Inhibition of cytokine production by methotrexate. Studies in healthy volunteers and patients with rheumatoid arthritis
de Lathouder, S.

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Chapter 2.

Inhibition of cytokine production by methotrexate
Studies in healthy volunteers and patients with rheumatoid arthritis.

Anderas H. Gerards, Sacha de Lathouder, Els de Groot, Ben A. C. Dijkmans and Lucien A. Aarden.

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Abstract

Objectives. To analyse whether the beneficial effects of methotrexate (MTX) in rheumatoid arthritis (RA) could be due to inhibition of inflammatory cytokine production.

Methods. Cytokine production was studied using whole blood (WB) and mononuclear cells (MNC) of healthy volunteers and RA patients. Cultures were stimulated with either bacterial products such as lipo-oligosaccharide (LOS) or Staphylococcus aureus Cowan I (SAC) to activate monocytes or with monoclonal antibodies to CD3 and CD28 to induce polyclonal T cell activation. We analysed the effect of MTX on cytokine production in these systems.

Results. We showed that MTX inhibits production of cytokines induced by T cell activation. Among the cytokines inhibited were IL-4, IL-13, IFNγ, TNFα and GM-CSF. Inhibition was seen at concentrations easily achieved in plasma of RA patients taking the drug. IL-8 production was hardly influenced by MTX. Furthermore inhibition was dependent on the stimulus; IL-6, IL-8 IL-1β and TNFα production induced by LOS or SAC was only slightly decreased by MTX. The addition of folinic acid or thymidine and hypoxanthine reversed the inhibitory effects of MTX on cytokine production. Concentrations of MTX required for inhibition varied between donors. Oral intake of 10 mg MTX by RA patients led to marked inhibition of cytokine production in blood drawn after 2h.

Conclusions. MTX turns out to be an efficient inhibitor of cytokine production induced by T cell activation in freshly drawn blood. This inhibition is due to inhibition of the de novo synthesis of purines and pyrimidines. Cytokines produced by monocytes are hardly affected by MTX.
**Introduction**

Methotrexate (MTX) has become the most frequently used anti-rheumatic drug (1;2). However, the exact mechanism of action in rheumatoid arthritis (RA) is not yet clarified (3-5). After administration, the kidneys rapidly excrete MTX and only a small amount of the drug is transported into cells by folate receptors. Intracellular MTX and polyglutamated derivatives of MTX not only inhibit dihydrofolate reductase (DHFR) but also have marked affinity for other folate-dependent enzymes such as thymidylate synthase, AICAR (5-amino-imidazol-4-carboxamide ribonucleotide transformylase) and AICARFT (AICAR formyl-transferase). The inhibition of these enzymes affects purine, pyrimidine and homocysteine metabolism and DNA synthesis (6-8). MTX polyglutamate levels in circulating erythrocytes and polymorphs correlate with clinical efficacy in RA (9). Notwithstanding our knowledge of MTX as a folate antagonist, the mechanism by which weekly administered, low-dose MTX, attenuates the disease process in RA patients remains elusive. Nesher et al. showed that MTX inhibits pokeweed mitogen-induced proliferation and immunoglobulin synthesis of peripheral blood cells via reduction of polyamine synthesis(10). Cronstein has put forward the interesting hypothesis that MTX may act via adenosine (11). MTX increases adenosine levels by inhibition of AICAR. Adenosine is known to have anti-inflammatory properties (12;13). Indeed, in animal models it was shown that MTX inhibits neutrophil function via stimulation of adenosine release (11) and that it also affects leukocyte recruitment to inflamed tissue (14). However, other experiments in animal models using adenosine agonists and antagonists, as well as measurement of purine and pyrimidine levels in blood of MTX-treated patients did not support the idea that MTX acts via adenosine (7;15). In view of the efficacy of anti-TNF treatment in RA, inhibition of cytokine production is another candidate mechanism for MTX. Down-regulation of inflammatory cytokines such as TNFα and IL-1β in rheumatoid synovium has been observed during treatment with MTX (16;17). In addition, plasma levels of various inflammatory cytokines are decreased during MTX treatment (18-20). Recently it was shown that MTX treatment results in decreased number of T cells capable of TNFα production whereas the number of T cells producing IL-10 after polyclonal activation increased (21). MTX possibly suppresses TNFα-induced NF-κB activation (22). Surprisingly, reports on in vitro effects of MTX on cytokine production are scarce. Available data demonstrate little or no effect of MTX on IL-1β or TNFα production in vitro (3;18;23-27). Only a very high dose of a liposomal preparation of MTX reduced TNFα production in peripheral blood-derived monocytes (28). There is no effect of MTX on TNFα production in lipopolysacharide (LPS)-stimulated WB cultures (18) or on IL-1 production of LPS stimulated MNC (24). Seitz et al. noticed an enhanced in vitro production of IL-10 by MNC of RA patients treated with MTX(29). Recently it was shown that MTX inhibits TNFα production in primed T cells, cultured for an extended period in the presence IL-2 (30). In contrast no effect of MTX in primary cultures of activated T cells was observed (30;31). There is no unanimity about effects of MTX on T cells. Some authors claim that MTX selectively kills activated T cells and fibroblasts by apoptosis (32;33), and induces apoptosis in synovium (34). Fairbanks et al. found that MTX is cytostatic and not cytotoxic, halting proliferation at G1 phase of the cell cycle, by inhibition of amidophosphoribosyl-transferase (32).

