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INHIBITION OF CYTOKINE PRODUCTION BY METHOTREXATE

Studies in healthy volunteers and patients with rheumatoid arthritis.

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Voor Opa

Contents

abbreviations

Ab= antibody
AZA= azathioprine
BSE= erythrocyte sedimentation rate
cAdo= 2-chloroadenosine
CIA= collagen induced arthritis
CQ= Chloroquine
CsA= Cyclosporin A
DMARD=disease modifying anti-rheumatic drug
ELISA= Enzyme linked immunosorbent assay
FLS=fibroblast like synoviocyte
GM-CSF= granulocyte-macrophage-colony-stimulating factor
HCQ= hydroxychloroquine
IFN=interferon
IL= interleukin
mAb= monoclonal antibody
MNC= mononuclear cells
MPA= mycophenolic acid
mRNA= messenger RNA
MTX= methotrexate
NSAID= non-steroidal anti-inflammatory drug
PBMC= preiferal blood mononucear cell
RA= rheumatoid arthritis
RF= rheumatoid factor
SF= synovial fluid
SJC= swollen joint count
SLE= systemic lupus erythematosus
SSZ= Sulfasalazine
TJC= tender joint count
TNF= tumor necrosis factor
WB= Whole blood

Chapter 1

General introduction

Rheumatoid Arthritis

Rheumatoid arthritis is a systemic chronic inflammatory joint disease. The disease affects about 1% of the population world-wide. The reasons why inflammation starts and continues within joints is unknown (1).

The normal joint consists of two bone ends covered with cartilage and connected by a capsule. The inside of this capsule is covered with the synovial membrane (1-3 cell layers thick) and is filled with synovial fluid (SF). In the inflamed joint the synovial membrane is thickened and invaded with cells (macrophages and T-cells), the SF contains many neutrophils, cartilage is destructed by proteolytic enzymes produced by macrophages and fibroblast like synoviocytes (FLS), and in progressed stages also bone is destructed. Eventually, bone destruction leads to deformations and disability.

In the SF and synovial tissue of RA patients a multitude of cytokines can be detected, such as tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), IL-6 and IL-8. These cytokines, and especially TNFα and IL-1β play an impotant role in the ongoing inflammation. The actions of several cytokines will be discussed later in this chapter. Activated CD4+ T-cells stimulate monocytes, macrophages and fibroblast-like synoviocytes (FLS) present in the joint. When stimulated, these cells produce cytokines and matrix metalloproteinases. These soluble mediators can subsequently stimulate chondrocytes, endothelial cells and osteoclasts present in the synovium. In adittion, osteoclasts, chondrocytes and FLS can be stimulated by T-cells through expression of cell-surface molecules. Furthermore, CD4+ T cells stimulate B-cells to produce immunoglobulins such as rheumatoid factor.

Clues for the pathogenesis

It has been observed that infection with *Borrelia burgdorferi* can induce arthritis. Much research has been done to find other infections that provoke RA. However, evidence for the idea that RA is provoked by a preceding infection has not been found. Whether In response to infection or to autoantigens, it is clear that hemopoietic cells play an important role in the disease. There is an unsettled debate about which cells are important in the pathogenesis of RA. Association between susceptibility and severity of disease and certain HLA-DR4/DR1 epitopes (2) provides a strong argument for T-cells, since the primary role for the HLA-DR molecule is to activate T-cells. T-cells are also important in several animal models. Collagen-induced arthritis (CIA), for example, is an antigen-driven model where the antigen is type-II collagen. Recent identification of antibodies against deiminated fibrinogen detected in 70% of early RA patients (3,4) urged others to investigate the T-cell response to deiminated peptides. In mice, transgenic for one of the HLA-DR epitopes genetically associated with RA, deimination of peptides leads to a higher affinity with the MHC molecule and to CD4+ T-cell activation (5). Whether deiminated fibrinogen really is an autoantigen that can cause RA in humans remains to be investigated.

Since macrophages and fibroblast-like synoviocytes (FLS) produce the actual destructive mediators, such as matrix metalloproteinases, these cells are thought to play a central role (6,7). Evidence comes from the abundant expression of cytokines by these cells in synovial tissue, whereas there is hardly any detectable cytokine production by T-cells (8). Analysis of RA synovial tissue demonstrates that monocytes, FLS, and macrophages are activated. There are indications that FLS can act independent from T-cell control in the

destructive phase of RA (for references see (9)). Furthermore, mutations in the p53 tumor suppressor gene are found in RA patients. Therefore some authors believe that FLS of RA patients behave like transformed cells (7).
There are also indications that RA can develop independent from T-cells. K/BxN T-cell receptor transgenic mice spontaneously develop RA like symptoms (10). Injection of serum from sick mice induces arthritis in healthy mice. The presence of antibodies directed against glucose 6-phosphate isomerase, a glycolytic enzyme, is responsible for the induction of disease (11). A serological test frequently used for the assessment of RA is the determination of rheumatoid factor (RF). RF are autoantibodies directed to the Fc portion of multimerised IgG. RF can be of the IgM, IgG or IgA isotype and are present in 80% of the RA patients. Production of (auto) antibodies by B-cells can be a part of the pathology of RA. However, since B-cells need T-cell help for antibody production and isotype switching, the T-cell could still be the crucial cell type. Interest for the role of the B-cell has also been revived after reports stating improvement of the disease after treatment with B-cell depleting chimeric antibodies to CD20 (12,13).
Most likely different cell types are relevant at different stages of the disease. Although there is no evidence for antigen driven T-cell activation in RA, T-cells may be important in the perpetuation of inflammation in RA (9,14). A better insight in the function of several cell types will lead to a better understanding of the pathogenesis of RA, which will ultimately lead to better therapies to counteract the disease.

Cytokines
Cytokines are important mediators in the immune system, regulating both innate and adaptive immune response (15). Cytokines are secreted by immune cells upon activation and regulate growth, proliferation, activation, differentiation and cytokines can have chemotactic properties. Other cell types, such as endothelial cells and fibroblasts also secrete cytokines. Various cytokines have similar biological effects, and at the same time a single cytokine can have different effects on different target cells. Cytokines can perform their functions in a autocrine, paracrine or endocrine manner. Cytokines act through binding to specific receptors on their target cells. The expression of these receptors on target cells determines whether or not a cell can respond to that cytokine.
Normally the inflammatory process is tightly regulated by cytokines; there is a balance between cytokines that initiate and maintain the response, (pro-)inflammatory cytokines, and cytokines that suppress the response, anti-inflammatory cytokines. However, during chronic inflammation, such as in RA, the balance between the pro-and anti-inflammatory cytokines is not maintained. Imbalanced production of cytokines can be responsible for the clinical symptoms and tissue damage seen in affected joints of RA patients (16,17). Many cytokines are found in synovial fluid; however, the relevance to the pathogenesis remains unclear. Cytokine expression in synovial tissue is probably more relevant, as this is the site where the inflammatory reaction takes place. Immunohistochemic analysis and the analysis of mRNA expression in inflamed RA tissue shows expression of many proinflammatory cytokines, such as TNFα, IL-1β, IL-6, granulocyte-macrophage-colony-stimulating factor (GM-CSF) and the chemokine IL-8 (8,17,18). A short description of their actions will be given.

Inflammatory cytokines

TNFα and IL-1β are prototype inflammatory cytokines. TNFα is produced by macrophages, monocytes, and T-cells. TNFα can bind to two forms of the TNF receptor, p55 and p75. Both receptors are also found as soluble form (sTNFR). TNFα is a potent inducer of other cytokines, such as IL-1, IL-6, IL-8 and GM-CSF. TNFα can also stimulate fibroblasts to express adhesion molecules and indirectly activate osteoclasts which are responsible for bone degradation (for references see (16)). Intra-articular injection with TNFα results in accelerated onset and more severe arthritis in a mouse model of collagen induced arthritis (CIA) (19). In addition, administration of monoclonal antibodies against TNFα led to attenuation of the disease (20,21). A dominant role of TNFα in the pathology of RA is also likely in man, since therapeutic blockade of TNFα results in major clinical improvement (22-24).

IL-1β is also produced by macrophages and monocytes. IL-1β is a member of the IL-1 family, of which IL-1α, IL-18 and IL-1 receptor antagonist (IL-1RA) are other members. IL-1β can stimulate IL-6 production and the production of chemokines (25). Both TNFα and IL-1β are implicated in cartilage degradation. IL-1β can stimulate the release of matrix metalloproteinases from chondrocytes and fibroblasts (reviewed in ref. (26)). TNFα and IL-β increase the ratio collagenase (MMP)/ tissue inhibitor of MMP (TIMP), secreted by different cell types in vitro (27). Like TFNα, IL-1β can induce inflammation and cartilage degradation in synovial joints in animal models (28). IL-1 is produced as inactive precursor protein, it is activated upon cleavage by IL-1 converting enzyme (ICE, caspase 1). Intervention with IL-1β signaling can reduce arthritis in CIA models (29). The naturally occurring IL-1 receptor antagonist (IL-1RA) can antagonize the actions of IL-1β. By binding to the IL-1receptor, IL-1RA prevents binding of IL-1β or IL-1α and thus signaling through the receptor. Consequently, IL-1RA has anti-inflammatory properties and is presently tested for its efficacy to treat RA. Active IL-18 is also derived by cleavage of the precursor form by ICE. IL-18 is primarily produced by macrophages. The relevance of IL-18 in RA was demonstrated by the enhancement of CIA and, on the other hand, by IL-18 knock out mice that have a delayed onset and milder severity of CIA (31, reviewed in (30)). Enhancement of CIA by IL-18 may work via recruitment and activation of neutrophils (31,32). IL-18 production is found in synovial cells in RA tissue and is correlated to the expression of IL-1β and TNFα (30,33). Addition of IL-18 induced the production of several inflammatory cytokines in vitro synovial tissue cultures and IL-18 works synergistically with IL-12 to induce production of interferon-γ (IFNγ) in synovial T cells (30,33).

IL-6 is secreted by mainly by monocytes. IL-6 can stimulate B- and T-cell growth and differentiation and induces the production of antibodies. In addition, IL-6 activates osteoclasts. Although considered to be a pro-inflammatory cytokine, IL-6 decreases the MMP/TIMP ratio, resulting in a tissue protective effect (27). Furthermore, IL-6 is not arthritogenic. IL-6 is of importance in the pathogenesis of arthritis, since the onset of CIA is delayed and arthritis is attenuated in IL-6 knock out mice and ever resistant against antigen induced arthritis (34). Furthermore, anti-IL-6 and anti-IL-6 receptor therapy has been shown to work benificial in RA patients (35,36). IL-6 induces production of acute phase proteins such as CRP and can itself be considered an acute-phase protein. Like CRP it can be measured in human serum as marker for disease activity.

Also present in synovial fluid from RA patients is IL-8. IL-8 can be secreted by monocytes and granulocytes, fibroblasts and endothelial cells. It is a chemokine that is chemotactic

for neutrophilic granulocytes. In addition, IL-8 promotes angiogenesis. GM-CSF is produced by T-cells and monocytic cells. As its name indicates, GM-CSF stimulates growth and differentiation of granulocytes and macrophages, and induces cytokine production in these cells. IL-15 production was found in macrophages in synovial tissue of RA patients. IL-15 is a growth factor for T-cells (reviewed in (30)).

Anti-inflammatory cytokines.
IL-10 is secreted by T-cells and monocytes and most hemopoietic cells express the IL-10 receptor. IL-10 has several functions. Inhibition of cytokine production in T-cells, inhibition of the activation of monocytic cells, and consequently, inhibition of the co-stimulatory capacity of APC, are anti-inflammatory properties of IL-10. On B-cells IL-10 induces isotype switching, prolonged survival and stimulated proliferation (37) and references herein).
Transforming growth factor beta (TGF-β) is also considered to be anti-inflammatory. TGFβ has a wide variety of functions extending from the regulation of adhesion molecule expression by endothelial cells and leukocytes, to the modulation of macrophage function, and the control of lymphocyte activation and proliferation. TGFβ, injected into an inflammed joint, inhibits cartilage destruction, but increases inflammation (38). IL-4 and IL-13 are produced by T-cells. Both cytokines stimulate B-cells. IL-4 and IL-13 inhibit the formation of T-cells from the inflammatory Th1 subset. IL-4 inhibits the production of IL-6 and IL-8 (for references see (16)).

Treatment of patients with RA
In the last 2 decades therapeutic strategies for RA patients have been changed. Traditionally, RA patients were treated according to the 'pyramid approach'. In the pyramid approach treatment was started with non-steroidal anti inflammatory drugs (NSAIDs), to reduce the pain and inflammation. NSAIDs do not have the potential to prevent progression of the disease. When NSAIDs are insufficiently effective, disease modifying anti-rheumatic drug (DMARDs) are prescribed. DMARDs usually have a delayed onset of action and are able to modify the disease. First relatively mild DMARDs such as hydroxychloroquine was used, followed by stronger DMARDs such as MTX (39). Since the publication of Wilske and Healey on remodeling the pyramid (40), the pyramid approach is abandonned. More aggressive treatment of RA and an earlier start is advocated by many groups. Several randomized trails have been undertaken to prove that direct treatment with DMARDs gives better results than the pyramid approach (41-43). An example of aggressive treatment is the combination of two or more DMARDs. Six months of intensive combination therapy with prednisolone, MTX and sulfasalazine (SSZ, salicylazosulfapyridine) results in a better control of the disease and lower rate of radiologic progression, than SSZ alone (44). This effect sustained during the follow up 4 to 5 years later, independent of the therapy following the combination therapy (45). Efficiency of a drug can be measured by evaluation of different clinical parameters, reflecting the activity of inflammation present in the patient, such as tender or swollen joint count (TJC and SJC), or erythrocyte sedimentation rate (BSE). A combination of these and more parameters is included in the DAS28. Furthermore, rheumatologists can determine radiologic progression of the disease by scoring visible bone erosions on X-rays of hand an feet.

There are many DMARDs used for treatment of RA. Many DMARDs originate from other disciplines. Nevertheless, these drugs are effective against RA. A short overview of several DMARDs will be given here, the effects of these drugs on cytokine production will be addressed in another section of this introduction.

DMARDs

Gold has been used for treatment of many diseases for centuries. In 1935 the first trial in RA was reported with an improvement of 70-80% and more followed. There are several forms of gold available for either intramuscular or oral administration. Even though gold reduces inflammation and delays radiologic progression, it is seldom used nowadays. Chloroquine (CQ) and hydroxychloroquine (HCQ) are antimalarial drugs. These 4-aminoquinoline derivatives differ from each other by one hydroxyl group at the end of the side chain. Antimalarials are successful in combination therapy, for example with MTX (46). In spite of the fact that antimalarials can decrease inflammation, they can not delay radiologic progression. SSZ was frequently used for the treatment of inflammatory bowel disease. SSZ was rediscovered for treatment of RA in the eighties and is capable of delaying radiologic progression (47). SSZ is most effective in combination with MTX and HCQ (46). Cyclosporin A (CsA) is often used to prevent graft rejection after transplantation. CsA inhibits T-cell activation by inhibition of transcription factor NF-AT, which leads to immunosuppression. Effect on radiologic progression is also observed. CsA is more efficient in combination with MTX than alone (48). However, the use of the drug is limited due to toxicity. A relatively new DMARD is leflunomide (N-(4-trifluoromethylphenyl)-5-methylisoxazol-4-carboxaminde). Its efficacy was shown in placebo controlled studies in human (49,50). Leflunomide is a reversible inhibitior of dihydro-orotate-dehydrogenase, a rate limiting enzyme in the de novo pyrimidine synthesis. This action is unique among DMARDs; therefore its potential lies in combination with other drugs for example with MTX (51), because MTX inhibits purine synthesis and a late step in pyrimidine synthesis. MTX is considered as one of the most powerful DMARDs. MTX was developed for treatment of malignancies. MTX delays radiologic progression and diminishes inflammation. MTX has become the gold standard for RA treatment. Many other and newer DMARDs or combinations of DMARDs are compared to the efficacy of MTX alone. Another drug that inhibits purine synthesis is mycophenolic acid (MPA, mycophenolate mofetil). Its efficacy in RA treatment has been investigated (52,53).

In 1949 Hench et al. published that administration of cortisone to a patient with RA had favorable results, thereby discovering the therapeutic effects of glucocorticoids (54). Glucocorticoids have immediate and strong anti-inflammatory effects. These days the use of glucocorticoids is advocated for early aggressive treatment in combination with other DMARDs (reviewed in (55)). The mechanism of action of glucocorticoids is complex and involves regulation of gene transcription, via the transcription factors AP-1 and NF-κB.

These days there are several recombinant proteins available for treatment of RA, these proteins are sometimes called biologicals. Anti-TNF therapy is of most importance in the clinic. Anti-TNF was used after the realization that TNFα is one of the key players in the pathogenesis of RA. Notwithstanding the spectacular efficacy of anti-TNF therapy a major problem is that it is expensive and therefore only prescribed when other therapies fail.

The effect of anti-rheumatic drugs on cytokine production.

