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

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Chapter 8

General discussion
The effect of MTX on cytokine production

Methotrexate (MTX) is widely used for the treatment of patients with rheumatoid arthritis (RA). MTX was developed more than 50 years ago for the treatment of malignancies (1). MTX inhibits the folate metabolism by inhibition of dihydrofolate reductase (DHFR) and several folate-dependant enzymes (2-5). As a consequence purine and pyrimidine synthesis is inhibited. Although MTX has been used to treat patients with RA for many years, the mechanism of weekly low dose MTX administration is not clear. Furthermore MTX is effective in only 70% of the patients. We have tried to get better insight into the mechanism of action of MTX. We hoped that this could lead to development of assays to predict efficacy of MTX treatment in individual patients.

The importance of cytokines in inflammatory reactions led us to examine the effects of MTX on cytokine production. In initial experiments we observed that MTX was an efficient inhibitor of cytokine production. In isolated mononuclear cells inhibition by MTX was less convincing and less reproducible. As discussed in chapter 1, existing literature does not give a clear picture on the effect of MTX on cytokines. We studied cytokine production after polyclonal stimulation of T-cells using a combination of anti-CD3 and anti-CD28, or after stimulation of monocytes, using bacterial products. MTX proved to be a strong inhibitor of T-cell cytokine production, but not of cytokine production by monocytes (chapter 2 and 3)(6). The reason for the inhibition of cytokine production is that MTX induces apoptosis in proliferating T-cells, preventing the cytokine production from these cells (chapter 3). This observation is in accordance with the results from Genestier et al. (7,8) who argue that the mechanism of MTX is clonal deletion of activated T-cells. In contrast, Cronstein et al. hypothesis that MTX works via an increase of extracellular adenosine (9). We did not find evidence for a role of adenosine in the inhibition of cytokine production in WB cultures by MTX. IL-8 production was not inhibited by MTX, whereas 2-c-Ado, an adenosine analogue did inhibit production of IL-8 (chapter 4). Inhibition of IL-8 by c-Ado was not caused by a better inhibition of IL-8-inducing cytokines, TNFα and GM-CSF, because addition of these cytokines did not prevent inhibition of IL-8 production by 2-c-Ado.

From our in vitro studies we concluded that the target for MTX is most likely the activated T-cell. A disadvantage of the WB system is that monocytes do not proliferate. It is therefore not surprising that we did not find an effect of MTX on these cells in vitro.

The effect of MPA on cytokine production

The immunosuppressive drug mycophenolate mofetil (MMF) or its active compound mycophenolic acid (MPA) is another drug that interferes with the DNA and RNA synthesis. Therefore we were interested whether the in vitro effects of this drug were comparable to MTX. MPA has been used to prevent organ rejection after transplantation (10,11). Experimentally MPA has been used for the treatment of RA (12,13). MPA inhibits IMPDH an enzyme of the de novo purine synthesis, causing depletion of guanosine nucleotides in cells (14). Like MTX, MPA inhibits the cytokine production in WB cultures stimulated with anti-CD3 and anti-CD28 (chapter 3). However MPA has a different mechanism of action than MTX (15), T-cells are not deleted but their activation is suppressed. MPA induces a block in the G1 phase of the cell cycle (16). This block is removed when adenosine and guanosine are added to in vitro cultures (6,17,18). In vivo a decline in MPA plasma levels will probably be enough to let cells proliferate again. In contrast to MTX, we found a small
but significant inhibition of IL-6, IL-8 and TNFα produced by monocytes after bacterial stimulation in WB and isolated MNC (chapter 5). To our surprise the production of the highly inflammatory cytokine IL-1β was upregulated after stimulation of monocytes with LPS but not with SAC. This implicates that MPA indeed does have an effect on monocytes. Whether MPA increases IL-1β production in vivo, and whether this has clinical consequences remains to be investigated.

**MTX in RA patients**

Our in vitro studies suggest that the T-cell could be a major target of MTX. We observed that individual patients differed in their sensitivity for MTX. Therefore, we expected that patients with a that inhibition of T-cell cytokine production by MTX in WB of RA patients could give an indication of the clinical response of these patients. We expected that patients with a high inhibition of cytokine production would have a better clinical response than patients with a lower inhibition of cytokine production.

It is important to identify patients who will not respond to MTX at an early stage of treatment so treatment can be adjusted. Plasma levels of MTX quickly rise after oral intake and remain detectable for a couple of hours. To determine MTX plasma levels in RA patients we have developed a bioassay (chapter 6). With this bioassay it is possible to measure low levels of bioactive MTX in serum. Two hours after MTX intake we found MTX concentrations that ranged from 61-351 ng/ml. These concentrations are high enough to decrease T-cell cytokine production in vitro. This has been confirmed ex vivo, by inhibition of cytokine production seen in WB of RA patients 2 hours after MTX intake. Addition of the MTX-antagonist folinic acid to WB, drawn 2 hours after MTX intake, increased cytokine production to comparable amounts as before MTX intake.

