In vitro effects of methotrexate on peripheral blood monocytes: modulation by folinic acid and S-adenosylmethionine

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Abstract

The mechanism of action of low dose methotrexate in rheumatoid arthritis has not been established. It has been shown to have an anti-inflammatory effect and to inhibit neutrophil chemotaxis, but the effect on monocytes has not been widely studied.

Normal donor peripheral blood monocytes were incubated with methotrexate in vitro and their superoxide production, chemotaxis, and phagocytosis subsequently assessed. Additionally, the influence of different culture media, and of folinic acid, and the methyl donor S-adenosylmethionine, and spermidine on the methotrexate mediated effects were evaluated.

It was found that methotrexate in low concentrations inhibited in vitro monocyte chemotaxis and superoxide production but only after prolonged incubation. This inhibition was augmented by incubation in medium containing a low methionine concentration and was abolished by folinic acid and S-adenosylmethionine, suggesting that methotrexate may interfere with specific methylation reactions.

The mechanism of action of low dose methotrexate in rheumatoid arthritis has not been defined. Initial studies failed to prove an immune modulating effect,¹⁻³ but subsequent studies reported suppression of B cell function,⁴ delayed type hypersensitivity,⁵ IgM rheumatoid factor synthesis,^{6,7} and mononuclear cell proliferation.⁷ An anti-inflammatory effect was suggested by others.⁸⁻¹⁰ This effect was not related to inhibition of cyclooxygenase or lipoxygenase.¹⁰ Furthermore, methotrexate inhibited neutrophil chemotaxis in vivo¹¹ and ex vivo,¹² though short in vitro incubations (30 minutes) of neutrophils with methotrexate failed to show such inhibition.¹²

Monocytes have a key role in chronic inflammatory conditions such as rheumatoid arthritis. Several slow acting antirheumatic drugs have been shown to modulate monocyte function,¹³⁻¹⁷ which may account, at least in part, for their beneficial effect in rheumatoid arthritis.

The effects of methotrexate on monocyte function have not been studied in detail. We studied in vitro the effects of methotrexate on isolated normal donor blood monocytes and the modulation of these effects by folinic acid and by the methyl donor S-adenosylmethionine.

Methods

SEPARATION OF MONONUCLEAR CELLS

Blood (30 ml) from healthy donors was diluted in 50 ml normal saline, layered over 12 ml Ficoll-Hypaque, and separated by centrifugation at 1200 rpm, room temperature, for 30 minutes. Mononuclear cells were collected and washed with RPMI-1640 (Sigma, St Louis, MO), medium-199 (BBL, Cockeysville, MD), or RPMI-1640 medium deficient, supplemented with L-glutamine (2 mmol/l), L-leucine (0.4 mmol/l), L-lysine (0.2 mmol/l), and L-methionine (0.03 or 0.06 mmol/l) (all from Sigma).

MONOCYTE SEPARATION AND INCUBATION

Monocytes were separated by the method of Freundlich and Avdolovic.¹⁸ Plastic Petri dishes (60×15 mm; Falcon, Becton-Dickinson, Oxnard, CA) were coated with 5 ml 2% gelatin (Sigma) and incubated for two hours at 37°C. The fluid was then removed and the dishes allowed to dry.

Five millilitres of a mononuclear cell suspension (2×10^6 cells/ml) were added to each Petri dish and incubated for 40 minutes at 37°C. The non-adherent cells were then removed by gentle suction. Adherent cells were 92 (SEM 3)% monocytes by latex phagocytosis.

Methotrexate (Sigma) was then added to the adherent cells, diluted in RPMI-1640, RPMI-1640 deficient, or medium-199, with 10% fetal calf serum to final concentrations of 10^{-8} to 10^{-5} mol/l. Controls were incubated in drug free media. Incubation times ranged from one to 64 hours.

The supernatant was then removed by gentle suction and 5 M EDTA (Sigma) in RPMI-1640, RPMI-1640 deficient, or medium-199, diluted 1:1 in 0.15 M phosphate buffered saline pH 7.3, was added for 10 minutes. After washing, the cells were resuspended in RPMI-1640, RPMI-1640 deficient, or medium-199 and incubated at 37°C for one hour with various concentrations of folinic acid (Sigma), S-adenosylmethionine (Sigma), spermidine (Sigma), or medium alone. The cells were then washed and resuspended in phenol red-free balanced salt solution (Sigma) for the superoxide assay or in RPMI-1640, medium-199, or RPMI-1640 deficient for the chemotaxis assay. Cell viability was evaluated by erythrosin B (Bio-Rad, Richmond, CA) dye exclusion.