Understanding the role of various cytokines can lead to a better insight into the pathogenesis and perpetuation of diseases such as RA. Knowledge of the role that certain cytokines play has lead to the development of successful anti-cytokine therapies such as anti-TNFα treatment. It is known that many conventional DMARDs have an effect on cytokine production. In the following section several commonly used anti-rheumatic drugs are described with a focus on their effects on cytokine production.

Gold

Many reports have described the interference of gold compounds with monocyte and macrophage derived cytokines induced by bacterial products (56-58). However, there is a debate on which cytokines are inhibited and which are not. In rheumatoid synovial cells stimulated with IL-1β, incubation with gold decreases the production of IL-6 and IL-8 but not of GM-CSF (59). Inhibition was due to the fact that gold interferes with the DNA binding of NF-κB (59,60).

In a more recent report it is observed that in vitro incubation of patients or normal PBMC with gold results in an increased number of cells producing IL-6 and IL-10 measured by ELISPOT. Remarkably, the level of these cytokines in the supernatant was unchanged (61). Possible involvement of NF-κB was not investigated in this paper.

Antimalarials

CQ and HCQ are antimalarial drugs often used for the treatment of RA. Onset of clinical improvement is very slow, maximal improvement may not be achieved before 6 months of treatment (62). Antimalarials are often used in combination with other DMARDs such as MTX, which generally improves the efficacy (46,63). These agents accumulate, and probably perform part of their actions, in the acidous lysosomes and golgi complex, where many enzyme reactions are inhibited. This may affect various pathways of inflammation.

It is observed that NK cell activity is inhibited and lymphocytes of CQ treated patients show reduced responsiveness to PHA. Both agents inhibit the production of inflammatory cytokines produced by monocytes and by T cells (36,64). Recently the mechanism by which CQ decreases TNFα production has been attributed to the inhibition of mitogen-activated protein (MAP) kinase signaling (65). Inhibition of NF-κB activation was not observed, nor was TNFα inhibition caused by the actions of CQ in the lysosomes (66). The MAP kinase pathway is not only required for TNFα transcription, it can be used for the transduction of many signals, therefore inhibition of this pathway may also lead to various other cellular effects reported for CQ. Because of the resemblance of the drugs, HCQ can work via a similar mechanism however, this has not been reported.

Sulfasalazine

In the colon SSZ is degraded into 5-aminosalicylic acid and sulfapyridine by the colonic bacteria. Interestingly several reports show that these compounds often do not have the same properties as SSZ (67). Similar to the drugs described before, the immunomodulatory action of SSZ is not clear. Most papers have focussed on the effects of the drug on macrophages.

SSZ inhibits IL-12 production in macrophages stimulated with LPS in vitro (68). In vitro, macrophages pretreated with SSZ (in vitro and in vivo) stimulated T-cells to produce more

IL-4 and less IFNγ (68). Inhibition of macrophage activation has also been observed, leading to decreased stimulation of T-cells (69,70). A reason for decreased macrophage activity could be that SSZ induced apoptosis (71). Furthermore, SSZ was found to inhibit translocation of NF-kB to the nucleus in a cell line and in macrophages (68,72). However, the role of NF-kB is disputed by others who found inhibition of leukocyte accumulation in both wild-type and in NF-kB knock out mice. They claim that the effects of SSZ are mediated by increased adenosine release (73).

Cyclosporin A

CsA is a T-cell specific drug. CsA binds to an immunophilin and this complex can inhibit calcineurin. Cacineurin is a phosphatase required for activation of transcription factor NF-AT and its translocation to the nucleus. Several cytokine genes are not activated in the presence of CsA. One of the cytokines genes regulated by NF-AT is IL-2. In the presence of CsA the IL-2 gene is not transcribed and IL-2 is not produced. Consequently proliferation of surrounding T-cells can not be induced. Another cytokine under regulation of NF-AT is IFNγ. Intracellular concentration of IFNγ is decreased in CD4 T-cells from patients treated with CsA (74).

Some cytokines are indirectly inhibited by the intracellular actions of CsA. M-CSF for example is decreased by the depletion of IL-2 since IL-2 or IL-2R agonist could restore the production in the presence of CsA (75). In a study with RA patients, changes in cytokine levels from baseline to 16 weeks of treatment were compared between one group that received CsA in combination with MTX and a group that received MTX alone. Circulating levels of IL-2, IL-12,TNFα, IFNγ were reduced in the combination group, IL-10 was elevated compared to the group that received only MTX (76).

Another calcineurin inhibitor, FK506, also inhibits T cell proliferation (77). In addition, FK506 inhibits TNFα and IL-1β production by PBMC after anti-CD3/CD28 stimulation (78). The use of FK506 for RA is experimental; positive clinical results are described in a phase II trial (79).

Leflunomide

The primary action of leflunomide is the inhibition an early step in pyrimidine synthesis. This was demonstrated by the fact that the anti-proliferative action could be antagonized by the addition of uridine or cytidine to the cell cultures (see references in (80)). At high concentrations of leflunomide this was only partially effective. A reason for this is given by the observation that leflunomide can stimulate TGFβ production and inhibits IL-2 production in vitro. Both actions may lead to suppressed immune reactions (81). Two recent reports demonstrate that leflunomide inhibits IL-6, IL-1β, TNFα, MMP-1 and MMP-3 production in cultures of human synovial tissue cells (82,83). Furthermore leflunomide is a potent inhibitor of the transcription factor NF-κB. In an open study 24 weeks of treatment with leflunomide and MTX of patients already on MTX therapy at the start of the study leads to a lower plasma concentration and a reduction in mRNA expression of several chemokines (MCP-1, TARC, and MDC) in PBMC (84).

Glucocorticoids

Glucocorticoids are often used in the treatment of rheumatic diseases. The production of many inflammatory cytokines is downregulated by glucocorticoids. Glucocorticoids bind to

intracellular glucocorticoid receptors and this complex can bind to glucocorticoid responsive elements on the DNA, affecting transcription either positively or negatively. But interaction between glucocorticoid-receptor and transcriptions factors may also be important. In mice, inhibition of cytokine production induced by TNFα by dexamethasone seems to be dependent on NF-κB activation, whereas in human FLS, taken form RA patients, inhibition by dexamethasone (Dex) seems to be independent of NF-κB (85-87). Production of IL-10 was increased in PBMC isolated from RA patients, 42 days after treatment with Dex (88). Both IL-4 and IFNγ production by PBMC were decreased by in vitro addition of Dex. However, the ratio IL-4/IFNγ increased, resulting in an immunosuppressive effect (88). Inhibition of collagenase expression by glucocorticoids has been ascribed to interaction between the glucocorticoid receptor and transcription factor AP-1 (89).

Mycophenolic acid

The immunosuppressive drug mycophenolate mofetil (MMF) or its active compound MPA is a product of several *penicillium* species. MPA is a reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH) (90). Inhibition of IMPDH leads to inhibition of guanosine synthesis and subsequent guanosine depletion from the cells which leads to impaired DNA synthesis (91). The immunosuppressive properties of MPA have been well established for prevention of rejection after organ transplantation (92-94). Recently MPA has attracted interest as therapeutic agent for inflammatory diseases, because it has shown to reduce lupus nephritis in mouse models and humans (93,95,96).

Nagy et al. have shown that MPA inhibits superantigen-induced cytokine production in human T cells whereas it had no effect on LPS- or mitogen-induced cytokine production (97). In the mouse, in vitro as well as in vivo, MPA inhibited TNFα and IFNγ production whereas it did not affect IL-6 production (98). Furthermore, no effect on IL-2 and IL-10 mRNA expression was seen after ConA stimulation of mouse cells (99). In human cells MPA blocked lymphocyte proliferation at the G1/S transition (100,101). It has been reported that MPA blocks proliferative responses in human T cells but that IL-2 production is not changed (99,102,103).

Therapeutic proteins

Strategies to inhibit TNFα

TNFα has been identified as a major player in the pathogenesis of RA. Successful blockade of TNF signaling in mouse models (29,104) have led to the development of anti-TNF therapies. At this moment there are two different approached available to inhibit the action of TNFα; by administration of anti-TNF monoclonal antibodies or administration of soluble TNF receptors. Both approaches are clinical effective (23). Anti-TNF therapy in combination with MTX is more effective than MTX alone (24,105,106). Several groups have investigated the effect of anti-TNF Ab treatment on cytokine levels (reviewed in (33)). Treatment with a single anti-TNF Ab infusion results in a rapid decline of serum concentrations IL-6 and IL-1β. The extent of the decrease differs between the studies (107-109). An increase of TNFα was noted, for up to 30 days with a high dose of anti-TNF Ab (108). Furthermore decreases in IL-8 and other chemokines have been described after histological analysis of synovial tissue (109).

Strategies to inhibit other cytokines

IL-1 blockade has been attempted by administration of IL-1RA. Placebo-controlled trials demonstrate clinical improvement (110,111). The combination of anakinra and MTX yields better clinical results than MTX alone (112). Invasion of the synovial membrane by mononuclear cells is reduced after 24 weeks of treatment (113).

In an open trial, anti-IL-6 receptor Ab (MRA) was efficient in reducing RA (114), larger randomized trials are currently performed by the same group and by others (115). Endothelial growth factor VEGF was reduced in patients using IL-6R Ab (116). Beneficial results with anti-IL-6 have also been reported (35). Polyclonal anti-IFNγ Abs have also been observed to be beneficial in RA in a small placebo controlled trial (117). All other pro-inflammatory cytokines involved in RA are considered to be potential targets.

Strategies to deplete inflammatory cells

Efficacy of several anti-CD4 antibodies have been tested in open and placebo controlled trials (118-120). The outcome of these trials varies. Despite depletion of CD4+ cells no significant clinical improvement was found (119,120). In another trial beneficial effects have been reported, although these effects were not related to the decrease in CD4+ cells (118). Analysis of long term beneficial effects of an relatively non-depleting anti-CD4 mAb was stopped due to 'unacceptable CD4 lymphopenia' according to the authors (121). Effects on cytokine production were not investigated in these trials. Blocking T-cell function rather than deleting T-cells is another approach to diminish inflammatory responses. Currently under investigation is CTLA4-Ig that blocks costimulation via CD28 on the T-cell.

Also under investigation is the effect of B-cell depletion with anti-CD20 mAbs (rituximab) In two open trials improvement of clinical parameters was reported (12,122). Deletion of macrophages has been considered. Therapeutic benefits of local deletion and systemic deletion are investigated (122,123)

MTX

MTX (amethopterin, 4-amino 4-deoxy-N10-methylpteroyl-glutamic acid) is a folate antagonist. It was developed together with several other anti-folates more that 50 years ago for the treatment of malignancies (124). The target of MTX proved to be dihydrofolate reductase (DHFR) (125). Folic acid is a water soluble vitamin that can not be synthesized by the human body, hence dietary intake is essential. Folic acid is one of the many folate forms that are part of the folate metabolism. The main folate form in plasma is 5-methyl tetra-hydrofolate (THF). Folates, and also antifolates such as methotrexate, can enter the cell via the reduced folate carrier (RFC) or the folate receptor (FR). RFC has a greater affinity for MTX than for folic acid for the FR the reverse is true (126,127). Like MTX, folinic acid (5-formyl-THF) is mainly transported by the RFC. Once MTX enters the cell MTX is rapidly polyglutamated by folylpolyglutamyl synthetase. This can have several advances. First polyglutamates are preferentially retained intracellular and second, there is evidence for an increased affinity to folate-dependent enzymes (128), reviewed in (129). While MTX-polyglutamates were detected in liver cells and erythrocytes, their presence in lymphocytes has not been convincingly demonstrated (130,131).

Several THF forms serve as one carbon donors for reactions in the purine and pyrimidine synthesis and methionine-homocysteine metabolism. Inhibition of DHFR by MTX results in

the depletion of reduced folates available for these reactions. Besides the inhibition of DHFR, MTX is responsible for the direct or indirect inhibition of several other folate-dependent enzymes involved in the *de novo* pathway of DNA synthesis such as thymidine synthetase (132), AICAR transformylase (133), and amidophosphoribosyltransferase (134).

In 1951 the related drug aminopterin was found to inhibit cell proliferation in psoriasis and RA (135). But it was not until the 1980s that MTX was tested in placebo controlled trails for the treatment of RA (136-138). MTX is usually given in a weekly dose of 7.5-20 mg and administered either orally of by parenteral injection. Despite frequent adverse reactions to MTX, serious toxicity has been rarely reported. Gastero-intestinal problems, such as nausea or diarrhea, are most frequently seen (139). Folic acid and sometimes folinic acid supplementation are given to reduce these side effects. Indeed, there is less discontinuation through adverse effects of MTX treatment in patient groups treated with folic acid and folinic acid and only a slight increased dose of MTX was given to obtain similar responses in the patient groups (140). MTX is effective in 80% of the RA patients, it is unknown why some patients do not respond to therapy. In order to improve efficacy, decrease side effects and identify those patients that will not respond, elucidation of the mechanism of MTX is essential.

Although many target enzymes of MTX are known, there is a debate on the mechanism by which MTX works in a low, weekly dose. Several reports on the effects of cytokine production appeared. Immunohistochemical analysis of synovial biopsies show that IL-1β and TNFα, but not IFNγ and IL-1β were reduced after MTX treatment (141). In bone marrow derived mononuclear cells but not in peripheral blood derived mononuclear cells (PBMC) of RA patients, MTX enhanced IL-1RA secretion and inhibit IL-1β secretion in in vitro cultures (142). Since the cells were not stimulated for cytokine production, IL-1β production levels are very low in this report. Barrera *et al.* show that a single dose of MTX given to RA patients leads to a decrease in IL-1β production and not in TNFα production in PBMC, but not in WB, when the cells are stimulated with LPS, ex vivo, 48 hours after intake of MTX (143). A lack of effect on TNFα expression after LPS stimulation by MTX, in vitro, has also been demonstrated at transcriptional level in macrophages isolated from healthy individuals (71). Patients responding to 24 weeks of MTX treatment had higher levels of IL-10 after LPS stimulation ex vivo than non responding patients (144). These reports show that there is no consensus about the effect of MTX on monocyte derived cytokines. None of the reports use similar methods or measures similar cytokines, therefore it is not possible to draw a conclusion.

Circulating cytokines have been measured. Inflammatory cytokines such as TNFα, IL-6 and IL-1β, are higher in RA patients than in healthy subjects. Soluble cytokine receptors (TNFR p55, p75 and IL-2R) are measured as a reflection of the activation of the immune system in a patient. Unfortunately, besides the reduction of serum IL-6 there does not seem to be an unequivocal effect of MTX treatment on these cytokines and soluble receptors (145-147).

Modulation of T cell cytokine production by MTX also received attention. Ex vivo experiments show that, after more than 6 months of MTX treatment, the percentage of TNFα-producing CD4+ T cells declined, while an increase of IL-10-producing CD4+ cells was found (148). Similarly, in vitro stimulation of PBMC with PHA in the presence of MTX, leads to decreased mRNA levels of IL-2 and IFNγ, whereas mRNA levels of IL-10 and IL-4

increased under these conditions (149). Murine spleen cells cultured in the presence of MTX show a reduction in IFNγ and TNFα levels (98) Mice treated with MTX produce less IFNγ in one study (98) but not in another (150). In the latter TNFα production is reduced on protein level but not on mRNA level. In SLE mice, IL-1 and TNFα were decreased by MTX, whereas IL-2 and IFNγ were increased, resulting in a restoration of cytokine levels as in control mice (151)

The group of Cronstein has hypothesized that MTX inhibits inflammation because its use results in intracellular accumulation of intermediates of the purine synthesis leading to an increase of extracellular adenosine (152). Indeed adenosine has several immunosuppressive properties such as inhibition of neutrophil degranulation (153) and inhibition of expression of adhesion molecules (reviewed in (152)). Furthermore, in monocytes, adenosine increases IL-10 production, whereas it decreases TNFα production (51,154). Evidence for this hypothesis comes from animal models. A combination of two non-selective adenosine receptor antagonists, theophiline and caffeine, reversed the therapeutic effect of MTX in a rat adjuvant arthritis model. Furthermore, MTX decreased lymphocyte accumulation at inflamed sites in wild type mice but not in adenosine receptor knock-out mice (138). Others argue that the main target of MTX are T-cells, since MTX induces apoptosis in activated T-cells (155,156). However, induction of apoptosis by MTX has been disputed (134,157). MTX was found to be cytostatic, halting the cells in the G1 phase of the cell cycle (134).

Scope of this thesis.

The scope of this thesis is to elucidate the mechanism of action of low dose methotrexate in RA. We have used an in vitro whole blood system to study the effects on cytokine production. A better insight into the mechanism of action is important for the improvement of efficacy of the drug and for the reduction of the side-effects and toxicity that MTX causes in some patients. Our research has focussed on effects of MTX on cytokine production by various immune cells. Besides MTX, we have also investigated the effects on cytokine production of another drug that inhibits DNA synthesis, MPA.

