Methotrexate inhibits neutrophil function by stimulating adenosine release from connective tissue cells

(adenosine receptor/purine receptor)

BRUCE N. CRONSTEIN*, MARK A. EBERLE*, HARRY E. GRUBER†, AND RICHARD I. LEVIN*

*Department of Medicine, Divisions of Rheumatology and Cardiology, New York University Medical Center, 550 First Avenue, New York, NY 10016; and 
†Genesis Pharmaceuticals, Inc., 11025 Roselle Street, San Diego, CA 92121-1207

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ABSTRACT Although commonly used to control a variety of inflammatory diseases, the mechanism of action of a low dose of methotrexate remains a mystery. Methotrexate accumulates intracellularly where it may interfere with purine metabolism. Therefore, we determined whether a 48-hr pretreatment with methotrexate affected adenosine release from [14C]adenine-labeled human fibroblasts and umbilical vein endothelial cells. Methotrexate significantly increased adenosine release by fibroblasts from 4 ± 1% to 31 ± 6% of total purine released (EC50, 1 nM) and by endothelial cells from 24 ± 4% to 42 ± 7%. Methotrexate-enhanced adenosine release from fibroblasts was further increased to 51 ± 4% (EC50, 6 nM) and from endothelial cells was increased to 58 ± 5% of total purine released by exposure to stimulated (Met-Leu-Phe at 0.1 μM) neutrophils. The effect of methotrexate on adenosine release was not due to cytotoxicity since cells treated with maximal concentrations of methotrexate took up [14C]adenine and released 14C-labeled purine (a measure of cell injury) in a manner identical to control cells. Methotrexate treatment of fibroblasts dramatically inhibited adherence to fibroblasts by both unstimulated neutrophils (IC90, 9 nM) and stimulated neutrophils (IC50, 13 nM). Methotrexate treatment inhibited neutrophil adherence by enhancing adenosine release from fibroblasts since digestion of extracellular adenosine by added adenosine deaminase completely abrogated the effect of methotrexate on neutrophil adherence without, itself, affecting adherence. One hypothesis that explains the effect of methotrexate on adenosine release is that, by inhibition of 5-aminomimidazole-4-carboxamide ribonucleotide (AICAR) transformylase, methotrexate induces the accumulation of AICAR, the nucleoside precursor of which (5-aminomimidazole-4-carboxamide ribonucleoside referred to hereafter as acadesine) has previously been shown to cause adenosine release from ischemic cardiac tissue. We found that acadesine also promotes adenosine release from and inhibits neutrophil adherence to connective tissue cells. The observation that the antiinflammatory actions of methotrexate are due to the capacity of methotrexate to induce adenosine release may form the basis for the development of an additional class of antiinflammatory drugs.

First reported to be useful in the treatment of rheumatoid arthritis (1), methotrexate is now widely used to treat a variety of inflammatory diseases, most notably rheumatoid arthritis (for review, see ref. 2). The mechanism by which methotrexate modulates inflammation remains, however, a mystery. The antineoplastic (antiproliferative) effects of methotrexate are due to inhibition of dihydrofolate reductase with resulting inhibition of purine and pyrimidine synthesis. However, folate depletion probably does not account for the therapeutic effects of methotrexate in inflammatory disease. (i) At the doses of methotrexate administered, leukopenia due to inhibition of DNA synthesis, does not occur (2), finding not consistent with the hypothesis that methotrexate is antiinflammatory due to inhibition by methotrexate of dihydrofolate reductase. (ii) In two (3, 4) of three (5) published trials neither folate supplementation nor administration of reduced folate (folic acid) reversed the therapeutic effects of this agent (although both agents reduced toxicity), direct evidence against inhibition of dihydrofolate reductase.

Recent observations have suggested a different mechanism to explain the antiinflammatory characteristics of methotrexate. Methotrexate and its polyglutamated analogues are very potent inhibitors of 5-aminomimidazole-4-carboxamide ribonucleotide (AICAR) transformylase (6–8), an enzyme required for de novo purine synthesis. In a study of canine myocardial injury Gruber et al. (9) found that administration of 5-aminomimidazole-4-carboxamide ribonucleoside (acadesine), the nucleoside precursor of AICAR, increases adenosine release from, diminishes neutrophil accumulation in, and increases collateral flow into ischemic myocardium. Thus, methotrexate, by inhibiting AICAR transformylase, may increase the intracellular concentration of its substrate, AICAR, which would lead, in turn, to increased release of adenosine, a potent antiinflammatory autocoid, at sites of inflammation.

