Inhibition of cytokine production by methotrexate.

Studies in healthy volunteers and patients with rheumatoid arthritis

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Objectives. To analyse whether the beneficial effects of methotrexate 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 \textit{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 methotrexate on cytokine production in these systems.

Results. We showed that methotrexate inhibits production of cytokines induced by T-cell activation. Among the cytokines inhibited were interleukin 4 (IL-4), IL-13, IFN\textgamma, tumour necrosis factor-\textalpha (TNF\textalpha) and granulocyte–macrophage colony-stimulating factor. Inhibition was seen at concentrations easily achieved in plasma of RA patients taking the drug. IL-8 production was hardly influenced by methotrexate. Furthermore, inhibition was dependent on the stimulus; IL-6, IL-8, IL-1\textbeta and TNF\textalpha production induced by LOS or SAC was only slightly decreased by methotrexate. The addition of folinic acid or thymidine and hypoxanthine reversed the inhibitory effects of methotrexate on cytokine production. Concentrations of methotrexate required for inhibition varied between donors. Oral intake of 10 mg methotrexate by RA patients led to marked inhibition of cytokine production in blood drawn after 2 h.

Conclusions. Methotrexate turns out to be an efficient inhibitor of cytokine production induced by T-cell activation in freshly drawn blood. This is due to inhibition of the \textit{de novo} synthesis of purines and pyrimidines. Cytokines produced by monocytes are hardly affected by methotrexate.

Key words: Methotrexate, Cytokines, Tumour necrosis factor, Rheumatoid arthritis, Whole blood culture.

Methotrexate has become the most frequently used anti-rheumatic drug \cite{1,2}. However, the exact mechanism of action in rheumatoid arthritis (RA) is not yet clarified \cite{3–5}. After administration, the kidneys rapidly excrete methotrexate and only a small amount of the drug is transported into cells by folate receptors. Intracellular methotrexate and polyglutamated derivatives of methotrexate 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 formyltransferase). The inhibition of these enzymes affects purine, pyrimidine and homocysteine...
metabolism and DNA synthesis [6–8]. Methotrexate polyglutamate levels in circulating erythrocytes and polymers correlate with clinical efficacy in RA [9].

Notwithstanding our knowledge of methotrexate as a folate antagonist, the mechanism by which weekly administered, low-dose methotrexate attenuates the disease process in RA patients remains elusive. Nesher and Moore [10] showed that methotrexate inhibits pokeweed mitogen-induced proliferation and immunoglobulin synthesis of peripheral blood cells via reduction of polyamine synthesis. Cronstein et al. [11] have put forward the interesting hypothesis that methotrexate may act via adenosine. Methotrexate 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 methotrexate inhibits neutrophil function via stimulation of adenosine release [11] and that it also affects leucocyte 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 methotrexate-treated patients did not support the idea that methotrexate acts via adenosine [7,15].

In view of the efficacy of anti-tumour necrosis factor (anti-TNF) treatment in RA, inhibition of cytokine production is another candidate mechanism for methotrexate. Down-regulation of inflammatory cytokines such as TNFα and interleukin 1β (IL-1β) in rheumatoid synovium has been observed during treatment with methotrexate [16, 17]. In addition, plasma levels of various inflammatory cytokines are decreased during methotrexate treatment [18–20]. Recently, it was shown that methotrexate treatment results in a decreased number of T cells capable of TNFα production, whereas the number of T cells producing IL-10 after polyclonal activation increased [21]. Methotrexate possibly suppresses TNFα-induced NF-κB activation [22]. Surprisingly, reports on in vitro effects of methotrexate on cytokine production are scarce. Available data demonstrate little or no effect of methotrexate on IL-1β or TNFα production in vitro [3, 18, 23–27]. Only a very high dose of a liposomal preparation of methotrexate reduced TNFα production in peripheral blood-derived monocytes [28].

There is no effect of methotrexate on TNFα production in lipopolysaccharide (LPS)-stimulated whole blood cultures [18] or on IL-1 production of LPS-stimulated MNC [24]. Seitz et al. [29] noticed an enhanced in vitro production of IL-10 by MNC of RA patients treated with methotrexate. Recently, it was shown that methotrexate inhibits TNFα production in primed T cells, cultured for an extended period in the presence IL-2 [30]. In contrast, no effect of methotrexate in primary cultures of activated T cells was observed [30, 31]. There is no unanimity about effects of methotrexate on T cells. Some authors claim that methotrexate selectively kills activated T cells and fibroblasts by apoptosis [32, 33], and induces apoptosis in synovium [34]. Fairbanks et al. [32] found that methotrexate is cytostatic and not cytotoxic, halting proliferation at the G1 phase of the cell cycle, by inhibition of amidophosphoribosyltransferase.

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

### 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. Whole blood (WB) cultures were performed in flat-bottom microtitre plates (Nunc, Kamstrup, Denmark) by a method previously described in detail [20]. Heparinized 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 μg/ml), 0.1% endotoxin-free fetal calf serum (FCS), 50 mm 2-mercaptoethanol and 15 U/ml sodium heparin. Cultures were performed in duplicate except for experiments presented in Table 1 and Fig 6. It was essential to screen the batch of blood collection tubes as well as medium and FCS for absence of stimulatory material.