In the first chapters of this thesis the effects of MTX and MPA on in vitro cytokine production are investigated. In chapter 2 effects of MTX are investigated. The mechanism of inhibition of cytokine production by MTX and MPA is studied in chapter 3. Effects of adenosine and MTX are compared in chapter 4. In chapter 5 the effect of MPA on IL-1β production by monocytes is described. In vitro effects of MTX on cytokine production are compared to the clinical outcome of RA patients in chapter 6 in order to be able to predict the response to MTX treatment before or soon after the beginning of treatment. Finally, in chapter 7 a new method for MTX measurement in plasma is described. With this method bio-active MTX can be measured. The results of the previous chapters are discussed in chapter 8.

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Chapter 2.

Inhibition of cytokine production by methotrexate

Studies in healthy volunteers and patients with rheumatoid arthritis.

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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). Not withstanding 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 *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.

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 (100µg/ml), 0.1 % endotoxin-free fetal calf serum (FCS), 50 µM 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 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.

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_2O.

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°C until use. IL-1β, IL-2, IL-4, IL-6, IL-8, IL-12p40, IL-13, TNFα and IFNγ 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 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

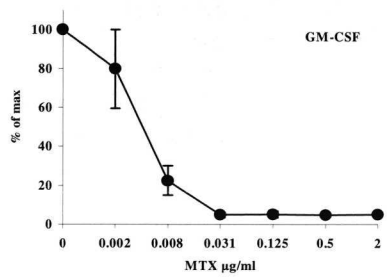
In vitro cytokine production in WB cultures.

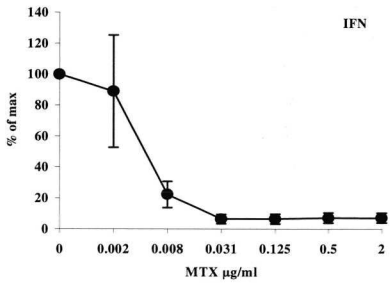
To assess cytokine production, WB cultures were stimulated with SAC. Table 1 shows a representative cytokine profile of a normal donor. It appeared that monokines (IL-1β, IL-6, IL-8, IL-12 and TNFα) are readily secreted into the supernatant. However T cell cytokines were not produced (IL-2, IL-13) or in minor quantities only (GM-CSF, IFNγ, table 1). With

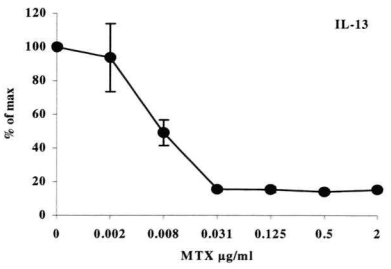
LOS similar results were obtained (not shown). Stimulation with a combination of anti-CD3 and anti-CD28 results in production of IL-2, IL-4, IL-13, GM-CSF, IFNγ and TNFα. Surprisingly also IL-8 is elevated (table 1). Polyclonal T cell stimulation of WB cultures leads to production of T cell cytokines and IL-8.

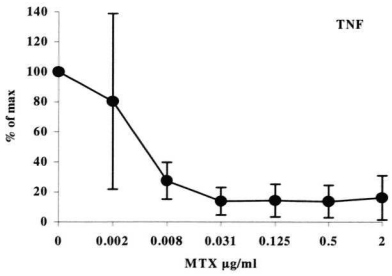
Table 1.	Cytokine production (pg/ml)		
	unstimulated	SAC	anti-CD3/28
IL-1β	10	1480	70
IL-6	-	5100	630
IL-8	60	38400	118100
IL-12p40	-	2150	710
TNF	-	3290	3550
IL-2	-	-	8480
IL-13	-	-	19100
GM-CSF	10	360	9300
IFNγ	-	1020	50700

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.









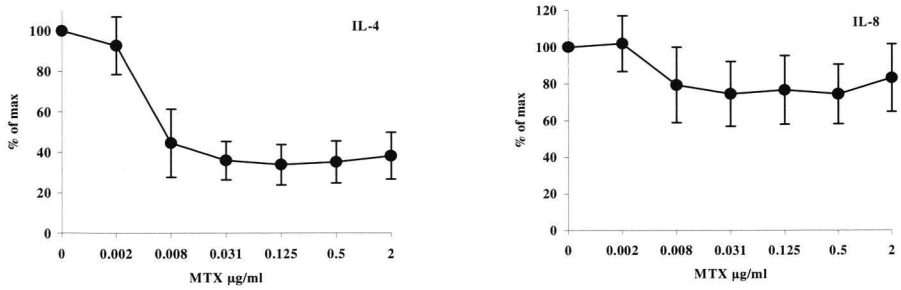


Figure 1. **Inhibition of T cell stimulated cytokine production by MTX.** WB cultures of 8 different blood donors were stimulated with anti-CD3 and anti-CD28. For each MTX concentration, cytokine production is expressed as % of production in the absence of MTX for each individual donor. The figure represents 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-depended 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.

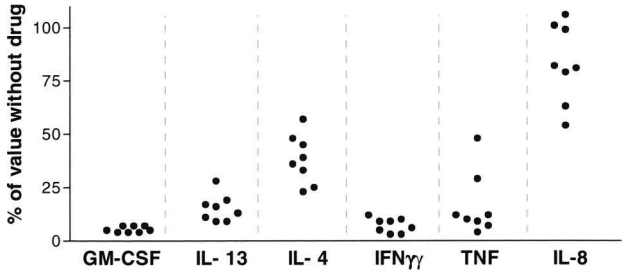


Figure 2. **Inhibition of T cell stimulated cytokine production by MTX of individual donors.** WB cultures of 8 different blood donors were stimulated with anti-CD3 and anti-CD28. Supernatants were harvested at day 4. Each dot represents cytokine production of a donor in the presence of 2 µg/ml MTX expressed as percentage of production in the absence of MTX. The range of cytokine production for the donors the in the absence of MTX is described in the legends of figure 1.

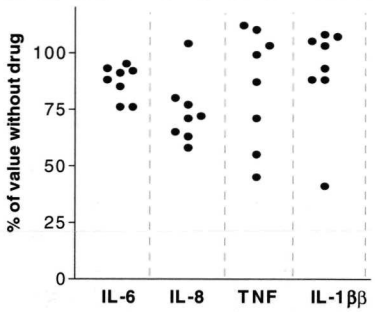


Figure 3. **Effect of MTX on LOS-induced cytokine production.** WB cultures of 8 different blood donors were stimulated with LOS (100 pg/ml). Each dot represents cytokine production in the presence of MTX (2 µg/ml) expressed as the percentage of production in the absence of MTX. These productions ranged 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.

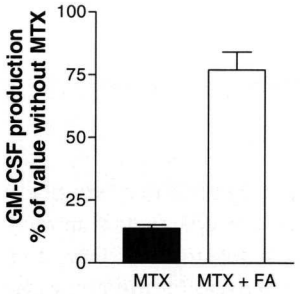


Figure 4. **Inhibition of cytokine production by MTX can be reversed by folinic acid**. WB cultures of 10 donors were stimulated by anti-CD3 and anti-CD28 with and without MTX in the presence (white bar) or absence (black bar) of 40µg/ml folinic acid. GM-CSF production is expressed as % of production 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 methabolism.

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.

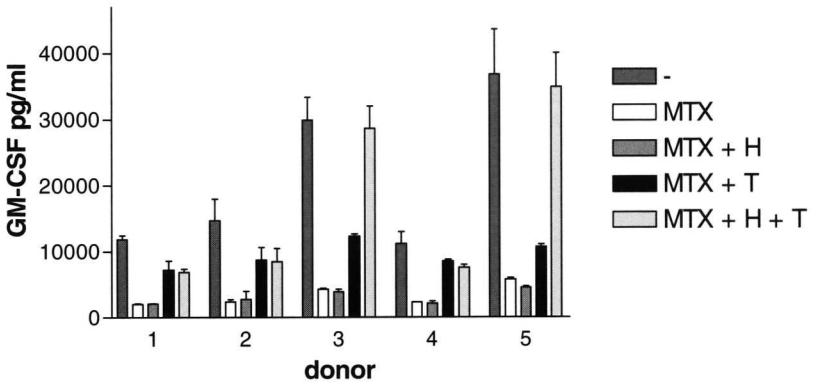


Figure 5. **Thymidine and hypoxanthine can reverse inhibition of cytokine production by MTX.** WB cultures of 5 donors were stimulated with anti-CD3 and anti-CD28 in the absence or presence of MTX (100 ng/ml). GM-CSF production is measured in the absence of MTX (light gray bar). Together with MTX we added nothing (white bar), 50 μM thymidine (dark gray bar), 100 μM hypoxanthine (black bar), or a combination of thymidine and hypoxanthine (striped bar). Error bars represent the SEM of duplicate cultures.

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).

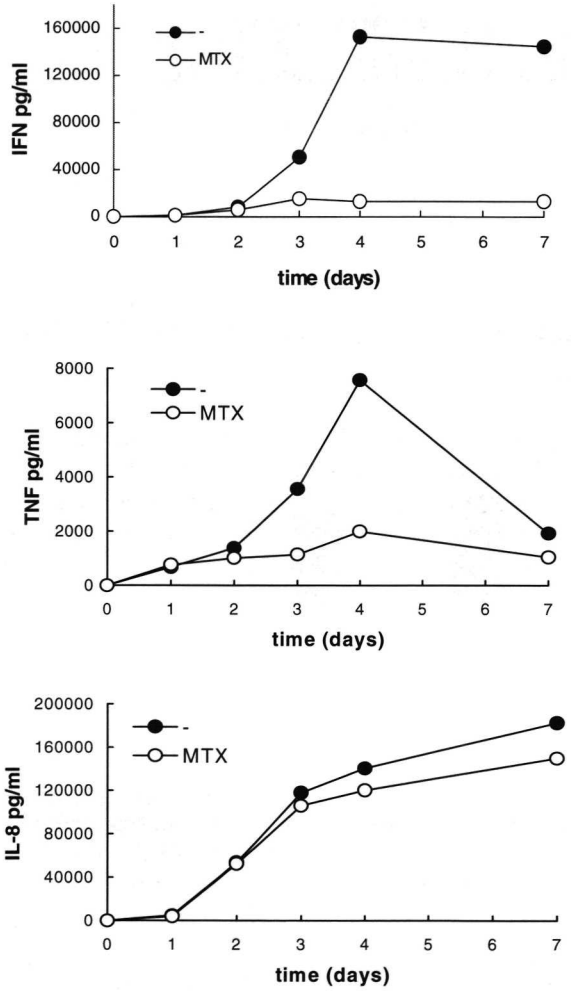


Figure 6. **Kinetics of inhibition of cytokine production by MTX.** Whole blood of a normal donor was stimulated with anti-CD3 and anti-CD28 in the presence (o-o) or in the absence (•-•) of MTX (100 ng/ml). Culture supernatants were harvested at indicated time points. This is a representative donor of 7 donors tested.

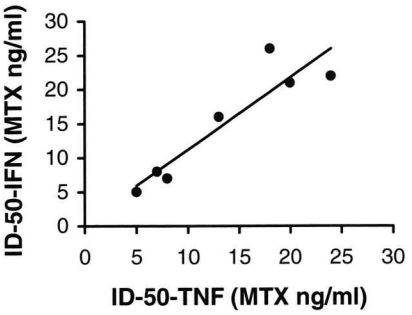


Figure 7. **TNFαand IFNγproduction are similarly affected by MTX.** WB cultures of 7 donors were stimulated with anti-CD3 and anti-CD28 in the presence of increasing amounts of MTX. Supernatants were tested for cytokine production and the amount of MTX required for 50% reduction (ID-50) was calculated for each cytokine. Here the results for IFNγ and TNFα are depicted.

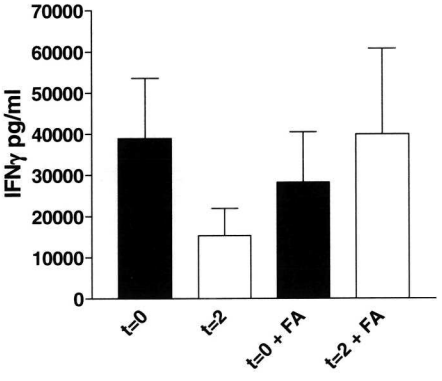


Figure 8. **Effect of oral uptake of MTX on ex vivo cytokine production.** Blood was obtained from 10 RA patients just before (t=0) and 2 hours after (t=2) the first oral application of MTX (10 mg). WB of 10 RA patients was stimulated with anti-CD3 and anti-CD28 in the absence or presence of 40μg/ml 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

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 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 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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Chapter 3.

Mycophenolic acid and methotrexate inhibit lymphocyte cytokine production via different mechanisms.

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Abstract

Mycophenolic acid (MPA) and methotrexate (MTX) are immunosuppressive drugs used for the treatment of various immunological disorders. MPA is an inhibitor of inosine monophosphate-dehydrogenase and MTX is a folate antagonist that inhibits tetrahydrofolate-reductase. Production of T cell cytokines in whole blood cultures as well as in PBMC cultures is inhibited by a low concentration of both drugs. Inhibition of cytokine production after monocyte stimulation was less evident. The mechanism by which inhibition is achieved is different for both drugs. Inhibition of T cell cytokine production by MPA was more profoundly and started earlier compared to the inhibition by MTX. MTX induced apoptosis in T cells that became activated, whereas MPA prevented activation of T cells by arresting the cells in the G0/G1 phase of the cell cycle. Addition of guanosine and adenosine can overcome this cell cycle arrest, even after several days. Furthermore MPA inhibited the expression of activation markers HLA-DR and CD71 on T cells.

The observation that MTX cannot prevent T cell activation but induces apoptosis in activated T cells and that MPA reversibly prevents activation of T cells could explain the immunosuppressive effects both drugs.

Introduction

Mycophenolate mofetyl and methotrexate (MTX) are immunosuppressive drugs used for treatment of immunological disorders. MTX has shown its efficacy in rheumatoid arthritis (RA) in placebo controlled trials (1,2) whereas the use of mycophenolate mofetyl or its active part mycophenolic acid (MPA) for treatment of RA is still experimental (3,4). MPA was reported to be an effective drug in preventing rejection in renal transplant patients (5,6). Recently it was suggested that MPA could be a useful drug to treat HIV infection since administration to HIV-infected subjects resulted in reduction of the number of activated T cells and of virus isolated from purified $CD4^+$ T cells (7). By which mechanism MPA and MTX are immunosuppressive is not clear. MPA is a selective inhibitor of inosine monophosphate dehydrogenase (IMPDH), the rate limiting step in the de novo synthesis of (d)GTP (8).

MTX is a folate antagonist. Addition of MTX to cell cultures will lead to the inhibition of de novo synthesis of purines and pyrimidines (9). In view of the extreme sensitivity of lymphocytes to inhibition of de novo nucleotide synthesis, T and B cells are likely candidates to be affected by the drugs (10). It has been reported that MPA blocks proliferative responses in human T cells but that IL-2 production and CD25 expression is not changed (11). Nagy et al. have shown that MPA inhibits superantigen-induced cytokine production in human T cells whereas it has no effect on LPS- or mitogen-induced cytokine production (12). In the mouse, in vitro as well as in vivo, MPA inhibited TNF-α and IFN-γ production by T cells whereas it did not affect IL-6 production (13). In another mouse study it was reported that in vivo application of MPA two hours before an LPS challenge inhibited TNF-α production, whereas TNF-α production induced by anti-CD3 was not inhibited or even increased (14). In splenocytes from SLE prone and normal mice MPA inhibited lymphocyte proliferation, the production of immunoglobulins and autoantibodies (15).

Mizoribine, another IMPDH inhibitor, has been found to block T cell proliferation in the G0/1 phase (16). Early events in T cell activation such as mRNA expression of c-myc, IL-2, c-myb, CD25 and cdc2 kinase were unaffected. Subsequently it has been reported that MPA also blocked lymphocyte proliferation in the G0/1 phase (17) (18). Conversely, it has been described that MPA can induce apoptosis in several human cell lines and in activated T cells (7,19). Recently we have shown that MTX, at low concentrations, effectively inhibits cytokine production after polyclonal T cell stimulation in human whole blood cultures, whereas MTX hardly effects cytokine production after stimulation with LPS or SAC (Gerards et al., submitted). This effect of MTX is due to induction of apoptosis in activated T cells (20). We decided to study the effect of MPA on cytokine production in human whole blood cultures. We observed that similar to MTX, MPA is a potent inhibitor of cytokine production but by a completely different mechanism.

Materials and methods

Cells and cell cultures.