We report that methotrexate, at pharmacologically relevant doses, induces adenosine release from human dermal fibroblasts and umbilical vein endothelial cells. The increase is most marked in the presence of neutrophils stimulated with the chemotactic agent Met-Leu-Phe (0.1 μM). In turn, the released adenosine inhibited neutrophil adhesion. Acadesine, in a manner similar to methotrexate, both adenosine release and neutrophil adherence.

MATERIALS AND METHODS

Materials. Tissue culture media [Dulbecco’s modified Eagle’s medium (DMEM) and medium 199] were obtained from Gibco. [14C]Adenine was purchased from NEN/DuPont and DEAE-cellulose thin layer chromatography plates were obtained from Eastman Kodak. The scintillant Filtron-X was supplied by National Diagnostics (Manville, NJ). Lymphoprep (Hypaque/Ficoll) was obtained from Nyegaard (Oslo, Norway) and Triethylamine was purchased from Aldrich Chemical (Orangeburg, NY) and Freon-113 was obtained from Matheson. Methotrexate, Met-Leu-Phe, AICAR, and all other reagents were obtained from Sigma. All reagents were of the highest quality available.

Endothelial Cell Cultures. Endothelial cells were cultured and grown as described by Jaffe et al. (10). Briefly, segments of freshly obtained human umbilical veins were treated with

Abbreviations: AICAR, 5-aminomimidazole-4-carboxamide ribonucleotide; ANOVA, analysis of variance; PMN, polymorphonuclear leukocyte.

To whom reprint requests should be addressed.
collagenase (0.1%), and the endothelial cells were collected and grown to confluence in gelatin-coated flasks containing medium 199/20% (vol/vol) fetal bovine serum at 37°C in a 5% CO2/95% air atmosphere. The endothelial cells were then passed as necessary and grown to confluence in gelatin-coated 96-well tissue culture plates in medium 199/20% fetal bovine serum. All cells were used in the third passage.

**Human Dermal Fibroblasts.** Normal human fibroblasts (GM08389) were obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository (Camden, NJ) and cell line HM was a generous gift of Frank Martiniuk (New York Univ. Medical Center, New York). The cells were grown to confluence in DMEM/20% fetal bovine serum and passed as necessary. All cells were used during passages 5–15. Nearly identical results were obtained when either cell line was used.

**Incubation of Cell Cultures with Methotrexate.** Fibroblasts or preconfluent cultures of endothelial cells were washed three times with medium and then incubated for 48 hr at 37°C in a 5% CO2/95% air atmosphere in fresh medium containing methotrexate at various concentrations. At the end of the incubation, cells were washed three times with fresh medium. When examined microscopically, there was no difference in cellular morphology between cells incubated with methotrexate and those treated with medium alone.

**Isolation of Neutrophils.** Human neutrophils were isolated from whole blood after centrifugation through Histopaque/Ficoll gradients, sedimentation through dextran, 6% (wt/vol), and hypotonic lysis of erythrocytes. This procedure allowed study of populations that were 98 ± 2% neutrophils with few contaminating erythrocytes or platelets. Neutrophils were suspended in a Hapes-buffered saline solution consisting of 150 mM Na+, 2.5 mM K+, 1.3 mM Ca2+, 1.2 mM Mg2+, 155 mM Cl−, and 10 mM Hapes (pH 7.45) (11).

**Labeling of Connective Tissue Cells with 14C]adenine.** After washing the cell cultures with fresh medium, cells were incubated in Hapes-buffered saline containing [14C]adenine (25 μCi/ml; 1 CI = 37 GBq) in a final volume of 250 μl per well for 3 hr at 37°C in a 5% CO2/95% air atmosphere. At the end of this incubation, wells were again washed three times with fresh medium before use in the experiments.