In short, the studies on MTX appear to be inconclusive regarding the effect on T cells, and although inflammatory cytokines diminish during MTX therapy, this effect was not
seen in \textit{in vitro} tests. The purpose of the present study is to assess whether MTX has an effect on T cell mediated production of inflammatory cytokines in vitro.

\textbf{Materials and Methods}

Blood samples were collected from a total of 20 healthy volunteers and 10 RA patients using 4 ml evacuated blood collection tubes (Greiner, Alphen a/d Rijn, the Netherlands), containing sodium heparin. WB cultures were performed in flat bottom microtiter plates (Nunc, Kamstrup, Denmark) by a method previously described in detail (20). Heparinised venous blood was used and cultured at a final 1:10 dilution at a final heparin concentration of 15 U/ml. In experiments performed with RA patients, whole blood was cultured at a final 1:4 dilution. All cultures were carried out in endotoxin-free Iscove's modified Dulbecco's medium (IMDM, BioWhittaker, Verviers, Belgium), supplemented with penicillin (100 IU/ml), streptomycin (100\mu g/ml), 0.1 % endotoxin-free fetal calf serum (FCS), 50 \mu M 2-mercaptoethanol, and 15 U/ml sodium heparin. Cultures were performed in duplicate except for experiments presented in table 1 and figure 6. It was essential to screen the batch of blood collection tubes as well as medium and FCS for absence of stimulatory material.

200 \mu l of diluted blood was stimulated with a combination of endotoxin-free anti-CD3 (CLB.T3/4.E, 1 \mu g/ml, Sanquin, Amsterdam, the Netherlands) and anti-CD28 (CLB.CD28/1, 1 \mu g/ml, Sanquin) or with LOS (100 pg/ml, derived from Neisseria Meningitidis, a kind gift of Dr J. Poolman, RIVM, Bilthoven, the Netherlands) or with SAC (Pansorbin, 1:4000, Calbiochem, La Jolla, CA). Cultures were incubated for 1 day (SAC and LOS) or 3 days (anti-CD3/anti-CD28) unless otherwise indicated.

MTX was obtained from AHP Pharma, Hoofddorp, the Netherlands. Folinic acid, folic acid, hypoxanthine and thymidine were obtained from Sigma (Sigma-Aldrich, Steinheim, Germany). Stock solutions of folinic acid (3 mg/ml), folic acid (3mg/ml), hypoxanthine (100 mM) and thymidine (100 mM) were prepared in H\textsubscript{2}O.

The production of cytokines was measured in the supernatant of the cell cultures in four serial dilutions. Supernatant was harvested at indicated times and tested directly by ELISA in various dilutions or stored at -20\degree C until use. IL-1\beta, IL-2, IL-4, IL-6, IL-8, IL-12p40, IL-13, TNF\alpha and IFN\gamma were measured with ELISA kits (PeliKine-compact, Sanquin) according to the protocol and are described before (20, 31, 35).

The GM-CSF ELISA was performed via the same protocol. The GM-CSF antibodies were a kind gift from Dr. G. Trinchieri (the Wistar Institute, Philadelphia, PA). In this assay the coating antibody was anti-GM-CSF 9.1 (used at 2 \mu g/ml), the biotinylated antibody was anti-GM-CSF 16.3 (0.1 \mu g/ml). Recombinant GM-CSF (Sandoz, Basel, Switzerland) was used for the preparation of a standard curve.