Heparinised blood collected from healthy volunteers was used for whole blood (WB) cultures and for isolation of peripheral blood mononuclear cells (PBMC). All cell cultures were performed in culture medium (Iscove's Modified Dulbecco's Medium (IMDM, Bio Whittaker, Verviers, Belgium) containing penicillin (100U/ml), streptomycin (100µg/ml) and 50 µM 2-mercaptoethanol). 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. PBMC were isolated from freshly drawn blood and separated over a Percoll gradient (d=1.078, Pharmacia Fine Chemicals AB, Uppsala, Sweden). PBMC were cultured ($2x10^5$ cells/ml) in culture medium supplemented with 5% FCS and 20 µg/ml human transferrin (Sigma-Aldrich, Steinheim, Germany). All cells were cultured in 200 µl at 37°C in the presence of 5% CO_2 in flat bottom plates (Nunc, Roskilde, Denmark). Stimuli and drugs were directly added to the cell culture, unless otherwise indicated. The duration of the cultures is indicated for each experiment.

Antibodies, stimuli and drugs.

Anti-CD3 (CLB.T3/4.E, CLB, Amsterdam, the Netherlands) and anti-CD28 (CLB.CD28/1) were used for stimulation of the cells at 1 µg/ml. Other stimuli were LPS (100 pg/ml, a gift from Dr. J. Poolman, RIVM, Bilthoven, the Netherlands) and Staphylococcus aureus cells (SAC, Pansorbin, 1:4000, Calbiochem, La Jolla, CA). Human recombinant IL-2 (Chiron corp., Emeryville, CA) was used at 100 units/ml. The drugs MPA (GibcoBRL, Grand Island, NY) and MTX (Lederle, Hoofddorp, the Netherlands) were freshly diluted from a 2.5 mg/ml stock in each experiment. For FACS analysis we used anti-TNF-α (CLB-TNF/5) anti-CD25, anti-HLA-DR, Ki-67 (all FITC-labeled), anti-CD69, anti-CD71 (both PE-labeled) anti-CD3 (PE-Cy5 labeled) and anti-CD2 (biotin-labeled) mAbs. These mAbs were purchased at the CLB, except for Ki-67 (Immunotech, Marseille, France). Guanosine and adenosine (cell culture tested) were obtained from Sigma-Aldrich.

Cytokine measurements.

The production of cytokines was measured in the supernatant of the cell cultures. Supernatant was harvested at indicated times and stored at –20°C until use. IL-8, IL-6, IL-4, IL-13, TNF-α and IFN-γ were measured with ELISA kits (PeliKine-compact, CLB) according to the protocol. In short, mAbs were coated on flat bottom microtiter 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 (HPE) buffer (CLB). Plates were washed 5 times (PBS, 0.02% Tween-20) and incubated for 30 minutes with streptavidin-labeled poly-horseradish peroxidase (CLB) 1:1000 diluted in PBS/2% skimmed milk. Plates were washed 5 times and developed with 0.003% H_2O_2, 100 µg/ml 3,5,3',5'-tetramethylbenzidine (Merck, Darmstadt, Germany) in 0.11 M Na-acetate, pH 5.5. The reaction was stopped with an equal volume (100 µl) of 2M H_2SO_4. Only for IL-13 an additional blocking step of 30 minutes with PBS/2% milk was required after the O/N coating step. GM-CSF was measured via a similar protocol as the other cytokines. The GM-CSF Abs were a kind gift from Dr. G. Trinchieri (the Wistar Institute, Philadelphia, PA). 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, Basel, Switzerland) was used for the preparation of a standard curve. For intracellular TNF-α staining $5x10^6$ cells/ml were cultured, to prevent secretion of cytokines 1µl/ml GolgiPlug (PharMingen, San Diego, CA) was added 18 hours before harvesting the cells.

FACS analysis.

Induction of apoptosis was measured by staining the cells with annexin-V (FITC-labeled, Bender Medsystems, Vienna, Austria). All incubations were performed at 4°C. Cells were washed 3 times with buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1

mM $MgCl_2$ and 0,5% (w/v) bovine serum albumin, pH 7.4) Cells were incubated for 20 minutes with annexin-V (1:400) and biotinylated anti-CD2 (1:10), washed 3 times, incubated with streptavidin-allophycocyanin (APC) (1:750, PharMingen) and measured on the FACScalibur (BectonDickinson, San Jose, CA). For the analysis of activation markers, stimulated or unstimulated cells, cultured with or without MPA, were analyzed after 24 hours for CD25 and CD69 expression and after 72 hours for Ki-67, HLA-DR and CD71 expression. After the cells were washed 3 times with PBS, 0.5% BSA and 0.02% Sodium azide (PBA), the cells were incubated with mAb's (1:10) for 20 minutes in the presence of 3 mg/ml human gammaglobulins (CLB), to block non-specific binding. Before staining with Ki-67, TNF-α and anti-CD3 mAb, cells were permeabilised by incubation for 10 minutes with FACS lysing solution and FACS permeabilising solution (Becton Dickinson) respectively, according to the manufacturers protocol, and washed 2 times. Thereafter cells were washed 3 times and measured on the FACScan or FACScalibur (Becton Dickinson). Appropriate isotype control mAbs (CLB) were used in each experiment.

Results

Effect of MPA and MTX on T cell cytokine production in WB and PBMC.

Whole blood of 8 different healthy donors was stimulated with a T cell stimulus (anti-CD3 plus anti-CD28 mAbs) in the presence of MPA and MTX for 4 days resulting in the production of T cell cytokines. As an example we show that MPA dose-dependently inhibited the GM-CSF production (Fig. 1A). The inhibition of GM-CSF production by MTX started at a lower concentration than that by MPA (Fig. 1B). In addition to the inhibition of GM-CSF production, MPA also inhibited the production of IL-4, IL-13, IL-8, IFN-γ and TNF-α (Fig. 2). Dose response curves of cytokine inhibition by MPA were similar for each cytokine. MTX inhibited the production of these cytokines as well, except that of IL-8, but the inhibition was not as strong as that observed with MPA (Fig. 2). In this experiment the amount of supernatant restricted the number of cytokines we could test. A prominent cytokine missing in this experiment is IL-2. In separate experiments we have amply tested the effect of MPA and MTX on IL-2 production. Indeed IL-2 is inhibited by both drugs at relatively early time points. However that IL-2 is a difficult cytokine to measure in supernatants because it is consumed by proliferating T cells. For that reason, at later time points IL-2 levels strongly decrease, also in cultures without drugs.

For the production of cytokines by T cells IL-2 is essential (15). Consequently, inhibition of cytokine production could be secondary to early depletion of IL-2. However addition of excess (100U/ml) of IL-2 at the start of the cultures did not alter the inhibition of either drug (not shown). The observed effects are therefore not caused by IL-2 starvation of the cells.

The kinetics of inhibition by MPA and MTX are different. At day one inhibition by MPA in 8 donors varied between 50 and 80% whereas MTX had no effect (fig. 3). At day two inhibition by MPA was more than 90% and inhibition by MTX between 40 and 75%. At day 3 both drugs efficiently inhibited cytokine production (fig. 3).

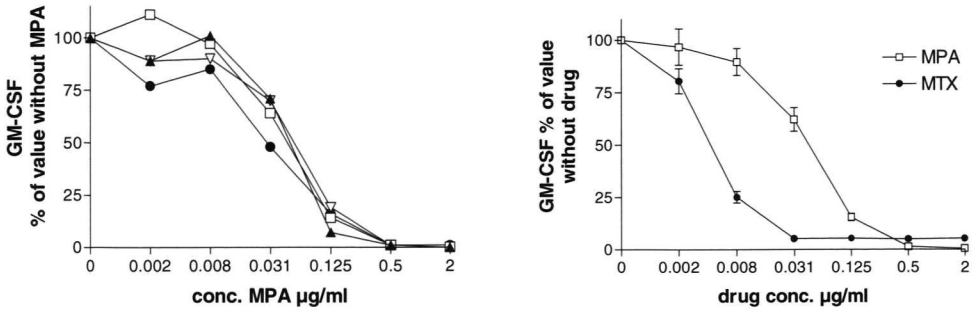


Figure 1: **The effect of MPA and MTX on the production of GM-CSF**. WB (1:10 diluted) of healthy donors was stimulated with anti-CD3/anti-CD28 and incubated with a concentration range of MPA and MTX for 4 days. Without addition of a drug, the GM-CSF production ranges from 37330-136050 pg/ml in the different donors. The values on the y-axis are percentages of the production in the absence of the drug. 1A, the dose dependent inhibition of GM-CSF production by MPA of 4 of the donors is shown. 1B, inhibition of GM-CSF production by MPA (□) is compared to the inhibition by MTX (•) (mean of 8 donors, +/- SEM).

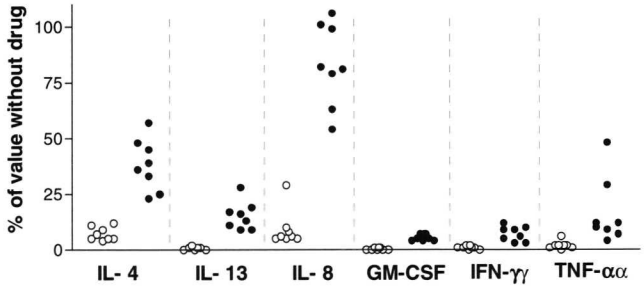


Figure 2: **The effect of MPA and MTX on cytokine production after T cell stimulation.** WB of 8 healthy donors was stimulated with anti-CD3/anti-CD28 and incubated with 2 µg/ml MPA (○) or 2 µg/ml MTX (•). Cytokine production was measured by ELISA after 4 days. Without addition of a drug, values of the different donors ranged from: 165-2500 pg/ml IL-4, 75250-394600 pg/ml IL-8, 3315-12075 pg/ml IL-13, 37330-136050 pg/ml GM-CSF, 47775-393050 pg/ml IFNγ and 470-10984 pg/ml TNF. The values on the y-axis are percentages of the production in the absence of the drug.

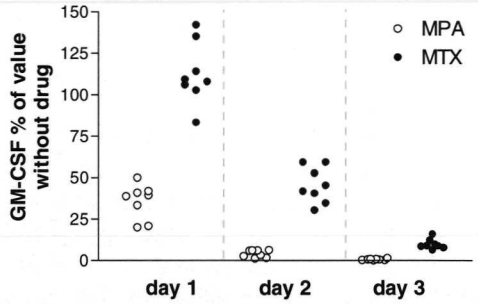


Figure 3: **The kinetics of GM-CSF inhibition by MPA and MTX.** WB of 8 healthy donors was stimulated by anti-CD3/anti-CD28 mAb without a drug or in the presence 2 μg/ml MPA (○) or 2 μg/ml MTX (●). At the indicated times the GM-CSF production was measured by ELISA. The values on the y-axis are percentages of the GM-CSF production in the absence of the drug. Without addition of a drug values of the different donors ranged from 370-1635 pg/ml at day 1, 2925-15940 pg/ml to day 2 and 20840-95470 pg/ml at day 3.

Effect of MPA and MTX on monocyte cytokine production.

Stimulation of WB with bacterial products LPS or SAC induces production of IL-6, IL-8 and TNF-α in monocytes. The production of these cytokines was hardly affected by MTX (Gerards et al., submitted). The LPS- or SAC-induced IL-6, IL-8 and TNF-α production was slightly inhibited by MPA (Fig. 4). The effects of MPA and MTX on cytokine production by PBMC were comparable to the effects of the drugs on the cytokine production by WB. In experiments with PBMC, the production of IL-8 was not measured, since isolated PBMC produce high levels of IL-8 in the absence of stimuli (not shown).

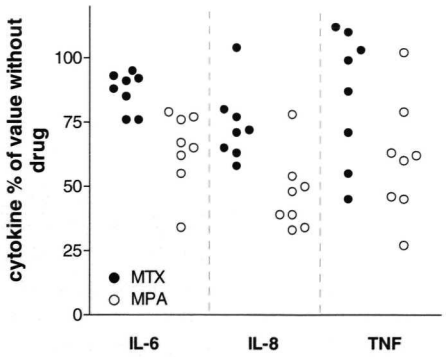


Figure 4: **The effect of MPA and MTX on LPS induced cytokine production.** WB of 8 healthy donors was incubated with LPS (100 pg/ml) in combination with 2 μg/ml MPA (○) or 2 μg/ml MTX (●). After 24 hours, the production of cytokines was measured by ELISA. The production of IL-6 ranges from 1853-3780 pg/ml, IL-8 from 5257-26030 pg/ml and TNF from 270-1053 pg/ml in the different donors. The values on the y-axis are percentages of the production in the absence of the drug.

Apoptosis induction.
It was reported before that both MTX and MPA induce apoptosis in T cells. We performed flow cytometric studies with PBMC to assess the effects of MPA and MTX on cell viability in low-density cultures. The morphology of the cells was analyzed after a 4-day culture period. Unstimulated cells remained small while anti-CD3/anti-CD28 stimulated cells formed blasts. Cells stimulated in the presence of MPA remained small, cells stimulated in the presence of MTX first formed blasts and then appeared to become apoptotic; they became more granular and shrunk (Fig. 5A). This notion was supported by annexin-V binding studies. In unstimulated cell cultures neither of the drugs induced annexin-V binding (Fig. 5B). Stimulated cells bound slightly more annexin-V, than resting cells did. Stimulation of the cells in the presence of MPA had no effect on annexin-V binding whereas stimulation in the presence of MTX led to extensive annexin-V binding (Fig. 5B). The induction of apoptosis starts after two days and the number of annexin-V positive cells increases in time.
The induction of apoptosis by MPA described in literature was found both on T cell lines (19) and on activated peripheral blood T cells (7). The PBMC used in our study were resting at the time MPA and the stimulus were added. Indeed, when MPA was added to PBMC stimulated earlier, MPA did induce annexin-V binding (Fig. 5C). MPA addition induced annexin-V binding to PBMC as soon as 24 hours after stimulation (not shown).

The effects of MPA are reversible.
Our data indicated that, rather than killing the cells, MPA prevents proliferation of freshly isolated cells. We next studied whether the effects of MPA might be reversible. Addition of 100 μM guanosine and 200 μM adenosine, together with the stimulus and MPA, was found to prevent the inhibitory effects of the drug (not shown). To see whether it is not only possible to prevent the inhibitory effects of MPA but also to *reverse* the inhibition, we stimulated PBMC in the presence of MPA and cultured them for various periods before addition of guanosine and adenosine. Both proliferation and cytokine production were restored in the cells cultured with anti-CD3/anti-CD28 and MPA after addition of guanosine and adenosine at day 4 (Fig. 6). Untreated PBMC were cultured in the same plate. These were stimulated at the day of guanosine and adenosine addition and served as a control for the capacity of the cells to proliferate and produce cytokines. We established that the proliferation and cytokine production in response to anti-CD3/anti-CD28 of PBMC that have been cultured in medium for 4 days prior to stimulation is comparable to when the stimulus is given directly. It was possible to reverse the effects of MPA by guanosine and adenosine addition for up to 14 days (not shown).

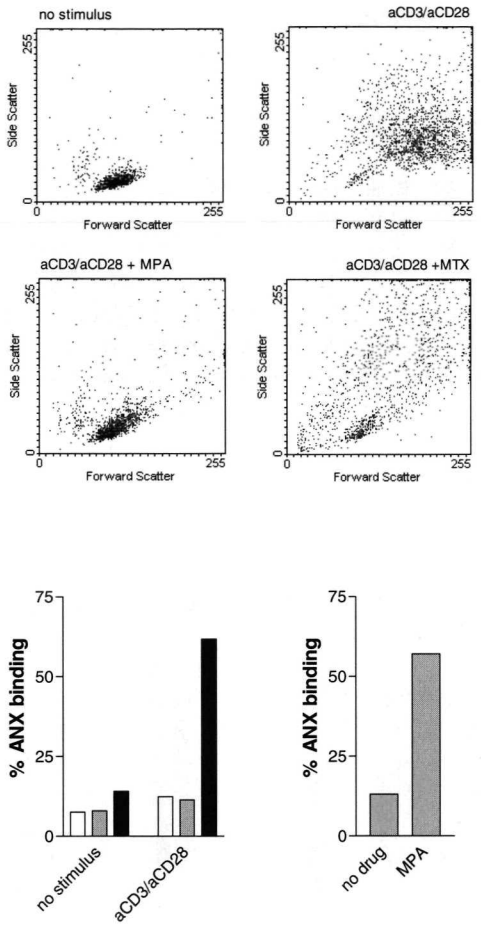
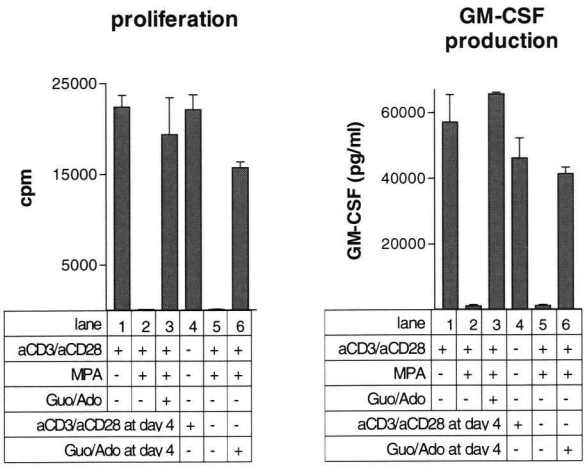


Figure 5: **A differential effect of MPA and MTX on the induction of apoptosis.** PBMC were not stimulated or stimulated with anti-CD3/anti-CD28 in the presence of MPA or MTX. After 4 days apoptosis was measured by staining the cells with annexin-V. 5A: FACS scatter plots of: non-stimulated PBMC (upper left panel), PBMC stimulated with anti-CD3/anti-CD28 (upper right panel) and stimulated in the presence of MTX (lower left panel) or MPA (lower right panel). 5B. Annexin-V binding to PBMC. The percentage annexin-V binding cells within the CD2 population is depicted on the Y-axis. Unstimulated PBMC (left three bars) and PBMC stimulated with antiCD3/antiCD28 (right three bars) in the presence of; no drug (white), MPA (gray) and MTX (black). Similar results were obtained in 3 separate experiments. 5C: MPA induces apoptosis in activated PBMC. PBMC are stimulated with anti-CD3/anti-CD28. At day 3 cells were washed and cultured with 100 U/ml IL-2, without MPA (white) or with 2 µg/ml MPA (gray). At day 6 cells were stained with annexin-V and were analyzed on the FACS. The percentage annexin-V binding cells within the CD2 population is depicted on the y-axis. Similar results were obtained in 2 separate experiments.



lane	1	2	3	4	5	6
aCD3/aCD28	+	+	+	-	+	+
MPA	-	+	+	-	+	+
Guo/Ado	-	-	+	-	-	-
aCD3/aCD28 at day 4	+	-	-			
Guo/Ado at day 4	-	-	+			

lane	1	2	3	4	5	6
aCD3/aCD28	+	+	+	-	+	+
MPA	-	+	+	-	+	+
Guo/Ado	-	-	+	-	-	-
aCD3/aCD28 at day 4	+	-	-			
Guo/Ado at day 4	-	-	+			

Figure 6: **Adenosine and Guanosine reverse the effects of MPA.** PBMC are cultured with the additions indicated. At day 8 3H-thymidine incorporation and cytokine pro-duction are assessed. Similar results are obtained in 4 separate experiments. Error bars indicate the SEM of triplicate cultures.