MONOCYTE CHEMOTAXIS ASSAY

Zymosan activated serum was prepared by incubating normal human serum with zymosan

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Accepted for publication 1 August 1990

(Sigma) (10 mg/ml) for 30 minutes at 37°C. After removal of the zymosan by centrifugation (2000 rpm for 10 minutes at 4°C) the supernatant was stored at -70°C until used.

One micromole of *N*-formyl-methionyl-leucyl-phenylalanine (Sigma) or 10% zymosan activated serum in medium was added to the lower compartments of Boyden chambers. A suspension of monocytes (10⁶/ml) was placed in the upper compartment. The two compartments were separated by a 5 µm pore size nitrocellulose filter (Millipore, Bedford, MA). Incubations were carried out at 37°C for 90 minutes.

Filters were then removed, fixed in isopropanol, stained with 8% Giemsa stain, washed in water, dehydrated in 70% and 100% isopropanol sequentially, cleared in xylenes, and mounted in Permunt. Random migration was determined by measuring migration towards medium alone. Chemotaxis was assayed by the leading front technique, measuring the distance travelled by the two most advanced cells in response to the zymosan activated serum, minus the random migration.

MONOCYTE SUPEROXIDE PRODUCTION ASSAY

Opsonised zymosan was prepared by incubating normal human serum with zymosan (10 mg/ml) for 30 minutes at 37°C. Opsonised zymosan was removed after centrifugation (2000 rpm at 4°C for 10 minutes), washed, resuspended in phenol red-free balanced salt solution, and stored at -70°C until used.

Superoxide production was measured by reduction of cytochrome *c*. Reaction mixtures contained 2 × 10⁵ monocytes/ml phenol red-free balanced salt solution, opsonised zymosan (2 mg/ml), and cytochrome *c* (2 mg/ml) (Sigma), with or without superoxide dismutase 30 U/ml (Sigma). Catalase (1000 U/ml, Sigma) was also added as oxidation of cytochrome *c* by hydrogen peroxide may underestimate superoxide production.¹⁹ Controls included reaction mixtures without cells and mixtures without stimulants.

Incubations were carried out at 37°C for 30 minutes. The extent of cytochrome *c* reduction in the supernatant was measured as the change in absorbance at 550 nm against controls (reaction mixtures without cells) which was inhibited by superoxide dismutase. The molar extinction coefficients of ferricytochrome *c* and ferrocyanochrome *c* (8.9 × 10³ and 29.9 × 10³ M⁻¹ cm⁻¹ respectively) were used in the calculations.

STATISTICAL ANALYSIS

Assays were performed in duplicate. Results are

the means of three to eight assays. The significance of the difference between the means was evaluated by Student's *t* test.

Results

CELL VIABILITY AND FUNCTION

Cell viability and function were checked after the incubation period by the erythrosin B exclusion assay. Adherent cell viability was >90% after incubations of up to 40 hours. A significant decrease in cell viability was noted at 64 hours' incubation (42 (SEM 12)% viability). Cell viability was not significantly affected by methotrexate in concentrations of 10⁻⁸ to 10⁻⁵ mol/l.

Random migration, chemotaxis, and superoxide production were likewise affected by the duration of incubation, showing good function during the initial 40 hours of incubation but deterioration of these functions with more prolonged incubations (tables 1, 2, and 3).

EFFECT OF METHOTREXATE ON MONOCYTE

RANDOM MIGRATION

Random migration was decreased by 25% after preincubation for 40 hours with methotrexate at 10⁻⁶ and 10⁻⁵ mol/l, but this did not reach statistical significance. Lower methotrexate concentrations did not affect random migration.

EFFECT OF METHOTREXATE ON MONOCYTE

CHEMOTAXIS

Preincubations with methotrexate at 10⁻⁸ and 10⁻⁷ mol/l did not affect monocyte chemotaxis. At 10⁻⁶ mol/l incubations of 40 hours resulted in 60% inhibition of chemotaxis (table 1). Increasing methotrexate concentration to 10⁻⁵ mol/l resulted in more rapid inhibition of chemotaxis (20% at six hours, 53% at 16 hours of preincubation), but the degree of inhibition was still 60% at 40 hours.