\textbf{Results}

\textbf{In vitro cytokine production in WB cultures.}

To assess cytokine production, WB cultures were stimulated with SAC. Table 1 shows a representative cytokine profile of a normal donor. It appeared that monokines (IL-1\beta, IL-6, IL-8, IL-12 and TNF\alpha) are readily secreted into the supernatant. However T cell cytokines were not produced (IL-2, IL-13) or in minor quantities only (GM-CSF, IFN\gamma, table 1). With
LOS similar results were obtained (not shown). Stimulation with a combination of anti-CD3 and anti-CD28 results in production of IL-2, IL-4, IL-13, GM-CSF, IFNγ and TNFα. Surprisingly also IL-8 is elevated (table 1). Polyclonal T cell stimulation of WB cultures leads to production of T cell cytokines and IL-8.

Table 1. Cytokine production (pg/ml)

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>unstimulated</th>
<th>SAC</th>
<th>anti-CD3/28</th>
</tr>
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<tbody>
<tr>
<td>IL-1β</td>
<td>10</td>
<td>1480</td>
<td>70</td>
</tr>
<tr>
<td>IL-6</td>
<td>-</td>
<td>5100</td>
<td>630</td>
</tr>
<tr>
<td>IL-8</td>
<td>60</td>
<td>38400</td>
<td>118100</td>
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<tr>
<td>IL-12p40</td>
<td>-</td>
<td>2150</td>
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<td>TNF</td>
<td>-</td>
<td>3290</td>
<td>3550</td>
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<tr>
<td>IL-2</td>
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<td>-</td>
<td>8480</td>
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<tr>
<td>IL-13</td>
<td>-</td>
<td>-</td>
<td>19100</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>10</td>
<td>360</td>
<td>9300</td>
</tr>
<tr>
<td>IFNγ</td>
<td>-</td>
<td>1020</td>
<td>50700</td>
</tr>
</tbody>
</table>

Table 1. Induction of cytokines in WB of a normal donor. Supernatants were harvested at day 1 after SAC stimulation and at day 3 after T cell stimulation; - = not detectable. This is a representative cytokine profile of one donor, each cytokine was measured in at least 3 healthy donors with similar results.
Inhibition of T cell stimulated cytokine production by MTX.

For each MTX concentration, cytokine production is expressed as % of production in the absence of MTX for each individual donor. The figure represents the mean +/- the 95% interval of these 8 donors. Supernatants were harvested at day 4. For the various donors the range of cytokine production in the absence of MTX was: IL-4, 170 - 2360 pg/ml; IL-13, 2700 - 10900; IL-8, 76000 - 276000 pg/ml; GM-CSF, 37300 - 123000 pg/ml; IFNγ, 47000 - 333000 pg/ml and TNFα, 470 - 11000 pg/ml.

Inhibition of cytokine production by MTX.

The next step was to study the influence of MTX on LOS-, SAC- or anti-CD3/anti-CD28-activated WB cultures. Addition of MTX to T cell stimulated cultures results in major inhibition of all cytokines tested, except IL-8. Even at high doses of MTX, IL-8 production is not affected whereas dose-dependent inhibition of the other cytokines is similar (fig. 1). Figure 2 shows the inhibition of cytokine production of each donor. SAC induced production of IL-6, IL-8, TNFα, IL-1β and IL-12 is not influenced by as much as 2 μg/ml MTX (not shown). LOS-induced cytokine production is slightly inhibited by high dose MTX (fig. 3). Similar results were obtained using MNC or purified T cells. However effects seen in purified cells were less profound and more variable compared to those in WB cultures (not shown).

These experiments show that MTX inhibits in vitro cytokine production (except IL-8) after T cell stimulation in WB, MNC and T cells, and not after stimulation with SAC or LOS.

Inhibition of T cell stimulated cytokine production by MTX of individual donors. WB cultures of 8 different blood donors were stimulated with anti-CD3 and anti-CD28. Supernatants were harvested at day 4. Each dot represents cytokine production of a donor in the presence of 2 μg/ml MTX expressed as percentage of production in the absence of MTX. The range of cytokine production for the donors the in absence of MTX is described in the legends of figure 1.
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Figure 3. Effect of MTX on LOS-induced cytokine production. WB cultures of 8 different blood donors were stimulated with LOS (100 pg/ml). Each dot represents cytokine production in the presence of MTX (2 μg/ml) expressed as the percentage of production in the absence of MTX. These productions ranged for IL-6 from 1800 - 3860 pg/ml; IL-8 from 4650 - 26100 pg/ml; TNFα from 220 - 1100 pg/ml and IL-1β from 580-2160 pg/ml.