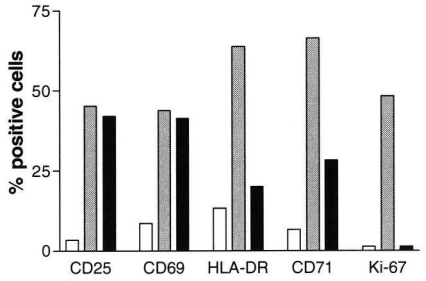


Figure 7: **The effect of MPA on activation markers.** PBMC are not stimulated (white bars) stimulated with anti-CD3/anti-CD28 in the absence (gray bars) and presence (black bars) of MPA. After 24 hours the expression of CD25 and CD69 was measured, after 72 hours the expression of HLA-DR, CD71 and Ki-67 was measured on the FACS. Similar results are obtained in 3 separate experiments.

The effect of MPA on activation markers.

In T cells, MPA induces an arrest in the G0/1 phase of the cell cycle (not shown) (18) (17). To more precisely define the point in the cell cycle, at which the cell is arrested by MPA, the expression of T cell activation markers was measured. CD69 and CD25 appear on the cell surface a few hours after stimulation whereas HLA-DR and CD71 appear after 2 days. It has been shown that the nuclear antigen Ki-67 is expressed in the late G1 phase just before cells enter the S phase (21). In our cultures Ki-67 positive cells appear 2 days after stimulation.

For optimal detection the expression of the early activation markers CD69 and CD25 was determined after 24 hours. Expression of late activation markers HLA-DR and CD71 and the nuclear antigen detected by Ki-67 was determined after 72 hours. The expression of CD69 and CD25 was not influenced by MPA, whereas the expression of HLA-DR and CD71 was decreased in the presence of MPA (Fig. 7). Ki-67 staining was completely inhibited in the presence of MPA (Fig. 7). Similar results were obtained when the cells were pre-incubated with MPA for 48 hours prior to stimulation with anti-CD3/anti-CD28. As expected MTX had no effect on the expression of either activation marker (not shown).

The effect of MPA and MTX on intracellular TNF-α staining.

From the results described above we concluded that MPA inhibits cytokine production independent of proliferation and MTX does not. To further investigate this hypothesis we stained PBMC intracellularly for TNF-α production at an early time point when the cells were not proliferating. After stimulation for two days, 46% of the T cells produce TNF-α; these are all CD69 positive cells. MTX did not influence intracellular TNF-α staining. In the presence of MPA only 10% of the T cells produced TNF-α (Fig. 8).

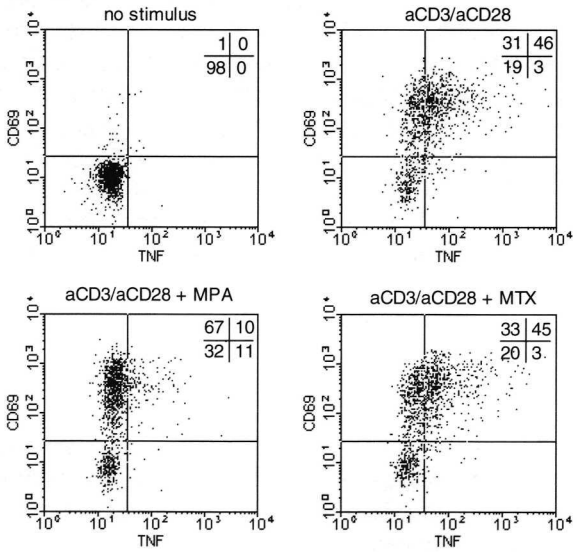


Figure 8. **Intracellular TNF-α staining.** PBMC were not stimulated or stimulated with anti-CD3/anti-CD28 in the presence of MPA or MTX. GolgiPlug was added after 28 hours, 16 hours later the cells were harvested and stained for CD3 and TNF-α after permeabilisation. Non-stimulated PBMC (upper left panel), PBMC stimulated with anti-CD3/anti-CD28 (upper right panel) and stimulated in the presence of MTX (lower left panel) or MPA (lower right panel). CD3 positive cells are shown in dotplots, with CD69 staining on the y-axis and TNF-α staining on the x-axis. The percentage of cells in each quadrant is indicated in the upper right corner of every plot. Similar results are obtained in 3 separate experiments.

Discussion.

MPA and MTX are used as immunosuppressive drugs but the mechanism by which the immunosuppression is achieved is disputed. Here we show that MPA, like MTX, strongly inhibited T cell cytokine production in whole blood-and PBMC cultures. Furthermore, production of IL-6, IL-8 and TNF-α induced by monocyte activation is slightly decreased by MPA, and even less affected by MTX. The mechanism by which these drugs affect cytokine production turns out to be different. Addition of MPA to PBMC prevents blastogenesis but does not lead to apoptosis. In contrast, addition of MTX induces apoptosis in activated T cells as shown before (20).

It has previously been reported that also MPA does induce apoptosis (7,10,19). The reason for this discrepancy is that we studied resting cells rather than proliferating cells or (continuously cycling) cell lines. Indeed, when MPA was given after stimulation, MPA did induce apoptosis in PBMC. This demonstrates that the effect of MPA on initially resting cells is different from the effect on proliferating cells.

MPA is a better inhibitor of cytokine production than MTX. This is probably due to the differences in kinetics. MTX targets blasts only and its kinetics of inhibition coincides with the kinetics of blastogenesis. MPA prevents blastogenesis; consequently, it works at an earlier stage than MTX. This is clearly demonstrated by the inhibition of intracellular TNF-α expression, at a time point where blasts are not yet present. Another remarkable difference in the inhibition induced by MPA and MTX is the effect on IL-8 production after T cell activation. MPA inhibited the IL-8 production but MTX did not. We found that IL-8 production depended on the endogenous production of TNF-α and GM-CSF by T cells and that IL-8 is not produced by T cells. MPA is a better inhibitor of both TNF-α and GM-CSF production than MTX. The residual (10%) TNF-α and GM-CSF production in the presence of MTX is sufficient to support full IL-8 production by a yet unidentified cell.

When activation marker expression is analyzed after anti-CD3/anti-CD28 stimulation, the inhibition of the expression by MPA follows the same kinetics as the inhibition of cytokine production. The expression of the activation markers appearing within hours was not affected by MPA, whereas the expression of markers that appear later (CD71, HLA-DR and Ki-67) was decreased. These results are in agreement with the observation that MPA blocks rat lymphocytes in the G0/1 phase, but has no effect on CD25 expression and with the finding that MPA blocks human PHA-activated T cells in G1 (17,18).

There are many reports showing that the inhibitory effects of MPA are prevented by addition of different guanosine nucleotides to the MPA-treated cell cultures (8,11,17,19,22,23). We used a combination of guanosine and adenosine to restore proliferation in PBMC cultured with MPA and anti-CD3/anti-CD28, as measured by cytokine production and [^{3}H]-thymidine incorporation. The use of adenosine was necessary, since guanosine alone did not completely restore proliferation of the cells. The recovery of proliferation and cytokine production in cells even after 14 days of culture with MPA and anti-CD3/anti-CD28 proves that under these conditions apoptosis was not induced. The cells are stopped in their

activation process, before entering the S-phase and the process is completely reversible. This observation correlated well with the observation that peripheral blood mononuclear cells, isolated from patients treated with MPA respond normally to mitogenic stimulation in vitro (10).

It has been shown before that depletion of (deoxy) guanosine nucleotides by inhibition of IMPDH is the reason for impaired DNA synthesis (10,11). Lymphocytes contain high levels of IMPDH and upon activation they express the IMPDH type II isoform, which is more sensitive to MPA than type I. Therefore, lymphocytes are a primary target for MPA (10). Although we show that MPA effectively inhibits T cell cytokine production, the exact mechanism remains to be elucidated.

Inhibition of proliferation will of course decrease the number of cytokine producing cells. However on day 2 T cell proliferation is hardly initiated whereas cytokine inhibition is already considerable. This means that inhibition of proliferation cannot be the sole explanation for the observed inhibition. In addition, the reduced production of monokines supports the notion that additional inhibitory mechanisms might be operational. Possibly, changes in metabolic pathways requiring guanosine nucleotides, such as RNA synthesis or G-protein-linked events and diminished expression of adhesion molecules (24) are important in the decreased production of cytokines.

In conclusion we show that MPA, effectively inhibits cytokine production by T cells. It does so by preventing T cells to become fully activated. In activated T cells MPA induces apoptosis; thus treatment with MPA may result in depletion of the in vivo activated T cells. Whether the reversible prevention of activation of resting T cells by MPA might have any consequences for the immune response remains to be investigated.

In the light of the successes of anti-TNF treatment in RA and Crohn's disease, our observation that TNF-α production by both T cells and monocytes is inhibited by MPA renders MPA an attractive drug for RA treatment.

Our findings lead to the conclusion that MPA prevents T cell activation without depleting them. Its efficacy in graft-versus-host-disease or transplantation could be due to this mechanism. Early in the disease MPA will work by inhibiting T cell activation. At a later stage of treatment, when the acute inflammation is over the treatment with MPA can be discontinued because in the absence of the pro-inflammatory signals alloantigen presentation will probably results in a (tolerogenic) Th2 type response in the remaining alloresponsive T cells.

Acknowledgments.

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Chapter 4.

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

Sacha de Lathouder, Els de Groot, Jonathan Coutinho
and Lucien Aarden.

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, theophiline 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% CO_2 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 H_2O (5mM) and used at con-centrations 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 microtiter 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% H_2O_2, 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 H_2SO_4 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)		
cytokine	no drug	MTX
IL-4	1463 (187)	506 (82)
IL-13	6908 (660)	1017 (155)
IL-8	202585 (23781)	164311 (22082)
GM-CSF	73785 (8186)	4281 (806)
IFN-γ	203267 (25492)	17272 (4228)
TNF-α	3317 (867)	451 (180)

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.

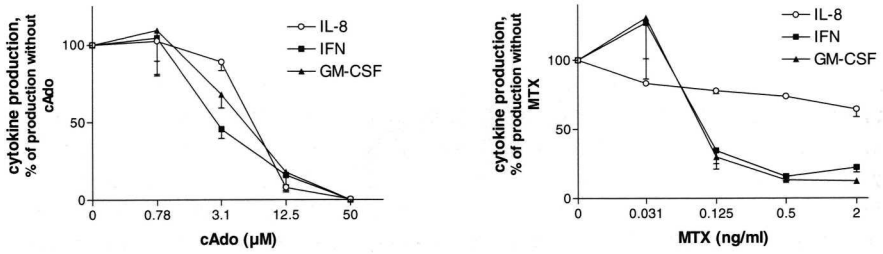

Figure 1. **Effect of cAdo and MTX on anti-CD3/anti-CD28 induced cytokine production.** Duplicate cultures of 1:10 diluted WB of 3 healthy donors was stimulated with anti-CD3/anti-CD28 with various concentrations of (A) cAdo or (B) MTX. After 3 days supernatants were harvested and tested for the presence 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 we 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).

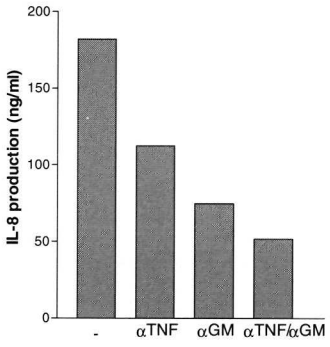

Figure 2. **Effect of anti-GM-CSF and anti-TNF antibodies.** 1:10 diluted WB was stimulated with anti-CD3/anti-CD28 in the presence of anti-TNF (aTNF) anti-GM-CSF (aGM) or both (aTNF/aGM). After 3 days IL-8 was measured in the supernatant by ELISA.

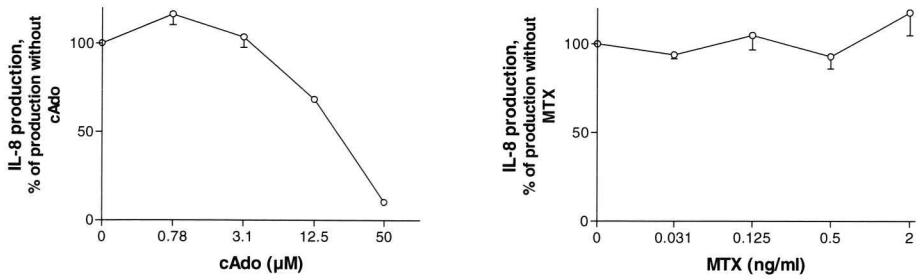

Figure 3. **Effect of cAdo and MTX on GM-CSF/TNF -induced IL-8 production.** Duplicate cultures of 1:10 diluted WB of 3 healthy donors was stimulated with GM-CSF/TNF with various concentrations of (A) 2-CAdo or (B) MTX. After 3 days supernatants were harvested and tested for the presence of IL-8. Data 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.

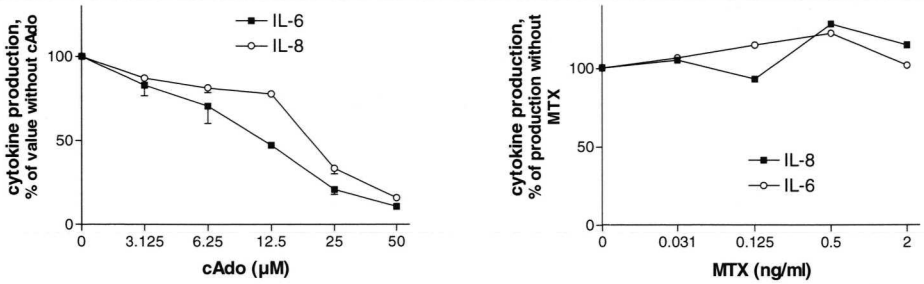

Figure 4. **cAdo inhibits monocyte cytokine production, MTX does not.** Duplicate cultures of 1:10 diluted WB were stimulated with LPS (100 pg/ml) and various concentrations of cAdo (A) or MTX (B). After 1 day supernatants were harvested and analysed for the presence of IL-8 and IL-6. Data are presented 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

adenosine 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 rue 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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Chapter 5

A differential effect of MPA on monokine production, evidence for induction os IL-1β production

Sacha de Lathouder, Els de Groot, Miriam Karsten and Lucien Aarden

Abstract

Mycophenolic acid (MPA) is an immunosuppressive drug used for the prevention of organ rejection after transplantation. We have investigated the effect of MPA on cytokine production by monocytes. MPA inhibited both SAC- and LPS- induced production of TNF, IL-8 and IL-6. LPS-induced IL-1β production was increased by MPA, whereas SAC induced IL-1β production was not affected. Elevated IL-1β production was not due to an increase of mRNA levels. In the presence of Z-VAD MPA does not increase IL-1β production. Therfore we suggest that MPA increases IL-1β processing rather than its secretion.

Introduction

The drug mycophenolate mofetil (MMF) or its active compound mycophenolic acid (MPA) is a reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH) (1). It is widely used as an immunosuppressive drug. The immuno-suppressive properties have been well established for prevention of rejection after organ transplantation (2-4). Recently it has attracted interest as therapeutic agent for other diseases as well, because MPA has shown to reduce lupus nephritis in mouse models and in humans (3,5,6).

