhesion of neutrophils to the endothelium (43). In the model under study, the apparent effect of adenosine was to diminish extravasation of leukocytes into an inflammatory exudate. There may be an additional beneficial effect of methotrexate therapy for the synovial tissues of patients treated with methotrexate; the concentration of adenosine present in the inflammatory pouch exudates is more than sufficient to inhibit generation of toxic oxygen metabolites by stimulated leukocytes. Thus, treatment with methotrexate may both diminish the number of leukocytes that accumulate in an inflammatory exudate and inhibit the destructive capacity of those leukocytes that do arrive at the inflamed site.

In the model studied here, inflammation was acute and was characterized by the accumulation of a neutrophilic infiltrate in both the air pouch and the surrounding tissues. Although it is likely that the adenosine released is acting directly on neutrophil adenosine receptors, it is also possible that adenosine inhibits the generation of cytokines or chemoattractants required for accumulation of the inflammatory exudate. Indeed, adenosine, probably acting at an A_2 receptor, inhibits synthesis of cytokines (TNFα) and other inflammatory proteins (complement C_2) by macrophages (44, 45). Moreover, adenosine, acting at its receptor, inhibits lymphocyte proliferation and induces suppressor activity in cultured lymphocytes (39). Thus, the antiinflammatory effects of methotrexate (acting via adenosine) are more general than those studied in this model of acute inflammation. Indeed, it is likely that the effects of methotrexate, acting via adenosine, on lymphocyte or monocyte function play a greater role in diminishing the chronic inflammation of rheumatoid arthritis than the effects on acute inflammation observed in this model.

We have demonstrated a novel biochemical mechanism of action of methotrexate. Low-dose weekly methotrexate therapy leads to intracellular accumulation of AICAR, which promotes increased adenosine release (and/or diminished adenosine uptake) at sites of inflammation. This increase in extracellular adenosine diminishes both the accumulation and function of leukocytes in inflamed sites. These findings suggest several novel approaches to the development of new agents that inhibit inflammation by increasing adenosine release: development of direct inhibitors of AICAR transformylase, inhibitors of adenosine deaminase and adenosine kinase, and adenosine uptake inhibitors.

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