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

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Chapter 7

In vitro T-cell activation is decreased two hours after MTX intake and does not predict clinical response.

Abstract.

Methotrexate (MTX) is frequently used for the treatment of rheumatoid arthritis (RA). However, not all RA patients respond to MTX. The aim of this study was to assess whether the effect of MTX on T-cell activation could predict the clinical response on MTX. Whole blood cultures (WBC) of 34 RA patients were analysed for T-cell cytokine production before and 2h after the first MTX dose. In vitro sensitivity to MTX was also determined. Several clinical parameters were followed in all patients during 12 weeks of MTX treatment. In vitro and ex vivo analysis of T-cell cytokine production can not be used to predict the clinical response.
CYTOKINE PRODUCTION AND CLINICAL RESPONSE

Introduction.
Methotrexate (MTX) is widely used for the treatment of rheumatoid arthritis (RA) (1,2). MTX is a folate antagonist and was initially developed for the treatment of malignancies (3). MTX inhibits dihydrofolate reductase and several other enzymes related to the folate metabolism. Inhibition of these enzymes leads to inhibition of the purine and pyrimidine synthesis. However the mechanism of action of low dose (10-20 mg/week) MTX for the treatment of RA patients remains elusive. Cronstein et al. argue that MTX works immunosuppressive by the increase of extracellular adenosine (4). Adenosine has anti-inflammatory actions such as the inhibition of neutrophil degranulation (5). Adenosine receptor antagonist and adenosine can diminish inflammation in mouse models (6,7). Others have proposed that MTX works via the deletion of activated T-cells by apoptosis and that this results in a decrease of cytokine production and inflammation (8,9).

Not all patients respond to MTX therapy. Only 40% of patients reach a ACR 50% response. It often takes months to identify patients who will not respond to MTX. It would be a major advantage to recognise these patients early, to initiate a progressive dosing schedule or treatment with other DMARDs. Recently, Seitz et al. observed that a low IL-1RA/IL-1β ratio in vitro before treatment was predictive of a good to excellent response to MTX (10). We published that MTX inhibits in vitro production of T-cell cytokines (9)(11). In the present study we aimed to determine whether the effect of MTX medication on in vitro T-cell cytokine production could predict the clinical response to MTX after 12 weeks in MTX-naïve RA patients.

Materials and Methods
Patients.
All 34 patients (see table 1) gave written informed consent and the local medical ethics committee approved the research protocol. Inclusion criteria were: rheumatoid arthritis (1987 American College of Rheumatology (ACR) criteria) and a swollen joint count of at least 6. Just before (t=0) and 2 hours after (t=2) the intake of the first oral dose of 10 mg MTX, blood was collected in endotoxin-free, 4 ml evacuated blood collection tubes (Greiner, Alphen a/d Rijn, the Netherlands) containing sodium heparin. Treatment of patients was continued with a weekly oral dose of 10 mg MTX for 12 weeks. Clinical parameters, determined before treatment and after 12 weeks, were: visual analogue scale (VAS) on disease activity (0-100), swollen joint count (SJC), tender joint count (TJC), erythrocyte sedimentation rate (ESR) and disease activity score on 28 joints (DAS28, (12)).
Whole blood cultures
Whole blood (WB) was diluted 1:4 in Iscove's Modified Dulbecco's Medium (IMDM, Bio Whittaker, Verviers, Belgium) containing penicillin (100 IU/ml), streptomycin (100 μg/ml), 15 U/ml heparin, 0.1% fetal bovine serum (FCS, Bodinco, Alkmaar, the Netherlands), and 50 μM 2-mercaptoethanol, all endotoxin-free. Anti-CD3 and anti-CD28 monoclonal antibodies (CLB.T3/4.E and CLB.CD28/1, Sanquin Reagents, Amsterdam, the Netherlands) were used at 1 μg/ml. WB was cultured in 200 μl cells at 37°C in the presence of 5% CO₂ in flat bottom plates (Nunc, Roskilde, Denmark). After 3 days of culture, supernatants were harvested and 5, 25, 125, 625 times diluted supernatants were tested for GM-CSF by ELISA as previously described (9).
**Measurement of MTX**