EFFECT OF METHOTREXATE ON MONOCYTE

SUPEROXIDE PRODUCTION

Methotrexate at 10⁻⁸ mol/l did not affect superoxide production (table 2). At 10⁻⁷ mol/l there was minimal suppression (17% at 16 hours, 24% at 40 hours). The inhibition was greater with 10⁻⁶ M and 10⁻⁵ M methotrexate, being 64% and 73% respectively at 40 hours.

INHIBITION BY METHOTREXATE

Effect of different media

We compared the effect of three different culture media on monocyte inhibition by methotrexate: RPMI-1640 containing 2.5 µM folic acid and 100 µM methionine, RPMI-1640 methionine deficient (Sigma) supplemented with 30 µM or 60 µM methionine, and medium-199 containing 0.025 µM folic acid and 200 µM methionine.

There was no significant difference between responses of monocytes incubated in RPMI-1640, medium-199, or RPMI-1640 deficient supplemented with 60 µM methionine. The

Table 1 Effect of methotrexate on monocyte chemotaxis toward 10% zymosan activated serum. Results are given as percentages (SEM) versus controls at one hour*

Methotrexate concentration (mol/l)	Time of preincubation with methotrexate (h)				
	1	6	16	40	64
10 ⁻⁵	97 (1)	80 (5)†	45 (10)†	41 (10)†	11 (8)
10 ⁻⁶	101 (3)	91 (3)	78 (8)	38 (3)†	3 (2)†
10 ⁻⁷	103 (5)	91 (23)	83 (4)	78 (6)	21 (3)
10 ⁻⁸	99 (2)	98 (2)	90 (2)	76 (3)	30 (4)
0	100 (5)	98 (5)	96 (6)	92 (8)	51 (18)

*100% = 68 µm, by the leading front method (see text).

†p < 0.05 v control without methotrexate.

Table 2 Effect of methotrexate on superoxide production by monocytes. Results are given as percentages (SEM) versus controls at one hour*

Methotrexate concentration (mol/l)	Time of preincubation with methotrexate (h)				
	1	6	16	40	64
10 ⁻⁵	98 (4)	86 (6)	30 (10)†	25 (8)†	12 (11)†
10 ⁻⁶	101 (2)	101 (2)	78 (6)†	33 (7)†	13 (5)†
10 ⁻⁷	99 (4)	96 (3)	80 (3)†	70 (8)†	30 (8)
10 ⁻⁸	105 (4)	98 (4)	88 (2)	79 (5)	41 (5)
0	100 (5)	101 (4)	96 (2)	92 (6)	67 (16)

*100%=4.8 nmol reduced cytochrome c/10⁶ monocytes×30 minutes.

†p<0.05 v control at respective incubation times.

Table 3 Effect of different media on inhibition of monocyte functions by methotrexate after 40 hours' incubation at 10⁻⁶ mol/l. Results are given as percentages (SEM) versus controls (monocytes incubated for 40 hours without methotrexate)*

Monocyte function	RPMI-1640	Medium-199	RPMI-1640 deficient†	RPMI-1640 deficient‡ + 10 ⁻⁴ M methionine
Chemotaxis	55 (5)	61 (9)	41 (3)§	84 (10)
Superoxide production	58 (7)	64 (8)	35 (7)§	83 (5)
Phagocytosis	89 (6)	90 (2)	87 (5)	82 (10)

*100%=63 µm (chemotaxis) and 4.4 nmol reduced cytochrome c/10⁶ monocytes×30 minutes (superoxide production).†Containing 3×10⁻⁵ M methionine.

‡Methionine was added for one hour after 40 hours' incubation with methotrexate.

§p<0.05 v cells in RPMI-1640.

||p<0.05 v cells in RPMI-1640 deficient.

degree of inhibition by methotrexate was not affected by changing the culture medium. When the cells were incubated in RPMI-1640 deficient medium supplemented with 30 µM methionine, however, slightly increased inhibitory effects of methotrexate on both chemotaxis and superoxide production were noted, which were reversible with addition of 10⁻⁴ mol/l methionine (table 3). Addition of glutamine at 10⁻³ mol/l, cysteine, serine, or glycine at 10⁻⁴ mol/l, however, did not significantly affect the methotrexate mediated inhibition. Consequently, all incubations were carried out in this medium.