An aliquot of 200 μl of diluted blood was stimulated with a combination of endotoxin-free anti-CD3 (CLB.T3/4,E, 1 μg/ml, Sanquin, Amsterdam, The Netherlands) and anti-CD28 (CLB.CD28/1, 1 μg/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.

Methotrexate was obtained from AHP Pharma, Hoofddorp, The Netherlands. Folinic acid, folate acid, hypoxanthine and thymidine were obtained from Sigma.

### Table 1. Induction of cytokines in whole blood of a normal donor

<table>
<thead>
<tr>
<th>Cytokine production (pg/ml) with stimulus</th>
<th>Unstimulated</th>
<th>SAC</th>
<th>anti-CD3/CD28</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>10</td>
<td>1480</td>
<td>70</td>
</tr>
<tr>
<td>IL-6</td>
<td>n.d.</td>
<td>5100</td>
<td>630</td>
</tr>
<tr>
<td>IL-8</td>
<td>60</td>
<td>38 400</td>
<td>118 100</td>
</tr>
<tr>
<td>IL-12 p40</td>
<td>n.d.</td>
<td>2150</td>
<td>710</td>
</tr>
<tr>
<td>TNFα</td>
<td>n.d.</td>
<td>3290</td>
<td>3550</td>
</tr>
<tr>
<td>IL-2</td>
<td>n.d.</td>
<td>n.d.</td>
<td>8480</td>
</tr>
<tr>
<td>IL-13</td>
<td>n.d.</td>
<td>n.d.</td>
<td>19 100</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>10</td>
<td>360</td>
<td>9300</td>
</tr>
<tr>
<td>IFNγ</td>
<td>n.d.</td>
<td>1020</td>
<td>50 700</td>
</tr>
</tbody>
</table>

Supernatants were harvested at day 1 after SAC stimulation and at day 3 after T-cell stimulation. This is a representative cytokine profile of one donor, each cytokine was measured in at least three healthy donors with similar results.

n.d., not detectable.
Inhibition of T-cell stimulated cytokine production by methotrexate. WB cultures of eight different blood donors were stimulated with anti-CD3 and anti-CD28. For each methotrexate concentration, cytokine production is expressed as the percentage of production in the absence of methotrexate for each individual donor. The figure represents the mean ± the 95% confidence interval of these eight donors. Supernatants were harvested at day 4. For the various donors the range of cytokine production in the absence of methotrexate was: IL-4, 170–2360 pg/ml; IL-13, 2700–10900 pg/ml; IL-8, 76000–276000 pg/ml; GM-CSF, 37300–123000 pg/ml; IFNγ, 47000–333000 pg/ml; and TNFα, 470–11000 pg/ml.

Fig. 1. Inhibition of T-cell stimulated cytokine production by methotrexate.
(Sigma-Aldrich, Steinheim, Germany). Stock solutions of folinic acid (3 mg/ml), folic acid (3 mg/ml), hypoxanthine (100 mM) and thymidine (100 mM) were prepared in H2O.

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 enzyme-linked immunosorbent assay (ELISA) in various dilutions or stored at −20 °C until use. IL-1β, IL-2, IL-4, IL-6, IL-8, IL-12p40, IL-13, TNFα and interferon-γ (IFNγ) were measured with ELISA kits (PeliKine-compact, Sanquin) according to the protocol and have been described previously [20, 31, 35]. The granulocyte–macrophage colony-stimulating factor (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 μg/ml), the biotinylated antibody was anti-GM-CSF 16.3 (0.1 μg/ml). Recombinant GM-CSF (Sandoz, Basel, Switzerland) was used for the preparation of a standard curve.

Results

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-1β, IL-6, IL-8, IL-12 and TNFα) are readily secreted into the supernatant. However, T-cell cytokines were not produced (IL-2, IL-13), or were in minor quantities only (GM-CSF, IFNγ, 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, IL-8 is also elevated (Table 1). Polyclonal T-cell stimulation of WB cultures leads to production of T-cell cytokines and IL-8.

Inhibition of cytokine production by methotrexate

The next step was to study the influence of methotrexate on LOS-, SAC- or anti-CD3/anti-CD28-activated WB cultures. Addition of methotrexate to T-cell stimulated cultures results in major inhibition of all cytokines tested, except IL-8. Even at high doses of methotrexate, 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 methotrexate (not shown). LOS-induced cytokine production is slightly inhibited by high-dose methotrexate (Fig. 3). Similar results were obtained using MNC or purified T cells. However, effects seen in purified cells were less profound and more variable than those in WB cultures (not shown).

These experiments show that methotrexate 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.