**Assay for 14C-Labeled Purine Release.** To study the effects of preincubation with methotrexate or incubation with adenosine on 14C-labeled purine release, endothelial cells or fibroblasts were incubated at 37°C in a 5% CO2/95% air atmosphere in the presence or absence of 1.25 x 10^4 neutrophils per ml with or without fMet-Leu-Phe (0.1 μM) and adenosine in a final volume of 200 μl. This concentration of neutrophils is 12.5% of that which we have found (12) to injure endothelial cells. Because in preliminary experiments preincubation of connective tissue cells with adenosine markedly reduced [14C]adenine uptake, adenosine was added during final incubations. In some experiments adenosine deaminase (0.125 international unit/ml), which had been dialyzed for 3–4 hr at 4°C against phosphate-buffered saline, was added to tissue culture wells. At the end of the incubation, samples of supernatant medium were collected, treated with 10% (vol/vol) trichloroacetic acid, and extracted with a mixture of Freon/triethylamine, 31:9 (vol/vol), before centrifugation at 10,000 x g. The aqueous layer was then collected and frozen (−20°C) until assayed for purine content. In some experiments the remaining supernatant medium was removed, the remaining cells were lysed by overnight incubation with water, and the lysates were collected for quantitation of radioactivity. All experimental conditions were performed in duplicate with <5% variation between replicates. In preliminary experiments we found that addition of the chemoattractant fMet-Leu-Phe (0.1 μM) in the absence of neutrophils did not affect adenosine release from connective tissue cells regardless of whether or not they were treated with methotrexate (100 μM) or adenosine (100 μM, data not shown).

**Separation and Quantitation of 14C-Labeled Purines.** A 50-μl portion of each sample was spotted onto DEAE-cellulose thin layer chromatography sheets. Separation was then carried out by chromatography in water/isobutanol/methanol/ammonium hydroxide in a ratio of 30:10:1:10 (vol/vol). After drying, the labeled purines and their carrier compounds (AMP, hypoxanthine, inosine, and adenosine, each at 500 mg/dl) were visualized under ultraviolet, cut out, and placed in scintillation vials. Radioactivity was quantitated in a Packard scintillation counter to an error of <0.2% (13).

**Neutrophil Adherence to Endothelial Cell or Fibroblast Monolayers.** After removal of medium for quantitation of purines, the monolayers and adherent neutrophils were fixed by addition of formaldehyde to 3.7% (vol/vol). Monolayers and their adherent neutrophils were then washed three times to remove nonadherent neutrophils and then stained with Weigert's hematoxylin. Adherent neutrophils were easily differentiated from underlying fibroblasts and endothelial cells on the basis of size and nuclear-staining characteristics (12). The number of neutrophils in three × 100 fields per well was quantified and the mean was calculated. Counts were performed on two replicate wells, which differed by <5%.

**Statistical Analysis.** All results represent the mean ± SEM, unless otherwise stated. The significance of the effects of agents and neutrophils and their interactions on adenosine release from and neutrophil adhesion to connective tissue cells was determined by the appropriate level of analysis of variance (ANOVA).

## RESULTS

Treatment of fibroblasts with methotrexate caused a dose-dependent increase in release of adenosine from 4 ± 1% to a maximum of 31 ± 6% of the total purine released (Fig. 1). Methotrexate was a surprisingly potent promoter of adenosine release with an EC50 of 1 nM. When fibroblasts were treated with methotrexate and then incubated with neutrophils, there was a nearly identical dose-dependent increase in release of adenosine from 5 ± 2% to 23 ± 5% of total purine released (Fig. 1, P < 0.01). However, treatment of fibroblasts with methotrexate followed by incubation with neutrophils stimulated with fMet-Leu-Phe (0.1 μM) markedly enhanced

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**Fig. 1.** Normal human fibroblasts were incubated with methotrexate at the indicated concentrations for 48 hr, washed, and labeled with [14C]adenine. After washing, the fibroblasts were incubated in the presence of medium alone, neutrophils, or stimulated (fMet-Leu-Phe at 0.1 μM) neutrophils. After 2 hr the supernatant was collected and analyzed by thin layer chromatography. Data are the mean ± SEM of four experiments performed in duplicate. Two-way ANOVA indicates that the percentage of purine released as adenosine varies with dose of methotrexate (P < 0.001) and with the presence of stimulated neutrophils (P < 0.003). PMN, polymorphonuclear leukocyte; fMLP, fMet-Leu-Phe.
adenosine release from 4 ± 1% to 51 ± 4% of total purine released (Fig. 1). The quantity of adenosine detected corresponds to ~400 nM, a concentration well within the effective physiologic range of activity for adenosine (14). Although stimulated neutrophils induced a greater shift in purine release from methotrexate-treated fibroblasts, there was no significant change in the concentration of methotrexate required to shift purine release (EC50, 6 nM). As shown (Fig. 1), neither unstimulated nor stimulated neutrophils altered basal adenosine release from fibroblasts. Moreover, methotrexate or neutrophils or their combination did not affect total purine release from fibroblasts (7 ± 2, 7 ± 1, 7 ± 1%, respectively, of total purine pool released vs. 7 ± 1% from control cells).