The clinical response to MTX treatment was followed in 34 RA patients, in order to investigate whether a good inhibition of cytokine production at t=2 would predict a good clinical response to MTX (chapter 7). The efficacy of treatment was determined by the change DAS28 and several clinical parameters over 12 weeks. T-cell cytokine production in WB, before and after MTX intake, was not predictive of the clinical response after 12 weeks of treatment with MTX. Nor was the in vitro sensitivity to MTX predictive of the clinical response. The percentage of inhibition of cytokine production, 2 hours after MTX intake was negatively correlated with the change in SJC. A good inhibition was related to a bad clinical response. This was quite the opposite from what we expected. A possible explanation for the observed correlation could be that in vitro both inhibitory and stimulatory factors determine total cytokine production. If an inhibiting cytokine such as IL-10 is more sensitive to MTX inhibition then the pro-inflammatory cytokines, the result would be that patients with an inherent high IL-10 production would show less inhibition of pro-inflammatory cytokine production. This would agree with the observation by Seitz et al who found that patients with high IL-10 production to IL-10 show the best clinical response to MTX (19).

The reason that we did not find (more) significant correlations could be due to the short follow up. After 12 weeks the improvement in this patient group was very low. Because MTX is given once a week, some argue that in order to be effective cellular retention is necessary. Indeed in erythrocytes, MTX is retained intracellularly by polyglutamation. Intracellular accumulation in the form of MTX-polyglutamates has also been found in liver cells (20) and several tumour cell lines (reviewed in (21). We have tried to find evidence
for accumulation of MTX in lymphocytes. Blood was drawn from several RA patients after 12 weeks of MTX treatment. T-cell cytokine production was compared to the production in the presence of MTX antagonist folinic acid or a combination of hypoxanthine and thymidine. When MTX would have accumulated in the cells, cytokine production in the presence of MTX antagonists should be higher than the production in the absence of MTX, however, we did not observe any effect of antagonising MTX. Suggesting that MTX was not accumulated in the T-cells.

**Immunosuppression by MTX**

Based on our in vitro studies we could hypothesise the following mechanism for the immunosuppression displayed by MTX in RA: The low weekly doses of MTX make plasma levels of MTX rise transiently. When MTX levels peak there is enough circulating MTX to induce apoptosis in T-cells that are proliferating at that moment. Resting T-cell are not deleted by MTX. Deletion of active T-cells is enough to ensure that the inflammatory reaction is dampened. Because T-cells can not produce inflammatory cytokines anymore nor instruct macrophages and other cells to proliferate and produce their harmful proteases. Because MTX works only on proliferating cells, it can induce apoptosis in just a part of the population. Therefore, in general, MTX-treated patients do not suffer from severe lymphopenia and opportunistic infections. We have no indications that MTX targets a specific subset of T-cells, apoptosis is induced in CD4+ as well as CD8+ T-cells (not shown) and Th-1 cytokines (IFNγ) as well as Th2 cytokines (IL-4) are inhibited by MTX (6,22). However, since T-cell cytokine production in RA patients treated with MTX is not related to the efficacy of treatment we can not rule out the possibility that other inflammatory cells are the target of MTX. Whether MTX targets cells in peripheral blood or in the synovium remains an unanswered question. It is clear that the effects of MTX can be reversed by the presence of thymidine and hypoxanthine (chapter 7 and (23))(7). When MTX inhibits the de novo synthesis, thymidine and hypoxanthine can be used by the cell for DNA/RNA synthesis via the salvage pathway for nucleotide synthesis. Dying cells are a rich source for these components of the salvage pathway. Since dying cells are numerous in inflamed joints there is a possibility that MTX does not work in the joint because of the presence of nucleotides.

**Nonresponsive patients**

For patients that do not respond to MTX therapy the underlying mechanism of non-responsiveness can be diverse. Alteration of MTX transport can lead to non-toxic intracellular levels. The cause of altered transport can be by change of influx, via reduced folate carriers, as well as efflux, via multi-drug resistance pumps (24). Mutation of the DHFR gene, can result in a gene-product with a low affinity for MTX. Overproduction of DHFR as a consequence of gene amplification is often observed in tumour cell lines. Research into MTX non-responsiveness has primarily focused on MTX-resistance during cancer treatment (reviewed in (25)), whether these mechanisms are also observed in treatment with low dose MTX is not clear. Due to the low amounts of MTX and the transient rise in serum levels, it is possible that subtle genetic variations in folate metabolism may influence the effectiveness of MTX treatment in RA. Furthermore, folate levels can be of importance, although this has not been confirmed in literature. Variations in hypoxanthine or thymidine serum levels in patients and the relation to efficacy have not
been determined. Van Eden et al. observed no relation between the efficacy of MTX and a mutation in the methyl-THFR gene (26), indicating that methionine metabolism is not the main route for efficacy of MTX. Although purine enzymes are elevated during MTX treatment, changes were not related to the efficacy of treatment (27).