Figure 4. Inhibition of cytokine production by MTX can be reversed by folinic acid. WB cultures of 10 donors were stimulated by anti-CD3 and anti-CD28 with and without MTX in the presence (white bar) or absence (black bar) of 40μg/ml folinic acid. GM-CSF production is expressed as % of production in the absence of MTX for each individual donor. GM-CSF production of the donors ranged from 11190-36765 pg/ml in the absence of MTX. Error bars indicate the SEM of 10 donors.

Interference of MTX with folate metabolism.
We then analysed whether this in vitro effect of MTX was due to interference with the folate metabolism. To evaluate the effects of MTX on folate metabolism, amethopterin, a stereoisomer of MTX incapable of inhibiting folate-dependent enzymes, was tested in WB cultures. Amethopterin was about 1000-fold less active then MTX in inhibiting anti-CD3/anti-CD28 induced cytokine production (not shown). We then investigated whether inhibition by MTX can be reversed by folinic acid or by folic acid. Indeed folinic acid reverses the inhibition by MTX (fig. 4) whereas high dose of folic acid had no effect (not shown). The effect of folinic acid on MTX treated cultures is significant (95% confidence interval 48% - 78%, p<0.001, paired t test on normaized data). Inhibition of cytokine production by MTX can also be reversed by addition of hypoxanthine and thymidine to the WB culture (fig. 5). In some donors addition of thymidine alone was sufficient. So it seems that MTX interferes with the folate metabolism and thereby with the synthesis of purines and pyrimidines.
Inhibition of cytokine production by MTX is a late phenomenon.

To explore the effects of MTX in our WB system in more detail, we evaluated cytokine production at different time points. Inhibition of IFNγ and TNFα production is only seen from day 3 on, similar results were seen for the other cytokines analysed, again with the exception of IL-8 (fig 6). In line with this late effect of MTX we observed that inhibition by MTX was identical when addition of MTX was delayed until 24 h after the start of the culture (not shown).

Sensitivity of donors to MTX.

We noticed that different donors needed different amounts of MTX to suppress cytokine production in WB cultures. To quantify this notion we determined the concentration of MTX required for 50% inhibition (ID-50) for each cytokine and in every individual. Dose-response curves of 7 donors were analysed. Figure 7 shows that TNFα and IFNγ in each donor are similarly affected by MTX and the same is true for the other cytokines (not shown). Additionally this experiment shows that between donors there is considerable variation in sensitivity for MTX.

MTX therapy leads to ex vivo inhibition of cytokine production.

MTX effectively inhibits cytokine production with an ID-50 between 5 and 25 ng/ml (fig. 7). Such levels are easily achieved in plasma, a couple of hours after oral application of MTX. To investigate whether plasma MTX levels are sufficient to inhibit cytokine production, we analysed WB cultures of 10 MTX-naive RA patients just before and 2 hours after their first administration of MTX (10 mg, orally). Indeed 2 hours after MTX
administration the mean IFN production in WB cultures was reduced from 21 ng/ml to 5.8 ng/ml (fig. 8), which corresponds to a mean ratio of 0.28 (95% confidence interval: 0.14-0.53; p<0.002 by paired t test on log-transformed data). The antagonistic effect of folinic acid was highly significant (p<0.002 by paired t test on log-transformed ratios). Similar results were obtained when GM-CSF was measured (not shown). As expected no change in IL-8 production was seen (not shown).

Figure 6. Kinetics of inhibition of cytokine production by MTX. Whole blood of a normal donor was stimulated with anti-CD3 and anti-CD28 in the presence (○-○) or in the absence (●-●) of MTX (100 ng/ml). Culture supernatants were harvested at indicated time points. This is a representative donor of 7 donors tested.
Figure 7. **TNFα and IFNγ production are similarly affected by MTX.** WB cultures of 7 donors were stimulated with anti-CD3 and anti-CD28 in the presence of increasing amounts of MTX. Supernatants were tested for cytokine production and the amount of MTX required for 50% reduction (ID-50) was calculated for each cytokine. Here the results for IFNγ and TNFα are depicted.

Figure 8. **Effect of oral uptake of MTX on ex vivo cytokine production.** Blood was obtained from 10 RA patients just before (t=0) and 2 hours after (t=2) the first oral application of MTX (10 mg). WB of 10 RA patients was stimulated with anti-CD3 and anti-CD28 in the absence or presence of 40μg/ml folinic acid (FA), without additional MTX.