MTX was measured in a bioassay as previously described (13). This bioassay uses inhibition of proliferation of B9.H cells as a measure for MTX concentration in serum. B9.H cells cultured in the presence of a dilution range of MTX (from 100 ng/ml to 0.1 ng/ml, Lederle, Hoofddorp, the Netherlands) was used as calibration curve.

**In vitro sensitivity for MTX**

For determination of sensitivity to MTX a concentration range (0-200 ng/ml) of MTX was added to 1:10 diluted WB stimulated with anti-CD3/anti-CD28. Inhibition of T-cell cytokine production by MTX was analysed after day 3. Cytokine production was plotted against MTX concentration, the area under the resulting curve (AUC) was calculated using GraphPad Prism. These values are used as a measure for the *in vitro* sensitivity to MTX.

**Statistics**

All statistical analysis was performed using GraphPad Prism software using non-parametric tests. For the determination of relations between the clinical response and laboratory parameters we used Spearman correlation.

**Results.**

**Effect of MTX intake on ex-vivo T cell activation**

Two hours after MTX intake GM-CSF production in whole blood (WB) cultures was reduced in 33 out of 34 patients. The mean GM-CSF production in WB cultures of all patients was reduced from 12 ng/ml (range 890-4253) to 5.5 ng/ml (range 550-18830) (figure 1). Inhibition of GM-CSF production at t=2 could be restored by addition of folinic acid to the WB cultures. In the presence of folinic acid the mean GM-CSF production at t=2 was 11 ng/ml (range 770-34080) (figure 1). Restoration of cytokine production by folinic acid suggests that the observed *in vitro* inhibition of GM-CSF production is mainly due to the presence of MTX in the plasma in whole blood cultures. Indeed, GM-CSF production by isolated mononuclear cells was identical between t=0 and t=2 samples (not shown). After MTX intake, MTX plasma concentration of all patients was measured. However, inhibition of GM-CSF production at t=2 was not correlated with MTX plasma concentration at t=2 (not shown).

**In-vitro sensitivity to MTX**

The effect of MTX on GM-CSF production was analysed on blood taken at t=0 of all 34 patients. MTX concentration was plotted against inhibition of cytokine production. The resulting curves were used to establish the *in vitro* sensitivity to MTX. The sensitivity to MTX was expressed as area under the curve (AUC). The mean AUC value is 15 (SD 6.1),
a high AUC value corresponds to a low sensitivity to MTX (figure 2 shows three patients with different sensitivities). UAC was not correlated with \textit{ex vivo} T cell activation. To account for individual pharmacokinetic variation we corrected MTX plasma levels at \( t=2 \) for the in vitro sensitivity of the patient for MTX. Plasma MTX level was divided by the AUC for each individual patient. Again no correlation between corrected MTX values and \textit{ex vivo} T cell activation was observed.

Figure 1. \textbf{Decrease in cytokine production 2 hours after MTX intake.} GM-CSF production, before (\( t=0 \)) and after (\( t=2 \)) MTX intake, was measured in WB as described. Folinic acid (FA) was added to the WB cultures at \( t=2 \). GM-CSF production of each patient is depicted. Mean (SD) production 12 (8.7) ng/ml at \( t=0 \), 5.5 (4.5) ng/ml at \( t=2 \), and 11 (8.2) ng/ml at \( t=2 + FA \). The mean is indicated by \( * \) and connected with a black line. The difference between the mean production at \( t=0 \) and \( t=2 \) was significant (\( P<0.05 \), Mann-Whitney). The difference between the mean production at \( t=0 \) and \( t=2 + FA \) is not significant (\( P=0.27 \), Mann-Whitney).

