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

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Chapter 4.

MTX induced inhibition of cytokine production is not mediated by adenosine.

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Abstract
The immunosuppressive drug methotrexate (MTX) is frequently used for the treatment of rheumatoid arthritis, however the mechanism of action is not completely clear. Whereas some argue that MTX works via the increase of adenosine, others think that MTX works via the deletion of T-cells. Here we show that MTX-induced inhibition of cytokine production is different from inhibition of cytokine production induced by adenosine analogue cAdo. Besides inhibition of cytokine production by T-cells, adenosine is also able to inhibit cytokines produced by monocytes. Therefore we conclude that MTX-induced inhibition of cytokine production is not mediated via adenosine.
introduction

Methotrexate (MTX) is the most frequently used disease-modifying anti-rheumatic drug for treatment of rheumatoid arthritis (1, 2). MTX is a folate antagonist and inhibits the enzyme dihydrofolate reductase (DHFR) and several other enzymes involved in the folate metabolism (3). In vitro inhibition of folate metabolism by MTX leads to the inhibition of de novo synthesis of purines and pyrimidines (4). Despite frequent use of the MTX, the mechanism of action of weekly low doses is not fully understood.

Cronstein et al. proposed that MTX acts via the increase of extracellular adenosine which is secreted by lymphocytes as a result of MTX-induced accumulation of purine intermediates (reviewed in ref. (5)). MTX increased adenosine secretion in human fibroblasts and their adherence to neutrophils was decreased, addition of adenosine deaminase (ADA) abolished these effects (6). The adenosine hypothesis is supported by the fact that in mouse models for arthritis MTX decreased lymphocyte accumulation at inflamed sites in wild-type mice but not in adenosine receptor knock-out mice (2). Furthermore, administration of the combination of two non-selective adenosine receptor inhibitors, theophylline and caffeine, reversed the therapeutic effect of MTX in these animal models (7). In humans adenosine indeed has anti-inflammatory properties. In whole blood adenosine inhibits neutrophil degranulation (8) and adenosine and adenosine receptor agonists inhibit TNFα expression in a human macrophage cell line (9). Human monocytes incubated with adenosine produce more IL-10 upon stimulation (10). IL-10 is a potent anti-inflammatory cytokine capable of inhibiting the production of pro-inflammatory cytokines such as TNFα and IL-1β (11).

There are also indications that MTX does not work via adenosine. Although in a small group of RA patients a high adenosine concentration in synovial fluid correlated with poor apoptosis induction of neutrophils, no relation was found between adenosine levels and drug treatment (12). Moreover, in one of the patients under MTX treatment, adenosine was undetectable in synovial fluid. In agreement with this, plasma levels of adenosine were not increased in RA patients 7 days after treatment with MTX, whereas uridine, hypoxanthine, and uric acid were decreased (4). In an antigen-induced arthritis model addition of adenosine receptor antagonists enhanced the beneficial effects of MTX (13). Genestier et al. showed that MTX induced apoptosis in activated T-cells in vitro, ADA did not inhibit this induction of apoptosis and adenosine itself only induced a slight amount of apoptosis (14). Suggesting that MTX at least partially works via inhibition of the T-cell response, independent from adenosine.

Our group has recently published that MTX inhibits T-cell cytokines but does not affect monocyte cytokines (15). The decrease of T-cell cytokine production by MTX was probably due to the induction of apoptosis in the T-cell population (16) confirming the results of Genestier et al (14). In this paper we investigated the role of adenosine in MTX-mediated cytokine inhibition in a whole blood system. The nonselective adenosine receptor agonist 2-chloroadenosine (cAdo) was used to for stimulation of the adenosine A1,2 and 3 receptors (8, 17). We present evidence that cytokine inhibition by MTX is independent from the immun-osuppressive properties of adenosine.
Materials and methods

Whole blood cultures

Heparinised blood collected from healthy volunteers was used for whole blood (WB) cultures. All cultures were performed in Iscove’s Modified Dulbecco’s Medium (IMDM, Bio Whittaker, Verviers, Belgium) containing penicillin (100U/ml), streptomycin (100μg/ml), 50 μM 2-mercaptoethanol. Whole blood (WB) was diluted 1:10 in culture medium supplemented with 0.1% fetal bovine serum (FCS, Bodinco, Alkmaar, the Netherlands) and 15 U/ml heparin, all endotoxin-free. WB was cultured in 200 μl at 37°C in the presence of 5% CO₂ in flat bottom plates (Nunc, Roskilde, Denmark). The duration of the cultures is indicated for each experiment. Supernatants are harvested and stored at -20°C until use.

Abs, stimuli and drugs.

Anti-CD3 (CLB.T3/4.E, Sanquin Reagents, Amsterdam, the Netherlands) and anti-CD28 (CLB.CD28/1, Sanquin Reagents) were used for stimulation of T-cells at 1 μg/ml (18,19). For stimulation of monocytes LPS (100 pg/ml, derived from Neisseria Meningitidis, a kind gift of Dr J. Poolman, RIVM, Bilthoven, the Netherlands) was used. MTX (Wyeth Pharmaceuticals BV, Hoofddorp, The Netherlands) was freshly diluted from a 2.5 mg/ml stock in each experiment.