We have previously reported that MPA profoundly inhibits cytokine production after T-cell stimulation (7). MPA achieves this inhibition by preventing T cells to go into cycle at the G1/S transition (7,8). In this article we have extended our research on the mechanism of action of MPA by investigation of the effect of MPA on monocytes, with a focus on IL-1β production.

IL-1β is a pro-inflammatory cytokine produced by activated monocytes and macrophages. IL-1β acts on many cell types and has many functions. It can increase lymphocyte responses, induce fever and increase expression of adhesion molecules (9,10). As a consequence, IL-1β is implicated in various diseases such as rheumatoid arthritis and inflammatory bowel disease (11). IL-1β is synthesised in the cytosol as inactive 31 kD protein without a signal peptide. It is secreted as biologically active 17 kD mature form. Cleavage of pro-IL-1β occurs by IL-1β converting enzyme (ICE, also called caspase-1) concurrently with secretion (12,13). The mechanism by which IL-1β is cleaved and secreted has only recently been elucidated (14,15). Stimulation of monocytes or macrophages with LPS results in pro-IL-1β synthesis, which is neither secreted nor processed to the mature form. Secretion and processing follows after a secondary stimulus with ATP, via stimulation of the purinergic $P2X_7$ receptor (14,16-20). The $P2X_7$ receptor belongs to a family of ATP-gated ion channels. Upon binding to extracellular ATP the channel opens and is permeable for ions, upon longer stimulation a pore is formed that is permeable for larger ions and low-molecular-weight solutes (reviewed in reference (21)). $P2X_7$ receptors are predominantly expressed on cells of the immune system, in contrast to the other P2X family members, which are more widely expressed.

Stimulation of the $P2X_7$ receptor with ATP leads to mature IL-1β production in LPS primed macrophages and monocytes (17,18). Blockade of the receptor with a monoclonal antibody results in inhibition of mature IL-1β production by monocytic THP-1 cell line after LPS and ATP (22). Further evidence for a role of the $P2X_7$ receptor in production of IL-1β comes from $P2X_7$ knock out mice. Macrophages of these mice are unable to make mature IL-1β upon stimulation with LPS and ATP (19).

The present study demonstrates that the anti-inflammatory drug MPA inhibits the production of IL-6, IL-8 and TNF after monocyte stimulation. However surprisingly IL-1β production is stimulated by MPA when monocytes are stimulated with LPS. This could have consequences for the application of MPA as an anti-inflammatory drug.

Materials and methods.

Heparinised blood collected from healthy volunteers was used for whole blood (WB) cultures and for isolation of peripheral blood mononuclear cells (MNC). All cell cultures were performed in culture medium (Iscove's Modified Dulbecco's Medium (IMDM, Bio Whittaker, Verviers, Belgium) containing penicillin (100U/ml), streptomycin (100µg/ml) and 50 µM 2-mercaptoethanol). 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. MNC were isolated from freshly drawn blood and separated over a Percoll gradient (d=1.078, Pharmacia Fine Chemicals AB, Uppsala, Sweden). MNC were cultured (2x105 cells/ml) in culture medium supplemented with 5% FCS and 20 µg/ml human transferrin (Sigma-Aldrich, Steinheim, Germany). All cells were cultured in 200 µl at 37°C in the presence of 5% CO_2 in 96 wells flat bottom plates (Nunc, Roskilde, Denmark) or in 1 ml in 24 well plates (Nunc) for mRNA isolation and immunoprecipitation. Stimuli and drugs were freshly diluted in culture medium supplemented with FCS and directly added to the cell culture. The duration of the cultures is indicated for each experiment.

Antibodies, stimuli and drugs.

To stimulate the cells we used LPS (100 pg/ml, derived from *Neisseria Meningitidis*, a gift from Dr. J. Poolman, RIVM, Bilthoven, the Netherlands) and Staphylococcus aureus cells (SAC, Pansorbin, 1:4000, Calbiochem, La Jolla, CA). The drug MPA (GibcoBRL, Grand Island, NY) was freshly diluted in culture medium from a 2.5 mg/ml stock and used at 1µg/ml. Z-VAD (Alexis, Lausen, Switzerland) was dissolved to 40mM in methanol and used at concentration of 40µM. At a 1:1000 dilution methanol has no effect on the cells.

Cytokine measurements.

Mature IL-1β, IL-6, IL-8 and TNFα (PeliKine-compact, Sanquin Reagents, Amsterdam, the Netherlands) was measured according to the manufacturers protocol. In short, mAbs were coated on flat bottom microtiter 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 (CLB) 1/1000 diluted in PBS 2% skimmed milk. Plates were washed 5 times and developed with 0.003% H_2O_2, 100 µg/ml 3,5,3',5'-tetramethylbenzidine (Merck, Darmstadt, Germany) in 0.11 M Na-acetate, pH 5.5. The reaction was stopped with an equal volume (100 µl) of 2M H_2SO_4 to the wells and was read at 450 nm.

For measurement of total IL-1β (pro IL-β + mature IL-1β), sheep anti-IL-1β polyclonal Abs were used (S77, a kind gift of Dr. S Poole, NIBSC, Hertfordshire, UK). 10 µg/ml aIL-1β was coated and 1 µg/ml biotin conjugated aIL-1β was used. As standard curve we used rIL-1β from the pro-IL-1β ELISA kit. To measure intracellular proIL-1β the supernatant was removed from the cells and replaced by an equal volume of culture medium and cells were lysed by repeated freezing and thawing.

Quantitative PCR.

$1x10^6$ MNC (0.5×10^6/ml) were cultured as described above. After 6 hours supernatant was harvested and tested for cytokine production. Cells were lysed in Trizol LS (Invitrogen, Carlsbad, CA) and RNA was extracted according to the manufacturers protocol. Subsequently cDNA was synthesised using Superscript and oligo-d(T) primers (Invitrogen). PCR was performed on a LightCycler machine (Roche Molecular Biochemicals, Mannheim, Germany) using a FastStart DNA master SYBRgreen I kit (Roche). The primer sequence for IL-1β was forward: GGATATGGAGCAACAAGTGG, reverse: ATGTACCAGTTGGGGAACTG and for IL-8 forward: TTGGCAGCCTTCCTG

ATTTC, reverse: AACTTCTCCACAACCCTCTG and for β2-microglobulin (β2M) forward: CCAGCAGAGAATGGAAAGTC, reverse: GATGCTGCTTACATGTCTCG.

Relative amount of target (IL-1β or IL-8) mRNA was calculated according to the formula for relative quantification with an external reference (β2M) described in the LightCycler manual; $E^{CpT(calibrator)-CpT(sample)} * E^{CpR(sample)-CpR(calibrator)}$. In which CpT stands for crossing point of target and CpR for crossing point of reference and E for the PCR efficiency that was set on 2. An unstimulated sample was used as calibrator.

IP and Western Blot.

S77 polyclonal antibody was coupled to CNBr-activated sepharose 4B (Amersham Phamacia Biotech AB, Uppsala, Sweden) following the instructions by the manufacturer and stored in PTA (PBS, 0.1% Tween, 0.01% NaN_3). Immunoprecipitation was carried out in 500µl sample (supernatant or lysed cells) in presence of 0.1% Tween O/N at 4°C. Sepharose was washed 3 times with PTA and 2 times with PBS and diluted in sample buffer (NuPage, Invitrogen, Groningen, the Netherlands). Electrophoresis and Western blot were carried out using the NuPage system (Invitrogen). Blots were blocked for minimal 1 hour in 0.01M Tris, 0.15M NaCl, 0.05% Tween, (TBST), 5% skimmed milk powder plus 2% sheep serum and subsequently stained with biotinylated S77 polyclonal antibody (1 µg/ml) in TBST 0.5% skimmed milk powder. After washing 3 times 10 min in TBST the blot was incubated with streptavidin-HRP (1:1000, Amersham biosciences, Buckinghamshire, England) for at least 30 min in TBST, 0.5% skimmed milk powder. The blot was developed with ECLplus (Amersham).

Statistical analysis

Statistical analysis was performed using GraphPad Prizm software. Wilcoxon signed rank test was used to determine differences in cytokine production in figure 1.

Results

Effect MPA on monocyte cytokine production.

In vitro production of monokines can be induced by SAC or LPS stimulation of a WB culture. MPA decreases IL-6, IL-8 and TNFα production induced by SAC by about 50% (figure 1a). This reduction by MPA is significant. IL-1β is not significantly reduced by MPA. Likewise, LPS-induced production of TNFα, IL-6 and IL-8 is decreased by MPA. Surprisingly IL-1β production induced by LPS is clearly increased in the presence of MPA (figure 1b). Similar results were obtained using MNC (not shown).

Figures 1a and 1b show cytokine production as a percentage of the production without MPA. In figure 2 the actual amounts of IL-1β production of the 8 donors are depicted after LPS and SAC stimulation. It shows that SAC is a more efficient stimulus for IL-1β production than LPS and that MPA increases LPS-induced IL-1β levels to those obtained by SAC stimulation.

To investigate whether changes in cytokine protein levels were a reflection of mRNA levels we have quantified IL-1β and IL-8 mRNA. Quantification of IL-8 mRNA was used as representative of the cytokines produced after LPS stimulation that are inhibited by MPA. Because the anti-coagulant heparin used in WB cultures can interfere with enzymes used in PCR and cDNA synthesis, we chose to use MNC instead. In contrast to the findings with IL-1β protein in the supernatant, we found an decrease in relative amount of IL-1β

mRNA in MPA treated MNC, when stimulated with LPS (figure 3). Relative IL-8 expression in LPS stimulated MNC is also decreased in MPA treated MNC (figure 3). Without LPS stimulation expression of IL-1β and IL-8 mRNA was less than 100 pg/ml (not shown).

The effect of MPA on intracellular IL-1β.

The IL-1β ELISA used for the experiments depicted in figure 1 and 2 measures the mature form of IL-1β only. To investigate whether MPA enhances the processing and secretion of proIL-1β we used an ELISA that can detect both mature- and pro-IL-1β. With this ELISA IL-1β was measured in supernatant and in cells (figure 4). As found before, MPA increased (mature) IL-1β production in the supernatant. In line with the notion that MPA might enhance processing of IL-1β, addition of MPA leads to a decrease of (pro)IL-1β in the cells.

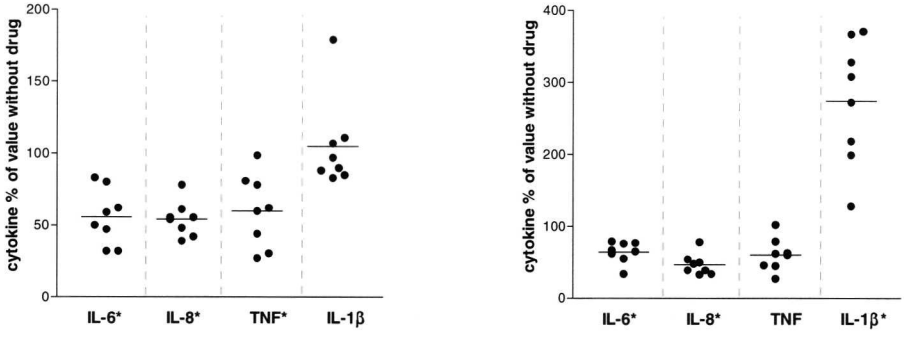

Figure 1. **Effect of MPA on cytokine production after monocyte stimulation.** WB (1:10) of 8 healthy donors is incubated for 24 hours with 1:4000 SAC (a) or 100 pg/ml LPS (b) with and without MPA. Cytokine production is depicted as percentage of the production in the absence of MPA. * $p < 0.05$, ns = not significantly different from production without MPA. Ranges of cytokine production after SAC stimulation in the absence of the drug are:IL-6 from 1453 - 5080 pg/ml, IL-8 from 8103 - 55810 pg/ml, TNF from 1197 - 4667 pg/ml and IL-1β from 1290 – 6020 pg/ml. After LPS stimulation ranges are: IL-6 from 1800 - 3860 pg/ml; IL-8 from 4650 - 26100 pg/ml; TNF from 220 - 1100 pg/ml and IL-1β from 580-2160 pg/ml.

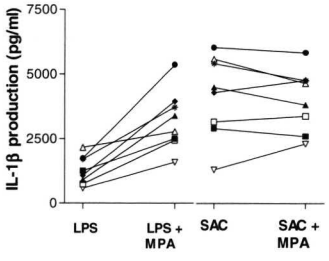

Figure 2. **Effect of MPA on IL-1β production.** WB (1:10) of 8 healthy donors is incubated for 24 hours with 100 pg/ml LPS or 1:4000 SAC in the absence of MPA or presence of MPA. Mature IL-1β production of each donor is depicted, each donor is represented by a symbol.

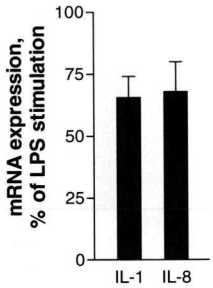

Figure 3. **mRNA expression in the presence of LPS and MPA.** Quantitative PCR was performed on PBMC that were cultured for 8 hours in the presence of LPS with or without MPA. Relative amount of IL-1β mRNA expression was calculated after the amount of mRNA in the samples was normalised on β2M mRNA expression. The amount of mRNA expressed after LPS stimulation was set to 100%, the percentage of mRNA experssion in the presence of LPS and MPA is depicted on the y-axis. Error bars represent the SEM of several PCR reactions performed on samples of three independent experiments. IL-1β production without LPS was not detectable in ELISA, when stimulated the increase by MPA was 547%, 238% and 224% of the value with LPS alone in three experiments (from 265 to 1449 pg/ml, from 1085 to 2580 pg/ml and from 1236 to 2774 pg/ml respectively.

Effect of Z-VAD on IL-1β production.

The experiments described indicate that the increase of mature IL-1β after LPS stimulation in the presence of MPA is caused by increased processing, secretion or both rather than increased transcription or translation. To discriminate between secretion and processing a broad range caspase inhibitor, Z-VAD, was used to inhibit ICE activity. As expected mature IL-1β is not detected in the supernatant of LPS-stimulated cultures in the presence of Z-VAD (figure 5). Production of other cytokines was not affected by Z-VAD (not shown). Supernatants were also tested in the ELISA that detects both pro and mature IL-1β. With this ELISA IL-1β was detected in the supernatant in the presence of Z-VAD. Indicating that the pro form is secreted, as described previously (18,23).

There is no difference in IL-1β production in the presence of Z-VAD between cultures incubated with or without MPA (figure 5). Immunoprecipitation and Western blot analysis of these supernatants confirms that in the presence of Z-VAD only proIL-1β is secreted into the supernatant of LPS stimulated cultures. In the presence of Z-VAD there is no difference in proIL-1β secretion between cultures incubated with or without MPA (figure 6). Suggesting that MPA only influences the processing but not the secretion of IL-1β.

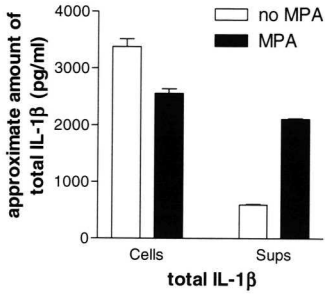

Figure 4. **Effect of MPA on mature and pro IL-1β production.** Total IL-1β production was measured with an ELISA detecting both in mature and pro IL-1β. Total IL-1β was detected in supernatant and cells as described in the materials and methods section. PBMC were cultured O/N with LPS in the presence and absence of MPA. In the absence of LPS cytokine values were not detectable. Error bars indicate the SEM of duplicate cultures. This is a representative of several experiments performed.

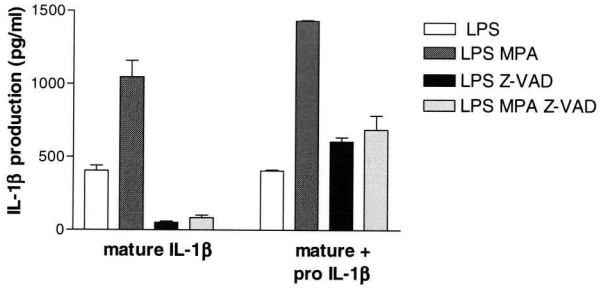

Figure 5. **Differences between mature and total IL-1β production after Z-VAD treatment.** Mature and total (mature and pro) IL-1β were measured in supernatant. PBMC were cultured O/N with LPS in the presence and absence of MPA and /or 40μM Z-VAD.