As compared to fibroblasts, endothelial cells, under control conditions, released a greater percentage of their purine as adenosine (24 ± 4% vs. 4 ± 1%, P < 0.01, n = 4). When endothelial cells were treated with methotrexate (100 μM), there was an increase in the percentage of adenosine released to 42 ± 7% of total purine released (P < 0.01, n = 4). As with fibroblasts, unstimulated neutrophils did not affect the percentage of purine released as adenosine from control or methotrexate-treated endothelial cells (20 ± 4% and 39 ± 8%, respectively, n = 4). Stimulated neutrophils also did not affect adenosine release (18 ± 3% of total purine released, n = 4) from endothelial cells but increased adenosine release from methotrexate-treated cells to 58 ± 5% of total purine released (P < 0.003 vs. control, n = 4).

To examine the hypothesis that inhibition of AICAR transformylase by methotrexate is responsible for the release of adenosine, we determined whether acadesine, the nucleoside precursor of AICAR, also increases adenosine release. Acadesine (100 μM) induced fibroblasts to release a greater percentage of purine as adenosine (from 3 ± 1% to 19 ± 5% of total purine released, P < 0.01, n = 4; Fig. 2). However, acadesine was far less potent than methotrexate. As with methotrexate, stimulated but not unstimulated neutrophils also enhanced the effect of acadesine (100 μM) on adenosine release (53 ± 7% and 22 ± 13% of total purine released, respectively, P < 0.001, n = 4; Fig. 2).

Acadesine (100 μM) treatment increased the percentage of purine released as adenosine from endothelial cells from 24 ± 4% to 39 ± 6% (n = 4, P < 0.01). Stimulated neutrophils further increased the percentage of purine released as adenosine to 62 ± 9% of total purine released (P < 0.001, n = 4).

To determine whether methotrexate or acadesine was toxic to endothelial cells or fibroblasts, we compared both uptake and release of purine by treated cells. Cells treated with methotrexate (100 μM) took up as much [14C]adenine as control cells (101 ± 7% of control uptake, n = 4) and did not release any greater percentage of the labeled purine pool during these experiments (7 ± 1 vs. 7 ± 2% of total label released from control and methotrexate-treated cells, n = 4).

Similarly, fibroblasts treated with acadesine (100 μM) or exposed to stimulated neutrophils plus methotrexate (100 μM) also released no more of their labeled purine pool than control cells (7 ± 1 and 7 ± 1% of total label released).

Moreover, no change in cell morphology was detected whether cells were treated with methotrexate, acadesine, stimulated neutrophils, or their combination. These results indicate that the increase in adenosine release from methotrexate-treated fibroblasts was not due to toxicity of methotrexate for fibroblasts. Similar results were obtained using endothelial cells (data not shown).

We next determined whether the release of adenosine from connective tissue cells treated with methotrexate was relevant to the antiinflammatory activity of methotrexate. We have previously demonstrated that adenosine, presumably acting at adenosine A2 receptors on neutrophils, inhibits neutrophil adherence to endothelial cells (12). Therefore, we determined whether adherence by unstimulated and stimulated neutrophils to connective tissue cells was affected by treatment of the connective tissue cells with methotrexate.

Treatment of connective tissue cells with methotrexate markedly inhibited adherence of both unstimulated and stimulated neutrophils to fibroblasts (EC50, 9 nM and 13 nM, respectively, P < 0.001; Fig. 3). Similarly, acadesine also inhibited unstimulated and stimulated neutrophil adherence to fibroblasts (EC50, 13 μM and 18 μM, respectively, P < 0.001; Fig. 4) at concentrations similar to those required for promotion of adenosine release. Methotrexate and acadesine inhibited neutrophil adherence to endothelial cells in a similar fashion (data not shown).