Effect of different stimulants

There was no significant difference between the chemotactic responses to zymosan activated serum and to *N*-formyl-methionyl-leucyl-phenylalanine. The degree of inhibition by

methotrexate was similar after either stimulation (data not shown).

METHOTREXATE INHIBITION OF CHEMOTAXIS AND SUPEROXIDE GENERATION

Effect of folic acid

Adherent mononuclear cells were incubated for 40 hours with 10⁻⁶ M methotrexate, then washed and incubated with folic acid at 10⁻⁸–10⁻⁴ mol/l for one hour before chemotaxis and superoxide generation assays. Folic acid at 10⁻⁶ to 10⁻⁴ mol/l reversed the inhibition by methotrexate, whereas lower concentrations had no effect (tables 4 and 5).

Effect of *S*-adenosylmethionine

Adherent mononuclear cells were incubated for 40 hours with 10⁻⁶ M methotrexate, then washed and incubated with *S*-adenosylmethionine at 10⁻⁸ to 10⁻⁴ mol/l. Reversal of the inhibition by methotrexate was achieved at *S*-adenosylmethionine concentrations of 10⁻⁶ to 10⁻⁴ mol/l, whereas lower concentrations were less effective (tables 4 and 5). *S*-adenosylmethionine itself did not significantly affect chemotaxis or superoxide production.

Effect of spermidine

To determine whether the effect of *S*-adenosylmethionine is mediated through increased synthesis of polyamines we incubated the cells with spermidine at 10⁻⁸ to 10⁻⁴ mol/l for one hour before the chemotaxis and superoxide production assays. As spermidine can be oxidised to aldehydes and hydrogen peroxide, catalase was added to some of the cultures at 1000 U/ml. Spermidine had no significant effect at any of the concentrations tested (tables 4 and 5).

Discussion

These results showed that methotrexate in concentrations up to 10⁻⁵ mol/l did not change random migration of normal peripheral blood

Table 4 Effect of folic acid, *S*-adenosylmethionine, and spermidine on methotrexate inhibition of monocyte chemotaxis. Results are given as percentages (SEM) versus controls (monocytes incubated for 40 hours in culture medium alone)*

	Concentration of <i>S</i> -adenosylmethionine/spermidine/folic acid (mol/l)					
	0 (methotrexate alone)	10 ⁻⁸	10 ⁻⁷	10 ⁻⁶	10 ⁻⁵	10 ⁻⁴
Methotrexate+ <i>S</i> -adenosylmethionine	41 (3)	62 (2)†	68 (2)†	68 (10)†	84 (5)†	92 (3)†
Methotrexate+spermidine	41 (3)	49 (3)	53 (6)	49 (5)	60 (8)	60 (10)
Methotrexate+folic acid	41 (3)	50 (3)	48 (4)	66 (3)†	80 (4)†	87 (6)†

*100%=63 µm by the leading front method (see text).

†p<0.05 v cells in methotrexate alone.

Table 5 Effect of folic acid, *S*-adenosylmethionine, and spermidine on methotrexate inhibition of monocyte superoxide production. Results are given as percentages (SEM) versus controls (monocytes incubated for 40 hours in culture medium alone)*

	Concentration of <i>S</i> -adenosylmethionine/spermidine/folic acid (mol/l)					
	0 (methotrexate alone)	10 ⁻⁸	10 ⁻⁷	10 ⁻⁶	10 ⁻⁵	10 ⁻⁴
Methotrexate+ <i>S</i> -adenosylmethionine	36 (7)	30 (6)	41 (3)	63 (5)†	73 (12)†	92 (6)†
Methotrexate+spermidine	36 (7)	35 (3)	30 (3)	29 (5)	47 (9)	56 (13)
Methotrexate+folic acid	36 (7)	39 (2)	44 (6)	60 (4)†	54 (8)†	85 (11)†

*100%=4.4 nmol reduced cytochrome c/10⁶ monocytes×30 minutes.