Recombinant GM-CSF (Sandoz, Basel, Switzerland) was used at 5 ng/ml and recombinant TNFα (Chiron Corporation, Emeryville, Ca) was used at 10 ng/ml for stimulation of neutrophils. 2-Chloroadenosine (cAdo) a nonselective adenosine agonist (RBI, Natick, MA) was dissolved in H₂O (5mM) and used at concentrations indicated in the figures. For the inhibition of IL-8 production, anti TNF (anti-TNF 5 and 7, Sanquin Reagents) and anti GM-CSF (16.3, was a kind gift from Dr. G. Trinchieri (the Wistar Institute, Philadelphia, PA) were used at 10 μg/ml.

Cytokine measurements.

The production of cytokines was measured in the supernatant of the cell cultures. IL-8, IL-6, and IFN-γ were measured with ELISA kits (PeliKine-compact, Sanquin Reagents) according to the protocol. In short, mAbs were coated on flat bottom microwell plates (Nunc, Maxisorb) overnight in 100 μl 0.1M Na-bicarbonate at pH 9.6. All incubations were performed in 100 μl at room temperature. Plates were washed 5 times with PBS 0.02%Tween-20 (Mallinckrodt Baker, Deventer, the Netherlands). Samples were incubated together with a biotinylated mAb for 2 hours in high performance ELISA buffer (HPE, Sanquin Reagents). Plates were washed 5 times (PBS, 0.02% Tween 20) and incubated for 30 minutes with streptavidine poly-horseradish peroxidase (Sanquin Reagents) 1/1000 diluted in PBS 2% skimmed milk. Plates were washed 5 times and developed with 0.003% H₂O₂, 100 μg/ml 3,5,3',5'-tetra-methylbenzidine (Merck, Darmstadt, Germany) in 0.11 M Na-acetate, pH 5.5. The reaction was stopped with an equal volume (100 μl) of 2M H₂SO₄ to the wells. Plates were read immediately at 450 and 540 nm. GM-CSF was measured via a similar protocol as the other cytokines. The GM-CSF Abs were a kind gift from Dr. G. Trinchieri. In this assay the coating Ab was anti-GM-CSF 9.1 (used at 2 μg/ml), the biotinylated Ab was anti-GM-CSF 16.3 (0.1 μg/ml). rGM-CSF (Sandoz) was used for the preparation of a standard curve.
**Table 1. Cytokine production in pg/ml (SEM)**

<table>
<thead>
<tr>
<th>cytokine</th>
<th>no drug</th>
<th>MTX</th>
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<tbody>
<tr>
<td>IL-4</td>
<td>1463 (187)</td>
<td>506 (82)</td>
</tr>
<tr>
<td>IL-13</td>
<td>6908 (660)</td>
<td>1017 (155)</td>
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<tr>
<td>IL-8</td>
<td>20258 (23781)</td>
<td>164311 (22082)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>73785 (8186)</td>
<td>4281 (806)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>203276 (25492)</td>
<td>17272 (4228)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>3317 (867)</td>
<td>451 (180)</td>
</tr>
</tbody>
</table>

Table 1. **Cytokine production.** WB of healthy donors was stimulated with anti-CD3/anti-CD28 and incubated with 2 μg/ml MTX. Cytokine production was measured by ELISA after 4 days.

**Figure 1. Effect of cAdo and MTX on anti-CD3/anti-CD28 induced cytokine production.** Duplicate cultures of 1:10 diluted WB of 3 healthy donors was stimulated with anti-CD3/anti-CD28 with various concentrations of (A) cAdo or (B) MTX. After 3 days supernatants were harvested and tested for the presence of IL-8 (open circle), IFNγ (closed square) and GM-CSF (closed triangle) by ELISA. Data are presented as percentage of the cytokine production with anti-CD3/anti-CD28 alone, error bars represent the SEM. Average IL-8 production in the absence of MTX or cAdo was 271600 pg/ml, IFNγ production was 63387 pg/ml and GM-CSF production was 7082 pg/ml.

**Results**

We have shown before that T-cell stimulation of whole blood by aCD3/aCD28 gives rise to production of various cytokines such as IL-2, IL-4, IL-13, GM-CSF, TNF, IFNγ and IL-8 (16). With the exception of IL-8, production of all cytokines is inhibited by MTX (table 1). To investigate the role of adenosine in the anti-inflammatory properties of MTX, we have compared the effect of MTX with that of (cAdo)(17).