To determine whether the diminished adherence of neutrophils was related to the increase in adenosine release from connective tissue cells, we determined whether addition of adenosine deaminase, which metabolizes adenosine to inosine, reverses the effect of methotrexate treatment on neutrophil adherence. Adenosine deaminase alone did not affect adherence of either unstimulated or stimulated neutrophils to either fibroblasts or endothelial cells (Figs. 5 and 6). In contrast, and as described above, treatment of connective tissue cells with methotrexate (100 μM) markedly inhibited neutrophil adherence to connective tissue cells and this inhibition was completely abolished by the addition of adenosine deaminase. Similarly, adenosine deaminase completely reversed the effect of acadesine on adherence to endothelial
The experiments performed in the washing cadaseine occupancy fields per pative tissue antiinflammatory (ADA, 0.125 otrexate and the in adenosine cellar released from otrexate diminishes neutrophils adundance varied in monolayers were were neutrophils to icant increase in adherence of both stimulated and unstimulated neutrophils to cadaseine-treated endothelial cells (P < 0.05).

DISCUSSION
The results of the experiments reported herein demonstrate an antinflammatory action of methotrexate: increased aden-
osine release. Treatment of both fibroblasts and endothelial cells with methotrexate at pharmacologically relevant doses increases adenosine release from these cells, an effect that is even more marked in the presence of stimulated neutrophils. The adenosine released from methotrexate-treated connective tissue cells, in turn, inhibits adhesion of neutrophils to connective tissue cells, a critical initial step for infiltration or injury by neutrophils of connective tissue cells. These observations suggest that this is a mechanism by which methotrexate diminishes inflammation in vivo.

We have shown herein that the concentration of adenosine released from methotrexate-treated cells that remains extra-
cellular (equivalent to a final concentration of 400–500 nM) inhibits neutrophil function but the antinflammatory effects of extracellular adenosine are not confined to neutrophil function. Previous studies have demonstrated that adenosine occupies adenosine A2 receptors on monocyte–macrophages (15–19) and lymphocytes (20–24), cells that play a major role in the pathogenesis of chronic inflammation. In general, occupancy of adenosine receptors on monocytes and lym-
phocytes inhibits their ability to induce tissue damage. It has been demonstrated (12, 14, 25–30) that adenosine occupies specific A2 receptors on the surface of neutrophils to inhibit the generation of toxic oxygen metabolites such as O2·-, H2O2, and adherence to endothelium. Thus, for example, increased release of adenosine from synovial cells could dampen both the acute and chronic inflammation present in the joints of patients with rheumatoid arthritis.

Although the functional effects of adenosine are not restricted to a single type of inflammatory cell, we would predict that the effects of the adenosine released from methotrexate-treated cells would be restricted to the areas most directly infiltrated by inflammatory cells. Adenosine is very short-lived in whole blood where it is rapidly taken up by erythrocytes or metabolized by adenosine deaminase (31). Moreover, at sites of tissue necrosis intracellular enzymes such as adenosine deaminase are released that can metab-
olize adenosine to the functionally inactive purine riboside inosine.

**FIG. 4.** Normal human fibroblasts were incubated in the presence of adenosine at the indicated concentrations with stimulated (fMet-Leu-Phe at 0.1 μM) or unstimulated neutrophils. After fixation and washing the monolayers were stained and the neutrophils were counted in three fields per well. Data are the mean (± SEM) of four experiments performed in duplicate. Two-way ANOVA demonstrates that neutrophil adherence varied significantly with the dose of adenosine (P < 0.01) and with stimulation (P < 0.001).

**FIG. 5.** Normal human fibroblasts were incubated with methotrexate (100 μM) for 48 hr, washed extensively, and then incubated for 2 hr with unstimulated or stimulated (fMet-Leu-Phe at 0.1 μM) neutrophils in the presence or absence of adenosine deaminase (ADA, 0.125 international unit/ml). After fixation and washing the monolayers were stained and the neutrophils were counted in three fields per well. Data are the mean (± SEM) of three experiments performed in duplicate. Two way ANOVA demonstrates that neutrophil adherence varied significantly with the presence of methotrexate (P < 0.01) and that adenosine deaminase induced a significant increase in adherence of both stimulated and unstimulated neutrophils to methotrexate-treated fibroblasts (P < 0.05).