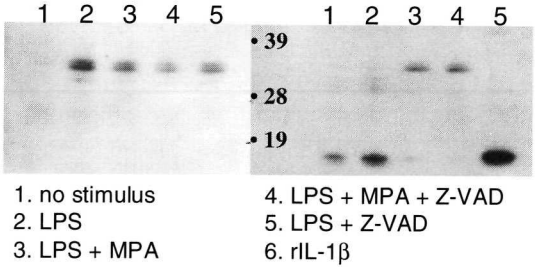

Figure 6. **Immunoprecipitation of both forms of IL-1β from cells and supernatant.** Pro and mature IL-1β are immunoprecipitated from supernatant and lysed cells with polyclonal anti-IL-1β coupled sepharose and analysed on western-blot as described in materials and methods. The blot was stained with the same polyclonal Ab. Culture conditions are indicated in the figure. Positions of pro IL-1β and mature IL-1β are indicated with a black and white arrow respectively. In lane M proteins of the sea-blue marker are indicated with their size in kDalton.

Discussion.

We demonstrated that MPA inhibits the production of inflammatory cytokines induced by SAC and LPS with the exception of IL-1β. MPA increases the production of LPS-induced mature IL-1β and does not influence SAC-induced IL-1β production. The increase of mature IL-1β protein levels induced by MPA was not caused by the increase in mRNA expression. Like IL-8 mRNA expression the expression of IL-1β mRNA was decreased to 65-70% of the expression induced by LPS alone.

In many recent reports it has been described that LPS induces only intracellular proIL-1β accumulation in monocytes and macrophages but does not induce much mature IL-1β secretion. For secretion of IL-1β from LPS treated monocytes, a secondary stimulus is necessary. This second stimulus can be given by multiple agents such as nigrecin, ATP or cell injury (16,17,24).

Recently, induction of secretion of IL-1β by ATP has received much attention. There are indications that this induction is a result of the activation of the $P2X_7$ receptor by ATP (14,18-20). As described previously (13) we do find considerable amounts of mature IL-1β in the supernatant after 6 to 24 hours stimulation with LPS alone. We do not see an increase of mature IL-1β secretion after an 80 minute ATP stimulation of our LPS primed cells (data not shown). This discrepancy can reflect a difference in culture conditions used (25).

As described before (26,27) we show that SAC is a better stimulus for the production of mature IL-1β than LPS. Furthermore, we showed that, in contrast to LPS-induced IL-1β production, SAC-induced IL-1β production is not increased by MPA. Because the intracellular activation pathways of LPS and SAC are different (28), it is possible that SAC is a better stimulus for the processing and subsequent secretion of IL-1β than LPS is. Indeed, stimulation with SAC at this concentration leads to complete secretion and processing of pro IL-1β. Therefore MPA does not have an additional effect on the production of mature IL-1β induced by SAC stimulation.

Stimulation of LPS primed monocytes with ATP in the presence of the caspase 1 inhibitor, Y-VAD, leads to secretion of pro IL-1β in the supernatant (18,23). These results indicate that the effect of ATP on the secretion of IL-1β is independent from the effect of IL-1β processing and thus that secretion is independent from processing. Our results point to an effect of MPA on the processing of pro IL-1β only. When processing of IL-1β is inhibited by Z-VAD, MPA does not increase the amount of pro IL-1β in our cultures. Only pro IL-1β is found in supernatants after Z-VAD treatment and it is the same amount for both LPS- and LPS plus MPA-treated cultures. This suggest that the secretion process is not affected by MPA.

The role of apoptosis in the maturation of IL-1β is not clear. Maturation can be induced by apoptosis (24). However, the induction of apoptosis is not necessary for the production of IL-1β as has been showen for LPS and ATP-induced IL-1β production (14). Another argument against the induction of apoptosis in the involvement of maturation of IL-1β is that the active form of caspase-3, one of the caspases responsible for the execution phase of apoptosis, is not detected after LPS plus ATP stimulation (29). Although MPA can induce apoptosis in activated cells (30,31) MPA does not induce apoptosis in initially resting (T-)cells (7). Therefore it is not expected that possible activation of ICE by MPA is a result of the induction of apoptosis in these cultures.

We conclude from these results that MPA can induce the processing of IL-1β but not its secretion. We think that MPA does so by activating ICE, although we do not present direct evidence for this hypothesis. Activation of ICE by MPA is remarkable and should be further investigated. Especially since ICE has been considered as new target to suppress inflammation. Besides processing IL-1, ICE also cleaves IL-18 another pro-inflammatory cytokine that induces IFNγ production. Mice treated with a caspase inhibitor or deficient in ICE are protected against experimental mucosal inflammation (32,33). The same ICE inhibitor is currently under investigation in osteoarthritis models (34).

We report an IL-1β inducing effect of the immunosuppressive drug MPA in vitro. In view of the development of new immunosupressants that inhibit the generation of mature IL-1β, one can question the use of MPA as anti-inflammatory drug. However, whether MPA can also induce mature IL-1β in vivo or whether this feature of MPA has clinical consequences remains to be investigated.

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Chapter 6

Bioassay for detection of methotrexate in serum.

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Scand J Rheumatology 2004:33

Abstract

Objective: A bioassay for the measurement of methotrexate (MTX) in serum is developed.

Methodes: The assay is based on the fact that MTX inhibits the proliferation of hypoxanthine-guanosine phosphoribosyl transferase (HGPRT) negative mouse B-cells (B9.H). HGPRT negative cells cannot use the salvage pathway of nucleotide synthesis to overcome inhibition by MTX.

Results: When B9.H cells are cultured with serial dilutions of serum, inhibition of proliferation is a measure for the amount of MTX in that serum. Circulating folates do not interfere with the assay.

Conclusion: This simple assay can detect low amounts of MTX in serum; it is therefore a useful assay to follow the pharmacodynamics of functional MTX after low dose MTX treatment.

Introduction

Methotrexate (MTX) inhibits the enzyme dihydrofolate reductase (DHFR) and several other enzymes, thereby inhibiting the folate metabolism and the *de novo* purine and pyrimidine synthesis (12;3). High-dose MTX therapy is well established for the treatment of several forms of cancer such as acute lymphoblastic leukemias (4). In addition, low-dose MTX therapy is widely used for the treatment of rheumatoid arthritis (RA) and other inflammatory diseases (5-8). The anti-inflammatory mechanism of low dose MTX treatment is still debated. Cronstein has suggested that MTX acts via the release of adenosine (9). We and others have proposed that MTX works via deletion of activated T-cells and the subsequent inhibition of cytokine production (10,11).

MTX levels in serum of RA patients show a very strong increase directly after administration of the drug and after 1 or 2 hours the levels decrease (1213-15). MTX is transported into cells by folate receptors, but the majority is excreted by the kidneys. Intracellular MTX is partially metabolised to polyglutamate forms leading to accumulation of these forms in the cell. MTX levels in erythrocytes are stable over a period of several days (15). For a better understanding of the pharmacodynamics of the drug and its (side) effects it is useful to monitor circulating levels of functional MTX in the patients.

Various assays are available for the detection of MTX in biological fluids (serum, plasma or cerebrospinal fluid) or erythrocytes (16,17). The fluorescence polarisation immunoassay (TDx) and enzyme multiplied immunoassay technique (EMIT) are commercially available antibody based assays used to measure MTX in serum and plasma. Another frequently used assay for the analysis of MTX is high-pressure liquid chromatography (HPLC) which can be used for analysis in various body fluids or cells. With this technique it is possible to distinguish between different polyglutamate forms of MTX that accumulate in erythrocytes (18). Two other methods are based on the ability of MTX to inhibit the enzyme DHFR. When dihydrofolate is reduced to tetrahydrofolate by DHFR, NADPH is oxidised to NADP, which can be monitored by measuring the absorbency. This reaction is inhibited by the addition of serum containing MTX (17;19). The radio-ligand binding assay is also based upon the fact that MTX forms a stable complex with DHFR in the presence of NADPH. DHFR is sequentially incubated with samples containing MTX and with [^{3}H]-MTX. Unbound [^{3}H]-MTX is removed by dextran coated charcoal. The amount of radioactivity in the supernatant is inversely proportional to the concentration MTX in the sample (20,21).

Although there are many different assays for the measurement of MTX in serum and plasma, none of these assays measures biologically active and thus functional MTX. Here we present a simple bioassay suitable for the measurement of functional MTX in serum and plasma. Furthermore this assay is sensitive enough to measure serum of RA patients, treated with low dose MTX.

Materials and methods

Cells and chemicals

B9 cells are IL-6 dependent mouse hybridoma cells, widely used to assay IL-6 activity ([22]). B9.H is subclone independent of IL-6 and negative for hypoxanthine-guanosine phosphoribosyl transferase (HGPRT, selected by Dr. S. Ebeling in our department).. B9 and B9.H cells were grown in culture medium: Iscove's Modified Dulbecco's Medium (IMDM, Bio Whittaker, Verviers, Belgium) containing penicillin (100U/ml), streptomycin (100µg/ml), 50 µM 2-mercaptoethanol) and 5% FCS (Bodinco, Alkmaar, the Netherlands), supplemented with 8 pg/ml rIL-6 (CLB, Amsterdam, the Netherlands) for B9 cells. When indicated, heat inactivated (30', 56°C) human pool serum (HPS) was used in addition to 5% FCS. Hypoxanthine and thymidine (cell culture tested) were obtained from Sigma (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). MTX was purchased from Lederle (Hoofddorp, the Netherlands). Polyglutamated MTX forms (Schircks, Jona, Switzerland) were a kind gift from Dr. G. Jansen (VU Medical Centre, Amsterdam, the Netherlands)

Proliferation assays

When proliferation was measured by cell counting 25,000 cells/well were cultured in 1ml for 3 days, at 37°C in the presence of 5% CO_2 in 24 well plates (Nunc, Roskilde, Denmark). The well was emptied and diluted in 9 ml isoton and counted on a Casy automated cell counter (Schärfe System GmbH, Reutingen, Germany).

For thymidine incorporation, cells (5000 cells/well) were cultured for 3 days in 200 µl at 37°C in the presence of 5% CO_2 in 96 well flat bottom plates (Nunc). [^{3}H]-Thymidine (2 Ci/mmole, Amersham, Bucks, UK) was added at a final concentration of 1 µCi/ml (37 kBq/ml) the last 4 hours of culture. The cells were then harvested on glass fibre filters (Wallac, Turku, Finland) and radioactivity was measured by liquid scintillation counting.

MTX bioassay.

For the measurement of MTX in sera from RA patients, sera were heated for 30 minutes at 56°C and stored at –20°C until use. Serum was added to B9.H (5000 cells/well) in two-fold dilutions, starting with 25% of the total culture volume. All dilutions were tested in duplicate cultures. MTX was freshly diluted (2-fold) as a standard curve (highest concentration 100 ng/ml) and added to B9.H cells. After 3 days proliferation was measured using [^{3}H]-thymidine incorporation as described above.

Patients

The study was approved by the local medical ethical committee and all patients gave written informed consent. Blood was taken from 23 RA patients, 2 hours after the first oral dose of MTX (10 mg). Serum was collected and heated for 30 minutes at 56°C to inactivate complement and stored at –20°C until use. MTX concentration in plasma stored at –20°C remains stable for at least 18 months (16).

Results

Effects of thymidine and hypoxanthine on the inhibition of proliferation by MTX.

MTX inhibits proliferation of a mouse B cell line, (figure 1a). Proliferation could be restored via the *salvage* pathway of nucleotide synthesis by addition of thymidine (T) and hypoxanthine (H) (figure 1a). Our aim was to measure MTX in serum and serum contains 1-2 mg/l H (23) When we analysed proliferation by ^{3}H-thymidine incorporation, we observed that the combination of serum H and the radioactive T led to decreased sensitivity for MTX.

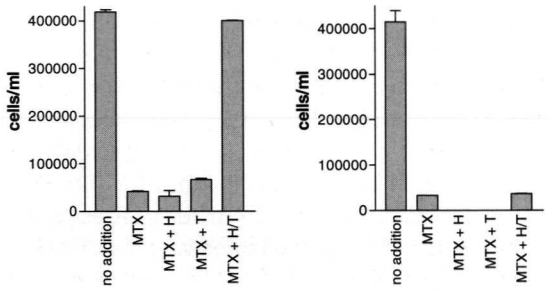

Figure 1. **Proliferation of cells in the presence of MTX.** B9 (A) and B9.H (B) cells (25000 cells/ml) were incubated with 1µg/ml MTX, MTX and 100µM hypoxanthine (MTX + H), MTX and 25µM thymidine (MTX + T), or MTX, hypoxanthine and thymidine together (MTX + H/T), as indicated on the x-axis. Cells were counted at day 3. This is a representative of several experiments performed. Error bars indicate the SEM of duplicate cultures.

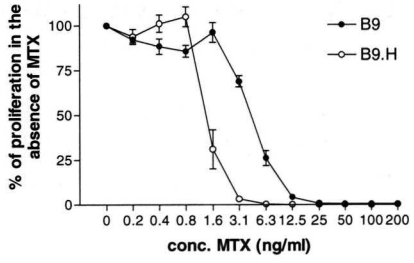

Figure 2. **Sensitivity of B9 and B9.H to MTX.** B9 (closed circles) and B9.H (open circles) cells were cultured in the presence of the indicated amounts of MTX. At day 3 [^{3}H]- thymidine was added for 4 hours and cells were harvested. Error bars represent the SEM of 2 separate experiments. Data are represented as the % of the value (cpm) without MTX.

Effects of MTX on HGPRT negative cell line.

To circumvent this difficulty we decided to use B9.H, a subclone of B9 that is negative for the enzyme HGPRT, an enzyme of the salvage pathway of nucleotide synthesis. As expected, the proliferation of this cell line could not be rescued by the addition of hypoxanthine and thymidine (figure 1b). Indeed we found that B9.H is more sensitive to MTX than the parental B9 (figure 2).

Recovery of MTX after heating.

Before testing patient sera, the recovery of MTX in different individual sera was investigated. Sera of 5 normal donors were spiked with 200 nM, 20 nM and 5 nM MTX

and heated for 30 minutes at 56°C to avoid complement-induced toxicity to the murine B9.H cells. The concentration of MTX was determined with the bioassay as described in the materials and methods section. The recovery of MTX in these sera is close to 100% (table 1). Two sera spiked with 5 nM were excluded because at a serum concentration of 25%, these sera proved to be toxic for the cells without addition of MTX. As this serum toxicity at high serum concentrations is a frequent finding this could limit the sensitivity of the assay. When full sensitivity is required we can eliminate toxicity of serum by boiling samples for 10 minutes. All (toxic) proteins are precipitated with this procedure, and, if needed, serum can be tested at 1:2 dilution. To asses the effects of this boiling procedure, various concentrations of MTX were added to 1ml HPS. We boiled 0.5 ml for 10 minutes and heated 0.5 ml at 56°C for 30 minutes. Precipitated proteins were spun down and in the supernatant, MTX was measured with the bioassay. As expected boiling did not affect MTX activity (figure 3).

Table 1	Sera spiked with MTX (nM)		
input	measured (mean)	SEM	n
200	208	4.7	5
20	24.1	0.3	5
5	5.96	0.4	3

Table 1. **Sera spiked with MTX.** Human sera were spiked with the indicated concentration of MTX and heated at 56°C for 30 minutes. MTX concentration was determined using the bioassay.

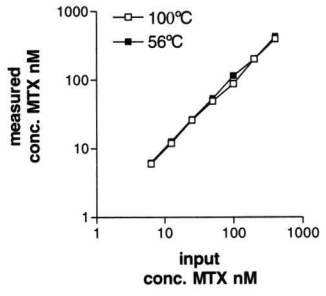

Figure 3. **Recovery of MTX after heating.** B9.H cells were cultured in the presence of heated sera containing different amounts of MTX. MTX was added to the sera before heating. The input is plotted against the values measured.

MTX measurement in sera from RA patients.

MTX was measured in 23 sera of RA patients. Sera were collected from these patients 2 hours after their first oral dose of MTX. The concentration MTX measured in the individual sera ranged from 32 to 269 ng/ml. The results of two independent measurements are plotted against each other in figure 4. The mean of the first measurement was 315 nM (SEM 25) and the second 270 nM (SEM 25).

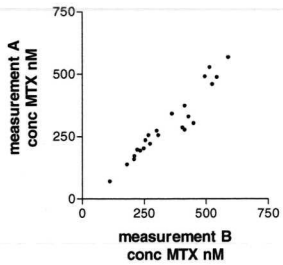

Figure 4. **MTX measurements in serum of RA patients.** Two hours after an oral dose of 10 mg MTX, serum MTX concentration of 23 RA patients was determined in two separate experiments using the bioassay.

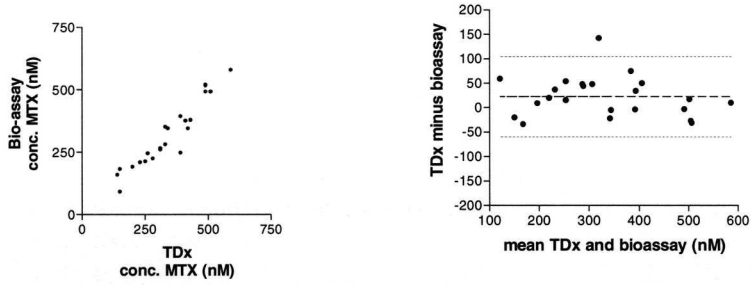

Figure 5. **Comparison of bioassay and TDx assay.** Two hours after an oral dose of 10 mg MTX, serum MTX concentration of 23 RA patients was determined using the bioassay and the commercially available TDx assay (Abbott). The results are also depicted in an Altman and Bland plot. Dotted lines indicate 2xSD from the mean difference (striped line).