Figure 2. \textbf{Examples of the sensitivity to MTX (AUC).} For each patient GM-CSF production was measured in WB in the presence of various concentrations of MTX. The area under the curve (AUC) is calculated from these graphs. 3 examples are shown, panel a: a high AUC value (24.1), panel b: an average AUC value (14.8) and panel c: a low AUC value (9.45). A high AUC value corresponds with a low sensitivity to MTX \textit{in vitro} and vice versa. Error bars indicate the SEM of duplicate measurements.
Table 2. Patient characteristics at baseline and after 12 weeks of treatment. Mean ± SD values of disease activity scale (DAS28), tender joint count (TJC), swollen joint count (SJC), visual analogue scale (VAS), and erythrocyte sedimentation rate (BSE in mm/hour) are measured before and after 12 weeks of treatment.

<table>
<thead>
<tr>
<th></th>
<th>DAS28</th>
<th>TJC</th>
<th>SJC</th>
<th>VAS</th>
<th>BSE</th>
</tr>
</thead>
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<tr>
<td>Mean score baseline (± SD)</td>
<td>6.1 ± 1.1</td>
<td>12.4 ± 6.7</td>
<td>10.6 ± 5.1</td>
<td>53.0 ± 24</td>
<td>45.1 ± 29</td>
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<tr>
<td>Mean score 12 weeks (± SD)</td>
<td>4.9 ± 1.4</td>
<td>7.3 ± 5.4</td>
<td>7.2 ± 5.1</td>
<td>41.4 ± 29</td>
<td>30.5 ± 22</td>
</tr>
<tr>
<td>Mean improvement</td>
<td>1.2</td>
<td>5.1</td>
<td>3.4</td>
<td>11.7</td>
<td>14.6</td>
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</tbody>
</table>

Table 3. Relation of change in clinical parameter to ex vivo decrease of T-cell cytokine production by MTX and sensitivity to MTX (AUC). Correlations between a change (Δ) in clinical parameters and the percentage of inhibition after MTX intake or the sensitivity to MTX (AUC) were analysed using nonparametric correlation (Spearman). P and r values are depicted.

<table>
<thead>
<tr>
<th></th>
<th>ΔDAS</th>
<th>ΔTJC</th>
<th>ΔSJC</th>
<th>ΔVAS</th>
<th>ΔBSE</th>
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<tr>
<td>ex vivo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>P value</td>
<td>0.051</td>
<td>0.088</td>
<td>0.026</td>
<td>0.358</td>
<td>0.387</td>
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<td>T cell activation</td>
<td></td>
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<tr>
<td>Spearman r</td>
<td>-0.34</td>
<td>-0.3</td>
<td>-0.38</td>
<td>-0.16</td>
<td>0.15</td>
</tr>
<tr>
<td>AUC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>0.864</td>
<td>0.341</td>
<td>0.986</td>
<td>0.711</td>
<td>0.723</td>
</tr>
<tr>
<td>Spearman r</td>
<td>-0.03</td>
<td>-0.17</td>
<td>0</td>
<td>0.07</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Relation with clinical response
The clinical response was determined by the change in several clinical parameters after 12 weeks of treatment (table 2). We analysed whether the ex vivo decrease in cytokine production by MTX was related to the clinical response after 12 weeks (change in DAS28, VAS, SJC, TJC and BSE table 3). The ex vivo effect of MTX correlated significantly with a change in SJC, but the relation between the two is weak (r=-0.38, figure 3). In vitro sensitivity to MTX (AUC), did not correlate with the change of any clinical parameter determined (table 3). In addition, cytokine production before and after MTX intake and MTX plasma concentration at t=2 neither showed a correlation to any clinical parameter (not shown).
Discussion.