**Discussion**

The in vitro inhibition of T cell cytokine production by MTX in freshly isolated human blood cells has not been reported before. WB cultures were predominantly used for this analysis. There are advantages in using WB cultures. The presence of erythrocytes protects against too much stress caused by oxygen radicals. Indeed WB cultures differ from isolated MNC. In WB cultures there is no background IL-8 production whereas, after stimulation, IL-12 en IFNγ production per cell is much higher than in MNC (35). MTX also inhibits cytokine production by purified T cells but this inhibition is less profound and more
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variable. Probably the higher activity of the salvage pathway as a result of the availability of nucleotides derived from dying cells and/or FCS added to the culture is responsible for this effect. We observed that activation of T cells in WB leads to production of a variety of T cell cytokines and of IL-8. This IL-8 production in WB cultures is surprising because isolated T cells produce very little IL-8 after anti-CD3 and anti-CD28 stimulation. Most likely activated T cells indirectly stimulate other cells such as monocytes or neutrophils. We analysed some of the possible mechanisms by which MTX inhibits TNFα, IFNγ, IL-2, IL-4, IL-13 and GM-CSF and not IL-8 production. It is unlikely that adenosine is involved in the effects seen in our cultures. We observed that adenosine or adenosine receptor agonists inhibit production of all cytokines, including IL-8 (not shown). In addition, adenosine antagonists had no effect on MTX inhibition. Already in 1990 Nesher et al. proposed that MTX might inhibit polyamine synthesis in MNC (10). In our system addition of polyamines failed to restore cytokine production in MTX-inhibited cultures. Moreover, our observation that inhibition of cytokine production by MTX can be reversed by a combination of hypoxanthine and thymidine shows that inhibition of purine and pyrimidine synthesis is the main mechanism by which cytokine production is inhibited. This observation is in agreement with the experiments by Genestier et al. (36). They observed that MTX induces apoptosis in activated T cells whereas not activated T cells are not touched. We have evidence that also in our cultures MTX leads to apoptosis in activated T cells as analysed by Annexin-V staining (not shown). Probably a lack of thymidine and/or purines during the transition from the G1 to the S phase leads to p53-mediated cell death. Monocytes are probably not inhibited by MTX because they hardly proliferate upon stimulation with SAC or LOS. Why Fairbanks et al. (32) using conditions very comparable to Genestier et al. did not find induction of apoptosis is not clear. Possibly salvage of nucleotides derived from dying cells in the high cell density culture could have influenced the outcome.

Recently Hildner et al. reported that cytokine production by long-term T cell cultures was inhibited by MTX (30). However, they did not see an effect of low dose MTX in primary cultures. This lack of effect can be ascribed to the choice to analyse cytokine production at day 2. We showed that inhibition of T cell cytokines does not occur on day 2, but is found from day 3 on.

Oral intake of 10 mg MTX leads to peak plasma levels of MTX around 50-100 ng/ml at 1-3h. We observed that in whole blood cultures of RA patients 2 hours after their first oral intake of MTX, plasma MTX levels are sufficient to inhibit cytokine production. Even after diluting the blood four times.

The main question to be addressed is whether our findings have any relation with the clinical situation. Possibly T cells are important targets for MTX but it is conceivable that other cells, for example in the synovial tissue, are the primary targets. If T cells are important, studying in vitro effects of MTX on T cells could be relevant for understanding its in vivo action. This would be in line with the observation of Rudwaleit et al. that during treatment with MTX the percentage of TNFα producing T cells decreases (21). If the real targets are other cells in the body, the experiments with T cells or WB cultures can still be clinically relevant. Various membrane receptors are involved in transport of MTX, folic acid and folinic acid into the cell. Moreover, in the cell the ratio of enzymes involved in polyglutamation and deglutamation can vary. Finally levels of purines and pyrimidines capable of salvaging the inhibition by MTX can differ from compartment to compartment.
and from individual to individual. In some donors, thymidine alone could reverse the inhibition of cytokine production by MTX. This is probably due to hypoxanthine release in the cultures by dying cells or by the presence of hypoxanthine in the plasma. Indeed HPLC analysis showed that up to 10 μM of free hypoxanthine could be present in WB supernatant after 1 day of culture. If the different sensitivity for MTX observed in our whole blood cultures is a reflection of (some of) these individual variations then sensitivity of cytokine production for MTX could be useful to predict clinical effectiveness of MTX in individual patients.

Conclusions

MTX is a specific inhibitor of pro-inflammatory cytokines in WB cultures after T cell stimulation. Inhibition is seen at MTX levels easily achieved in plasma after oral uptake of 10 mg MTX. The inhibition is due to interference with folate-dependent purine and pyrimidine synthesis. There is considerable variation between donors in sensitivity for these vitro effects of MTX. This could reflect the in vivo situation in which some patients respond to lower doses of MTX than other patients.

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