Interference of methotrexate with folate metabolism

We then analysed whether this in vitro effect of methotrexate was due to interference with the folate metabolism. To evaluate the effects of methotrexate on folate metabolism, amethopterin, a stereoisomer of methotrexate incapable of inhibiting folate-dependent enzymes, was tested in WB cultures. Amethopterin was about 1000-fold less active than methotrexate in inhibiting anti-CD3/anti-CD28-induced cytokine production (not shown). We then investigated whether inhibition by methotrexate can be reversed by folinic acid or by folic acid. Indeed, folinic acid reverses the inhibition by methotrexate (Fig. 4), whereas high doses of folic acid had no effect (not shown). The effect of folinic acid on methotrexate-treated cultures is significant (95% confidence interval 48–78%, P < 0.001, paired t-test on normalized data). Inhibition of cytokine production by methotrexate 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 methotrexate interferes with the folate metabolism and thereby with the synthesis of purines and pyrimidines.

Inhibition of cytokine production by methotrexate is a late phenomenon

To explore the effects of methotrexate 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 methotrexate, we observed that inhibition by methotrexate was identical when addition of methotrexate was delayed until 24 h after the start of the culture (not shown).

Sensitivity of donors to methotrexate

We noticed that different donors needed different amounts of methotrexate to suppress cytokine production in WB cultures. To quantify this notion we determined the concentration of methotrexate required for 50% inhibition (ID50) for each cytokine and in every individual. Dose–response curves of seven donors were analysed. Figure 7 shows that TNFα and IFNγ in each donor are similarly affected by methotrexate, and the same is true for the other cytokines (not shown). In addition, this experiment shows that between donors there is considerable variation in sensitivity to methotrexate.

Methotrexate therapy leads to ex vivo inhibition of cytokine production

Methotrexate effectively inhibits cytokine production with an ID50 between 5 and 25 ng/ml (Fig. 7). Such levels are easily achieved in plasma, a couple of hours after oral application of methotrexate. To investigate whether plasma methotrexate levels are sufficient to inhibit cytokine production, we analysed WB cultures
of 10 methotrexate-naive RA patients just before and 2 h after their first administration of methotrexate (10 mg, orally). Indeed, 2 h after methotrexate administration the mean IFN\(_\gamma\)/C13 production in WB cultures was reduced from 21 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).

**Discussion**

The *in vitro* inhibition of T-cell cytokine production by methotrexate in freshly isolated human blood cells has not been reported before. WB cultures were predomin-
antly 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 and IFNγ production per cell is much higher than in MNC [35]. Methotrexate also inhibits cytokine production by purified T cells, but this inhibition is less profound and more 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.

Fig. 6. Kinetics of inhibition of cytokine production by methotrexate. Whole blood of a normal donor was stimulated with anti-CD3 and anti-CD28 in the presence (○–○) or in the absence (●–●) of methotrexate (100 ng/ml). Culture supernatants were harvested at indicated time points. This is a representative donor of seven donors tested.

Fig. 7. TNFα and IFNγ production are similarly affected by methotrexate. WB cultures of seven donors were stimulated with anti-CD3 and anti-CD28 in the presence of increasing amounts of methotrexate. Supernatants were tested for cytokine production and the amount of methotrexate required for 50% reduction (ID50) was calculated for each cytokine.

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

We analysed some of the possible mechanisms by which methotrexate 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 methotrexate inhibition. In 1990, Nesher and Moore [10] proposed that methotrexate might inhibit polyamine synthesis in MNC. In our system, addition of polyamines failed to restore cytokine production in methotrexate-inhibited cultures. Moreover, our observation that inhibition of
cytokine production by methotrexate 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 methotrexate induces apoptosis in activated T cells, whereas non-activated T cells are not affected. We also have evidence that in our cultures methotrexate 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 a lack of thymidine and/or purines during the transition. Monoocytes are probably not inhibited by methotrexate because they hardly proliferate upon stimulation with SAC or LOS. Why Fairbanks et al. [32], using very similar conditions to Genestier et al., did not find induction of apoptosis is not clear. Possibly, salvage of nucleotides derived from dying cells in the high-density cell culture could have influenced the outcome.

Recently, Hildner et al. [30] reported that cytokine production by long-term T-cell cultures was inhibited by methotrexate. However, they did not see an effect of low-dose methotrexate 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 onwards.

Oral intake of 10 mg methotrexate leads to peak plasma levels of methotrexate around 50–100 ng/ml at 1–3 h. We observed that in WB cultures of RA patients 2 h after their first oral intake of methotrexate, plasma methotrexate 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 to the clinical situation. Possibly, T cells are important targets for methotrexate, 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 methotrexate on T cells could be relevant for understanding its *in vivo* action. This would be in line with the observation of Rudwaleit et al. [21] that during treatment with methotrexate the percentage of TNFα-producing T cells decreases. 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 methotrexate, folic acid and folic acid into the cell. Moreover, in the cell the ratio of enzymes involved in polyglutamation and de-glutamation can vary. Finally, levels of purines and pyrimidines capable of salvaging the inhibition by methotrexate can differ from compartment to compartment and from individual to individual. In some donors, thymidine alone could reverse the inhibition of cytokine production by methotrexate. This is probably due to hypoxanthine release in the cultures by dying blood 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 to methotrexate observed in our WB cultures is a reflection of (some of) these individual variations, then sensitivity of cytokine production to methotrexate could be useful in predicting clinical effectiveness of methotrexate in individual patients.

Conclusions

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

Conflict of interest

The authors have declared no conflicts of interest.

References

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