We observed that cAdo is an efficient inhibitor off all cytokines tested including IL-8 (figure 1). In view of the fact that purified T-cells do not produce IL-8 at all upon stimulation, the abundant production of IL-8 in whole blood after T-cell activation came as a surprise. We have now found that neutrophils and monocytes can produce IL-8 in WB after anti-CD3/anti-CD28 stimulation (Kikkert et al., manuscript in preparation). IL-8 production in these circumstances is secondary to GM-CSF and TNFα production by T-cells since monoclonal antibodies (mAbs) against these cytokines inhibit IL-8 production (figure 2).
To further dissect the differential effect of cAdo and MTX on IL-8 production we analysed their effect on whole blood stimulated with a combination of GM-CSF and TNFα. Again, MTX was not able to inhibit IL-8 production, whereas cAdo efficiently inhibited IL-8 production (figure 3).

From these experiments we concluded that cAdo not only targets T-cells but also neutrophils and/or monocytes. To test the efficacy of cAdo to inhibit cytokine production after monocyte stimulation WB was stimulated with LPS. In contrast to the inability of MTX to inhibit cytokine production of monocytes after stimulation with bacterial products ((15), figure 4), cAdo efficiently inhibits monocyte cytokine production (figure 4).

**Figure 2.** Effect of anti-GM-CSF and anti-TNF antibodies. 1:10 diluted WB was stimulated with anti-CD3/anti-CD28 in the presence of anti-TNF (aTNF) anti-GM-CSF (aGM) or both (aTNF/aGM). After 3 days IL-8 was measured in the supernatant by ELISA.

**Figure 3.** Effect of cAdo and MTX on GM-CSF/TNF -induced IL-8 production. Duplicate cultures of 1:10 diluted WB of 3 healthy donors was stimulated with GM-CSF/TNF with various concentrations of (A) 2-cAdo or (B) MTX. After 3 days supernatants were harvested and tested for the presence of IL-8. Data are presented as percentage of the IL-8 production in the presence of GM-CSF/TNF alone, error bars represent the SEM of duplicate cultures. The average IL-8 production in the absence of cAdo and MTX was 98523 pg/ml.
MTX AND ADENOSINE

Figure 4. cAdo inhibits monocyte cytokine production, MTX does not. Duplicate cultures of 1:10 diluted WB were stimulated with LPS (100 pg/ml) and various concentrations of cAdo (A) or MTX (B). After 1 day supernatants were harvested and analysed for the presence of IL-8 and IL-6. Data are presented as percentage of the production in the presence of LPS alone, error bars represent the SEM. Panel B depicts result of a separate experiment of the same donor, not preformed in duplo. Production in the absence of cAdo was 2190 and 5835 pg/ml for IL-6 and IL-8 respectively. In the absence of MTX the production was 1710 and 11560 pg/ml for IL-6 and IL-8 respectively.

Discussion

In this paper we demonstrate that in whole blood the immunosuppressive properties of MTX are different from the properties of the adenosine receptor agonist cAdo. An advantage for using cAdo instead of adenosine is that cAdo can not be metabolized. Both MTX and cAdo inhibit T-cell cytokine production. In contrast to MTX, cAdo efficiently inhibits IL-8 production. The production of IL-8 in whole blood after anti-CD3/anti-CD28 stimulation depends on the production of GM-CSF and TNFα since neutralising mAbs to GM-CSF and TNFα inhibit IL-8 production. Because MTX inhibits both GM-CSF and TNFα production one would expect that also IL-8 production would be inhibited. However inhibition of GM-CSF and TNFα production by MTX is not complete and the remaining levels in the presence of MTX are sufficient to induce a full blown IL-8 response.

One could argue that cAdo inhibits IL-8 because cAdo is a more efficient inhibitor of GM-CSF and TNFα production. However, cAdo also inhibits IL-8 production induced by exogenous addition of GM-CSF and TNFα, whereas there is no effect of MTX under these conditions. Hence cytokine inhibition via adenosine is clearly different from MTX inhibition.

We have shown before that MTX does not decrease cytokines produced by LPS or SAC-stimulated monocytes (5,16). Here we show that cAdo does inhibit cytokine production of monocytes in WB stimulated with LPS.

Our conclusion is that cAdo prevents IL-8 production by targeting the cells that produce IL-8, possibly the monocytes. Besides monocytes, also neutrophils can be responsible for IL-8 production in WB after anti-CD3/anti-CD28 stimulation. Isolated neutrophils can produce IL-8 in response to exogenous addition of GM-CSF and TNFα (Kikkert et al. manuscript in preparation). Inhibition of cytokine production from monocytes and macrophage cell lines has been described before (20). In addition, adenosine has several anti inflammatory effects on neutrophils, such as inhibition of degranulation (8).

The presented data show that adenosine has different immunosuppressive properties than MTX. In vitro, the main target for MTX seems to be the T-cell, whereas the targets for
adeno sine are probably T-cells, monocytes and neutrophils. Our results show adenosine is not an intermediate in the inhibition of cytokine production by MTX. Of course one can not rule out that the mechanism of immunosuppression of MTX in vivo differs from that observed in vitro, and that adenosine does have a role in the mechanism of MTX in vivo.

Acknowledgements.
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References