The immunosuppressive effect of MTX \textit{in vitro} is mainly targeted to T-cells. MTX specifically deletes activated T-cell by induction of apoptosis and therefore inhibits the T-cell cytokine production in WB. MTX hardly affects monokine production in WB (9,11). From the T cell cytokines we have analysed, which include IFNy, TNF, IL-2, IL-4 and IL-13, the GM-CSF production proved to be the most consistent and reproducible. Therefore, GM-CSF production was used as a read-out system for T-cell activation. Intake of MTX leads to strongly decreased T cell activation in whole blood, 2 hours after administration of the drug. The decrease in T-cell activation is due to MTX because cytokine production at t=2 can be restored by addition of folic acid. In addition to the lack of inhibition of cytokine production in isolated MNC’s, this suggests that at t=2 the MTX is present mainly in the plasma. Indeed, at that time point, bioactive MTX was found in plasma at concentrations ranging from 61 to 351 ng/ml. We expected inhibition of cytokine production at t=2 to be dependent on plasma MTX concentration and on the sensitivity of the donor for MTX. That latter function was measured at t=0 by inducing T cell activation in the presence of a concentration range of MTX. To our surprise the ex vivo inhibition of T cell activation was not correlated with the \textit{in vitro} sensitivity to MTX not even after correction for plasma MTX levels. It suggests that within the two hours of treatment, something other than the MTX concentration has changed in the whole blood sample.

With a single exception, significant correlations between \textit{in vitro} parameters and clinical parameters were not observed. Neither sensitivity to MTX, nor cytokine production before or after MTX intake, nor MTX plasma concentration correlated to the clinical response of the patients. Only the change in SJC is correlated with the inhibition of T cell activation at t=2. However the relation is weak (r=-0.38) and strongly dependent on a single patient that showed a stimulation of cytokine production in the 2h blood sample. Moreover it should be realised that the p value has not been corrected for multiple statistical testing. However, if further analysis would confirm the observation we have to explain why patients with the lowest ex vivo response to MTX are clinically the best responders. The best guess is that MTX not only inhibits production of pro-inflammatory cytokines but also of anti-inflammatory cytokines such as IL-10. It was shown before that patients with a good clinical response to MTX, have enhanced IL-10 production in PBMC stimulated with LPS (14). We therefore have investigated the effect of MTX on cytokine production also in the presence of a neutralizing anti-IL-10 monoclonal antibodies. We observed that anti-IL-10 slightly increases cytokine production at t=2 but that MTX still inhibits cytokine production in the presence of anti-IL-10. In these experiments we also observed that not only MTX, but also its counterpart FA can decrease GM-CSF production in WB before MTX intake. This inhibition is reversed by anti-IL-10 (data not shown). The inhibitory capacity of FA itself can explain why FA does not completely restore the GM-CSF production at t=2 to production levels at t=0. Further analysis of the effects of anti-IL-10 and FA and the relation to the clinical response is presently under investigation.

Recently Seitz et al. have published that the ratio of IL-1RA/IL-1β production by peripheral blood mononuclear cells (PBMC) predicts disease outcome (10). PBMC were isolated before the start of MTX treatment and cultured without stimulation. A low ratio of IL-1RA/IL-1β protein (<100) was found in the groups with a good or excellent response after 6 months of MTX treatment. Since we use strict endotoxin-free culture conditions,
there is no detectable cytokine production in un-stimulated WB cultures. Therefore we have not been able to confirm the claims made by Seitz et al.

The purpose of this study was to investigate whether the analysis of cytokine production in vitro can be used to predict clinical response to MTX treatment. This notion was based on the hypothesis that clonal deletion of activated T cells was instrumental in the efficacy of MTX in treating RA. Our results suggest that this idea might be wrong. Intake of MTX leads temporally to plasma MTX levels which are sufficient to inhibit T cell activation. However the in vitro and the ex vivo effect of MTX on T cell cytokine production has no or little relation with the clinical response. Therefore further investigation into predictors of disease outcome is warranted.

References