†p<0.05 v cells in methotrexate alone.

monocytes. Methotrexate inhibited monocyte chemotaxis and superoxide production in vitro, however. These effects were dependent on time and concentration. They were noted at 10^{-6} and 10^{-5} mol/l concentrations of methotrexate and prolonged incubation periods. This is in agreement with a previous study¹⁷ showing that 30 minute in vitro incubations inhibited monocyte chemotaxis only at methotrexate concentrations of 10^{-4} and 10^{-3} mol/l, whereas lower concentrations were not inhibitory. The importance of the time factor was also shown by the lack of methotrexate effect on neutrophil chemotaxis after short (30 minutes) in vitro incubations.¹²

Low dose pulse methotrexate results in peak serum concentrations at 10^{-6} mol/l, with trough concentrations below 10^{-8} mol/l.²⁰ The inability to show significant effect of methotrexate at this low concentration in our in vitro system raised the question as to whether such an effect on monocytes occurs in vivo. Although the answer to this is not apparent, evidently some important parameters in vitro are different in vivo. One such variable is the concentration of folic acid, which is 100-fold greater in RPMI-1640 than in the serum. Increased folate may interfere with the methotrexate effect owing to competition on the membrane receptor site, or on the enzyme dihydrofolate reductase. Moreover, methotrexate influx and efflux in cells may differ in vivo and in vitro.²¹ Possibly, the noted in vitro anti-inflammatory effects of methotrexate are achievable in vivo at lower concentrations with continual use.

This question of relevancy of the in vitro data to the in vivo situation might have been answered by an ex vivo study performed on monocytes of patients with rheumatoid arthritis treated with methotrexate alone. As the current recommended treatment for rheumatoid arthritis requires a combination of drugs, however, starting and maintaining patients with methotrexate alone for the sole purpose of this study was not feasible.

The inhibitory effects of methotrexate were reversed by addition of folinic acid, suggesting that inhibition of chemotaxis and superoxide production is mediated through inhibition of generation of reduced folates.

The methotrexate inhibitory effects were augmented by incubation in culture medium containing a low methionine concentration. This was reversed by addition of methionine and also by S-adenosylmethionine, and was dependent on concentration. The ability of S-adenosylmethionine to enter cells has been questioned because of its polarity. A recent study, however, reported doubling of S-adenosylmethionine content in L1210 cells incubated with 5×10^{-4} M S-adenosylmethionine.²² To evaluate whether the protective effect of S-adenosylmethionine is mediated through generation of polyamines we added spermidine to the cultures after the incubation with methotrexate. In contrast with S-adenosylmethionine, spermidine did not reverse the methotrexate effects on monocytes. Although polyamines (especially in high concentrations, 10^{-4} mol/l) can modulate leucocyte responses to stimuli,²³⁻²⁵ we found no such effect with the

concentrations tested. These combined data suggest that methotrexate may also inhibit methylation reactions dependent on S-adenosylmethionine.

Methylation reactions may have a role in the chemotactic and oxidative responses of monocytes.²⁶⁻²⁸ Generation of certain methyl donors might be affected by methotrexate. It primarily inhibits the generation of reduced folates through inhibition of dihydrofolate reductase,²⁹ and it may secondarily inhibit the synthesis of S-adenosylmethionine. S-adenosylmethionine is synthesised from methionine. In the cell methionine is regenerated from homocysteine, a reaction which requires both methionine synthase, methyl cobalamine, and methyl tetrahydrofolate, which is dependent on dihydrofolate reductase.³⁰ In addition to interference with intracellular methionine synthesis, methotrexate may also inhibit methionine transport into the cell.³¹

We are not aware of any report on the effect of methotrexate on S-adenosylmethionine synthesis, but additional indirect evidence suggests such an effect. Rats given nitrous oxide, which inhibits regeneration of methionine through inhibition of methylcobalamine and methionine synthase, had lower tissue concentrations of both methionine and S-adenosylmethionine.³² In this context it is of interest to note that nitrous oxide was reported to augment methotrexate toxicity in vivo³³ and to decrease neutrophil chemotaxis.³⁴ Furthermore, after methotrexate treatment patients had increased plasma and urine homocysteine concentrations³⁵ and decreased plasma methionine,³⁴ suggesting inhibition of methionine synthesis.

These data support the hypothesis that methotrexate has anti-inflammatory effects.⁸⁻¹⁰ We suggest that by inhibiting formation of methyl donors, such as reduced folates and, possibly, S-adenosylmethionine, methotrexate may inhibit methylation dependent processes that are essential for the inflammatory response.

Supported in part by a fellowship grant from the Lupus Foundation of America, Eastern Missouri Chapter (Dr Nesher), and grants from the Employees Charity Services of McDonnell-Douglas Corporation of St Louis, and Eastern Missouri Chapter of the Arthritis Foundation.

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