Side effects of MTX treatment

The main reason for discontinuation of MTX treatment is toxicity (28,29). Most side effects are displayed in the gastrointestinal (GI)-tract, probably due to high cell turnover. Common side effects are nausea, stomatitis, and diarrhoea (30). To reduce side effects of MTX, folic acid and, less frequently, folinic acid is prescribed. In vitro folic acid does not reverse the inhibitory effect of MTX (22). In patients, folic acid does not reduce the efficacy of MTX (31,32), although sometimes slightly higher doses of MTX are required (32). The reason why folates are able to reduce the toxicity without reducing the efficacy is not clear. Reduced folate forms such as folinic acid and MTX use the reduced folate carrier (RFC) to enter the cell, whereas folic acid uses the folate receptor. In theory, when given shortly after another, folinic acid can have an effect on MTX efficacy, because it competes with MTX for the RFC, interfering with cellular uptake of MTX. Since MTX levels peak directly after intake and decline rapidly after several hours, folinic acid given 24 after MTX intake does not interfere with MTX uptake. Another explanation for the observation that folic acid does not reduce the efficacy of MTX is that the cellular targets for them are different. For example, cells in the GI-tract can be more sensitive to MTX than potential target cells. When the dose of MTX is too low for an effect on T-cells, MTX can still cause toxicity in GI-tract, which can be prevented by folate supplementation. Furthermore it is possible that the mechanism of toxicity is different from the mechanism of efficacy. A mutation in an enzyme of the methionine metabolism does not seem to influence the efficacy of MTX (26). In contrast, the presence of this mutation was associated with an increased risk of discontinuation of MTX treatment due to toxicity (33). Therefore, toxicity might be caused by inhibition of methionine metabolism, whereas the efficacy is due to inhibition of purine and or pyrimidine synthesis.

Concluding remarks

Our work was based on the idea that is MTX might work via depletion of activated T-cells. However, depletion of T-cells with anti-CD4 antibodies was not very successful (34) in RA, although CD4+ T-cell counts dropped during therapy (35,36). The immune system is tightly controlled. An immune response is mounted by inflammatory cells and cytokines and at the same time it is repressed by other cells and mechanisms. Recently, interest in a subset of T-cells with repressive properties has been revived. In mice, these regulatory T-cells (Treg cells) have been characterised as part of the population of CD4+/CD25+ cells. Depletion of these cells leads to development of auto-immune diseases. This could be prevented by reconstitution with CD4+/CD25+ Treg cells (37). Treg cells are essential for the induction of self-tolerance and prevent transplant rejection and inflammatory bowel disease or colitis (38-40). Subsequently these cells were also found and characterised in healthy persons (41-45). Treg cells are able to suppress proliferation of the whole CD4+ population or CD4+/CD25- cells (41-45). When CD4+ cells are depleted logically Treg cells are also depleted. Although it is clear that inflammation suppression by Treg cells is insufficient in joints of RA patients, depletion of these cells can result in even more
incomplete suppression of inflammation. It is likely that MTX does not deplete $T_{reg}$ cells because $T_{reg}$ cells proliferate less than other CD4+ cells (46). Therefore these cells are less likely affected by MTX.

$T_{reg}$ cells can mediate their function via cell-cell interaction and via secretion of cytokines (39,41). TGFβ and IL-10 have been implicated. It would be interesting to extend our studies and to $T_{reg}$ cells and include measurements on production of the cytokines TGFβ and IL-10.

In conclusion, we have shown that in vitro, MTX induces apoptosis in activated T-cells at concentrations easily achieved in vivo. However we have not been able to show that the effect on T-cells is related to the clinical efficacy of MTX in RA patients. Therefore it has to be investigated whether other cellular targets are involved. Before concluding that MTX does not work via the T-cells at all the involvement of, and the effects of MTX on cytokines that have not been studied here should be explored. Such as the involvement of anti-inflammatory cytokines IL-10 and TGFβ. Furthermore knowledge of whether pharmacodynamic variables are responsible for the variable response to MTX treatment in RA patients should be studied.

References