**FIG. 6.** Preconfluent monolayers of human umbilical vein endothelial cells were incubated in the presence or absence of methotrexate (100 μM) for 48 hr and washed extensively. The monolayers were then incubated with neutrophils in the presence (stimulated) and absence (unstimulated) of fMet-Leu-Phe (0.1 μM) and adenosine deaminase (ADA, 0.125 international unit/ml) for 2 hr. After fixation and washing the monolayers were stained and the neutrophils were counted in three fields per well. Data are the mean (± SEM) of three experiments performed in duplicate. Two-way ANOVA demonstrates that neutrophil adherence varied significantly with the presence of methotrexate (P < 0.01) and that adenosine deaminase induced a significant change in adherence of both stimulated and unstimulated neutrophils to methotrexate-treated endothelial cells (P < 0.05).

**FIG. 7.** Normal human endothelial cells were incubated for 2 hr with unstimulated or stimulated (fMet-Leu-Phe at 0.1 μM) neutrophils in the presence or absence of adenosine (100 μM) and adenosine deaminase (ADA, 0.125 international unit/ml). After fixation and washing, the monolayers were stained and the neutrophils were counted in three fields per well. Data are the mean (± SEM) of three experiments performed in duplicate. Two-way ANOVA demonstrates that neutrophil adherence varied significantly with the presence of adenosine (P < 0.01) and that adenosine deaminase induced a significant change in adherence of both stimulated and unstimulated neutrophils to adenosine-treated endothelial cells (P < 0.03).
The molecular mechanism by which methotrexate and acadesine promote adenosine release from connective tissue cells remains unknown; however, our data suggest a possible pathway by which methotrexate may induce increased extracellular adenosine concentrations. Methotrexate and its polyglutamated derivatives are potent inhibitors of AICAR transformylase (8). Inhibition of AICAR transformylase could cause accumulation of its substrate, AICAR and adenosine, a compound previously shown to promote adenosine release by an unknown mechanism (9). Our data are consistent with this hypothesis. Thus, the parallel effects of acadesine and methotrexate on adenosine release and neutrophil adherence suggest that the effect of methotrexate on adenosine release is due to inhibition by methotrexate of AICAR transformylase with accumulation of AICAR.

An alternative pathway by which methotrexate could modulate inflammatory cell interactions is suggested by studies of Nesher and Moore (32), who found that methionine reverses the effects of methotrexate on in vitro immunoglobulin production and hypothesized that uptake of methionine leads to regeneration of S-adenosylmethionine, a methyl donor that may be depleted in methotrexate-treated cells due to inhibition of dihydrofolate reductase. Our results do not exclude the hypothesis of Nesher and Moore (32) but suggest an alternative interpretation of their studies. In methotrexate-treated cells exogenous methionine may degrade to homocysteine that could recondense with adenosine thereby "trapping" excess adenosine intracellularly as S-adenosylhomocysteine. If increased adenosine release contributes to the antiinflammatory activity of methotrexate, then intracellular "trapping" of adenosine would reverse the effects of methotrexate treatment. Alternatively, methotrexate may inhibit the function of various cell types by different mechanisms.

Our results show that, in contrast to untreated connective tissue cells, cells treated with either methotrexate or acadesine release more adenosine after exposure to stimulated neutrophils. The mechanism by which stimulated neutrophils enhance adenosine release only from cells treated with methotrexate or acadesine is unknown. However, it is well known that intracellular stores of reduced glutathione protect connective tissues from oxidant injury. Stimulated neutrophils release a variety of toxic oxygen metabolites that require detoxification and, ultimately, ATP turnover to regenerate reduced glutathione. Moreover, ATP is used to reestablish membrane ion gradients in connective tissue cells after exposure to the toxic products of neutrophils. Therefore, it is possible that neutrophils enhance adenosine release from connective tissue cells treated with methotrexate or acadesine because such treatment might diminish reutilization of adenosine generated during adenine nucleotide turnover.

Whereas our studies do not rule out a direct effect of methotrexate on neutrophil function, our results do indicate an antiinflammatory mechanism by which methotrexate may ameliorate rheumatoid arthritis; methotrexate increases adenosine release from connective tissue cells, specifically connective tissue cells under stress. Since the effects of adenosine are confined to those areas where the adenosine is released and because of the extremely rapid metabolism of adenosine in tissues and in the blood, the potential toxicity of excess adenosine release is reduced. Thus, the demonstration that agents capable of stimulating adenosine release at inflamed sites are antiinflammatory could lead to the development of an additional class of antiinflammatory drugs.

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