Comparison of bioassay with TDx assay.

To confirm the results obtained with the bioassay, we have compared our results with the results of the frequently used and commercially available TDx assay. All 23 RA sera that were tested in the bioassay were send to a facility were MTX concentration in serum is routinely measured with the TDx assay. The outcome of both measurements is depicted in figure 5a. With the TDx assay the mean MTX concentration was 343 nM (SEM 26) and with the bioassay the mean concentration was 290 nM (SEM 25). The Altman and Bland plot (fig 5b) shows that there is reasonable agreement between the methods and that the difference between the two is not dependent of the actual MTX concentration.

Effect of MTX polyglutamation on proliferation

In the cell the majority of the MTX is polyglutamated. We have tested the effects of polyglutamated forms of MTX on the proliferation of B9.H cells. Of the polyglutamated forms tested, only MTX-glu2 is capable of inhibiting B9.H proliferation but at approximately 5-fold higher concentrations than MTX (figure 6). The activity of the MTX-glu4 and -glu5 forms is 400-fold lower.

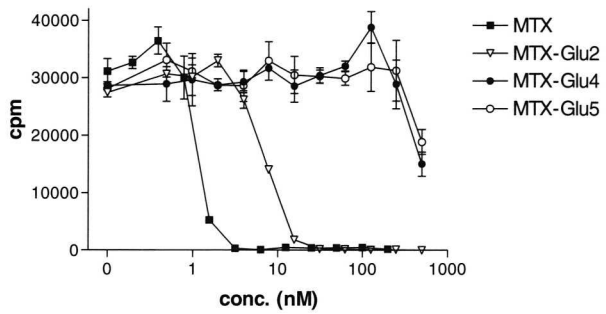

Figure 6. **Effect of MTX polyglutamation on proliferation.** Concentration curves of MTX and MTX-glu2, -glu4 and –glu5 were added to B9.H cells. At day 3 [^{3}H]- thymidine was added for 4 hours and cells were harvested. Error bars represent the SEM of duplicate cultures.

Discussion

For the measurement of MTX in human serum we have developed a bioassay. This assay makes use of mouse B9 cells that are negative for the enzyme HGPRT (B9.H); proliferation of these cells is measured by the incorporation of radiolabelled thymidine. MTX present in serum samples inhibits proliferation of B9.H cells. HGPRT-negative B9.H cells cannot use the salvage pathway of nucleotide synthesis. Consequently, the presence of nucleotides, either present in FCS, released by dying cells in the culture, or radiolabelled thymidine, can not disturb the assay. In addition, this clone proved to be more sensitive to MTX. Very likely the absence of HGPRT accounts for the increased sensitivity of B9.H cells compared to B9 cells. It has been reported that certain HGPRT-negative cells, derived from the cell line HL-60, are 3 times more sensitive to the effects of MTX than wild type cells (24).

Several groups have studied the pharmacodynamics of MTX for different reasons (12-15;26-31). Many have used the commercially available TDx method for measurement of MTX in plasma (1213;14;28-31). Although MTX dose and administration vary between the studies the reported mean plasma levels measured in the first two hours are similar. However, the half life of MTX that is reported is more variable, individuals that have a half life of more than 30 hours are reported (13,29). Several hours after intake of MTX an inactive metabolite, 7-OH-MTX, rises in plasma. This metabolite can cross react with the TDx assay. Although cross reactivity is reported to be lower than 1.5% (13) this can present a problem when MTX is measured at levels are around the sensitivity limit of the assay, for example at 24 hours after oral intake. A late time point, such as 24 hours, is especially important for the calculation of half-lives. It is possible that interference of 7-OH-MTX in the assay can be the reason for the discrepancy in half-lives that are reported in the studies mentioned before. The advantage of measurement of MTX with the bioassay is that only functional MTX can be measured. These measurements are therefore independent of differences in MTX metabolism of patients.

MTX serum levels were measured with the bioassay in 23 patients, two hours after they received their first oral dose of 10 mg MTX. These levels correlate well with the levels measured with the TDx assay. The mean MTX concentration we have measured lies within the reported range of MTX levels described by others (30,31).

With this bioassay it is possible to measure low amounts of MTX in serum especially after boiling of the serum samples. Here we showed that a spiked 5 nM could be recovered by testing the sample at a1:4 dilution. The EMIT has a sensitivity of 200 nM, the HPLC method of 30 nM and the TDx assay of 10 nM (16). The radio-ligand binding assay reaches a sensitivity of 1 nM (15,20) and is slightly more sensitive than the bioassay. The enzymatic assay has a comparable sensitivity (3 nM) (17). To achieve maximal sensitivity, without possible serum effects on the viability of B9.H cells, it is best to boil all serum samples before use. This results in precipitation of all serum proteins and allows testing in a 1:2 serum dilution shifting the theoretical detection limit to 2.5 nM. A concentration method as described by Sinnet et al. is also suitable for use in the bioassay (12). Concentration of the sample will lead to a further lowering of the detection limit.

In table 1 we reported about toxicity of serum even in the absence of MTX. These effects of were only observed at serum concentrations of more than 12.5% of the total culture volume. In this case it was known that the samples did not contain MTX, hence the observed toxicity was due to other factors. Occasionally it can be questionable whether toxicity is induced by contaminated serum or MTX. In that case it is possible to discriminate between the two because inhibition by MTX can be reversed by addition of folinic acid (32).

Folate supplementation is given to RA patients treated with MTX to reduce MTX related toxicity. In most cases folic acid is given (30,33;34). After 48 weeks of MTX therapy with folic acid supplementation mean total folate levels in serum rise to 59.7 nM (34). Folic acid, up to 90 µM, does not have an effect on proliferation of B9.H cells, nor on MTX induced inhibition of proliferation (not shown). Folinic acid supplementation is exceptional. However, folinic acid can prevent MTX induced inhibition of proliferation of B9.H cells at 0.32 µM, these concentrations are probably not reached in plasma after a weekly oral dose of 2.5 mg (34). Therefore, it is unlikely that folate supplementation of patients interferes with the bioassay. The bioassay described here is a simple and sensitive assay to measure MTX in serum and can be performed using techniques which are available at many rheumatological or immunological laboratories. Although thorough validation is necessary, this assay might be useful to study the pharmacodynamics of MTX in RA patients. Only future clinical studies will show if it has a place in routine monitoring.

Acknowledgements.

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Chapter 7

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

A.H. Gerards, S de Lathouder, E.R. de Groot, B.A.C. Dijkmans and L.A. Aarden

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 asses 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.

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% CO_2 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

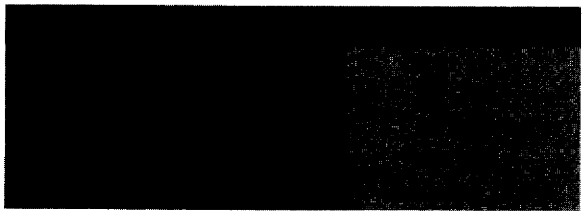
MTX was measured in a bioassay as previously described (13). This bioassay uses inhibition of proliferation of B9.H cells as a measure for MTX concentration in serum. B9.H cells cultured in the presence of a dilution range of MTX (from 100 ng/ml to 0.1 ng/ml, Lederle, Hoofddorp, the Netherlands) was 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) (figure1). 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 *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 *ex vivo* T cell activation was observed.

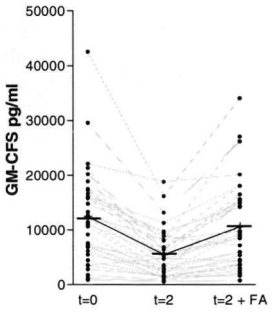


Figure 1. **Decrease in cytokine production 2 hours after MTX intake.** GM-CSF production, before (t=0) and after (t=2) MTX intake, was measured in WB as described. Folinic acid (FA) was added to the WB cultures at t=2. GM-CSF production of each patient is depicted. Mean (SD) production 12 (8.7) ng/ml 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 plus FA is not significant (P=0.27, Mann-Whitney).

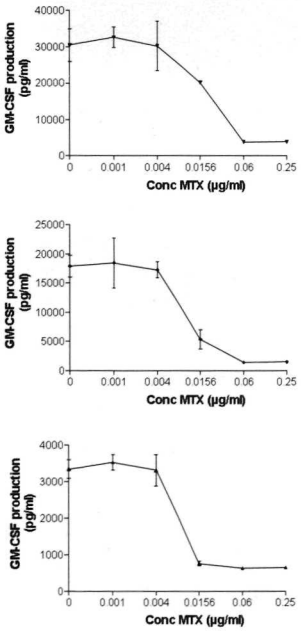


Figure 2. **Examples of the sensitivity to MTX (AUC).** For each patient GM-CSF production was measured in WB in the presence of various concentrations of MTX. The area under the curve (AUC) is calculated from these graphs. 3 examples are shown, panel a: a high AUC value (24.1), panel b: an average AUC value (14.8) and panel c: a low AUC value (9.45). A high AUC value corresponds with a low sensitivity to MTX *in vitro* and vice versa. Error bars indicate the SEM of duplicate measurements.

Table 2.	Clinical response				
	DAS28	TJC	SJC	VAS	BSE
Mean score baseline (± SD)	6.1 ± 1.1	12.4 ± 6.7	10.6 ± 5.1	53.0 ± 24	45.1 ± 29
Mean score 12 weeks (± SD)	4.9 ± 1.4	7.3 ± 5.4	7.2 ± 5.1	41.4 ± 29	30.5 ± 22
Mean improvement	1.2	5.1	3.4	11.7	14.6

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.

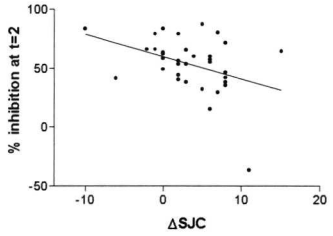


Figure 3. **Ex vivo effect of MTX correlates with a change in swollen joints.** The ex vivo effect of MTX is depicted by the percentage inhibition of GM-CSF production at t=2 on the y-axis. The change (Δ) in SJC is depicted on the x-axis, a positive value represents a clinical improvement or a decrease in the number of swollen joints ($P<0.05$ and Spearman $r = -0.38$).

Table 3.	Relation of cytokine production to clinical response					
		ΔDAS	ΔTJC	ΔSJC	ΔVAS	ΔBSE
ex vivo T cell activation	P value	0.051	0.088	0.026	0.358	0.387
	Spearman r	-0.34	-0.3	-0.38	-0.16	0.15
AUC	P value	0.864	0.341	0.986	0.711	0.723
	Spearman r	-0.03	-0.17	0	0.07	0.06

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.

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 *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 IFNγ, 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 folinic 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 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 *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.

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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.* hypothesise 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-cAdo, an adenosine analogue did inhibit production of IL-8 (chapter 4). Inhibition of IL-8 by cAdo 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-cAdo.

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 Ede 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 to 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 deletion 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 (T_{reg} 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+ T_{reg} cells (37). T_{reg} 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). T_{reg} cells are able to suppress proliferation of the whole CD4+ population or CD4+/CD25- cells (41-45). When CD4+ cells are depleted logically T_{reg} cells are also depleted. Although it is clear that inflammation suppression by T_{reg} 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 effcts 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. Furtheremore knowlage of whether pharmacodynamic variables are responsible for the variable response to MTX treatment in RA patients should be studied.

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Chapter 9

Summary

Rheumatoid arthritis (RA) is a chronic autoimmune disease, characterised by inflammatory reactions in the joints. In the Netherlands, 1-2% of the population suffers from RA and females are affected more often than males.
There are several cells that play a role in the inflammatory process. Synovial fibroblasts and macrophages in the joints are probably responsible for damage to these joints. Their actions can be coordinated by T-cells. The cells of the immune system communicate through interaction with molecular structures on the cell surface and by the production of cytokines. Cytokines are small proteins with a multitude of functions. Each cytokine has several functions, and several cytokines have similar functions. Some cytokines can stimulate cells to grow, to divide or to produce cytokines. Other cytokines inhibit cell growth and cell division. Predominantly pro-inflammatory cytokines are detected in the inflamed joints of RA patients.
Methorexate (MTX) is often used for treatment of RA patients. MTX was developed as an anti-cancer drug and has been used by RA patients, in low doses, since the eighties. Although clinically most patients respond positively to MTX, a lot of them discontinue treatment due to side effects. Some patients do not respond to MTX therapy.
MTX is a folate antagonist. The folate metabolism is important for many processes in the cell, such as methionine metabolism and purine and pyrimidine synthesis, necessary for synthesis of DNA and RNA. MTX's in RA patients is not completely known, although there are several theories. MTX can induce adenosine, which has an anti-inflammatory effect, especially on macrophages. Cronstein thinks the induction of adenosine is the main mechanism of MTX's working mechanism. It has been suggested that the T-cell is important for MTX to be effective, because MTX seems to induce apoptosis in T-cells, or, as others observe, halts T-cell growth.
Experiments described in this thesis were performed to increase our knowledge of the working mechanism of MTX. A better insight is important for the improvement of efficacy of MTX treatment and for the reduction of side effects.

A general introduction (**chapter 1**) is followed by a description of the in vitro effects of MTX in whole blood (**chapter 2**). MTX inhibits the production of all cytokines that are produced after stimulation of cells with anti-CD3/anti-CD28, with the exception of the production of interleukin (IL) -8. The inhibition caused by MTX can be arrested if folinic acid is added. MTX will not inhibit the production of cytokine if it is stimulated with bacterial products (SAC and LPS). We will show that the usage of MTX by RA patients causes an inhibition of their cytokine production. Evidence that this inhibition is caused by MTX is based on the observation that cytokine production is continued after folinic acid is added to the cell culture.
Chapter 3 studies the mechanism of inhibition of cytokine production by MTX. MTX is compared to mycophenolic acid (MPA), a medicine that is able to inhibit DNA synthesis. Like MTX, MPA inhibits the production of cytokine in whole blood after stimulation with anti-CD3/anti-CD28. MPA inhibits the production of cytokine slightly better than MTX does and, unlike MTX, it also inhibits IL-8 production. Inhibition of cytokine production by MPA is more rapid than inhibition by MTX. MTX inhibits cytokine by inducing apoptosis in the proliferating cells. MPA does not induce apoptosis, but stops the progression of the cell cycle and thus halts proliferation (and cytokine production).

As a result of these findings, we think that MTX kills proliferating T-cells and consequently, has an immunosuppressive effect. To investigate whether MTX works by increasing adenosine levels, we showed that MTX, in vitro, works differently than an adenosine analogue (cAdo) **in chapter 4**. Unlike than MTX, cAdo inhibits IL-8 production. IL-8 is created by monocytes and probably by granulocytes, not by T-cells. cAdo also differs from MTX in its feature to inhibit cytokine production after LPS stimulation.

While comparing studies on MTX and MPA, we deduced that MPA had an effect on the production of monokines. This was further investigated in **chapter 5**. MPA indeed inhibits the production of monokines IL-6, IL-8 and TNF, but stimulates the production of IL-1β. A higher expression of IL-1β mRNA in the presence of MPA is not the cause of this stimulation. Because MPA decreases proIL-1β levels in the cells, it seems that MPA stimulates conversion from proIL-1β to IL-1β. If the induction of IL-1β is present *in vivo*, and whether this has clinical consequences, needs to be examined further.

A new method for detecting MTX and bioactive MTX in serum is described in **chapter 6**. This method is more sensitive than current popular methods and is easy to perform in immunological/rheumatological laboratories, without any special equipment.

For treatment of RA patients it is important to asses the efficacy of treatment at an early stage. In **chapter 7** we correlated the in vitro effects of MTX with the clinical response of 34 RA patients. Blood samples were taken from these patients just before (t=0) and two hours (t=2) after they had taken MTX for the first time. The concentration of MTX was determined in t=2 samples. The t=0 samples were also used to determine the *in vitro* sensitivity of MTX. Cytokine production after T-cell stimulation was determined in each sample. The production of cytokine was significantly lower after usage of MTX than before. Unfortunately, *in vitro* sensitivity, cytokine production (before and after usage of MTX) and the plasma MTX concentration were not predictive in to the clinical response on MTX treatment. Because the clinical response was measured after 12 weeks, clinical improvement was low in this patient group. However, a negative relationship was found between the amount of inhibition of cytokine production after 2 hours and the decrease of swollen joints (SJC). This means that patients who show little inhibition of cytokine production at t=2 have a larger decrease of swollen joints. Nevertheless, this relationship is weak and possibly dependent on one single patient with a good clinical response and a higher cytokine production at t=2. More patients should be tested in order to verify this relationship. If true, we could explain such a relationship by stating that MTX also inhibits anti-inflammatory cytokines such as IL-10.

Chapter 8 of this thesis is a general discussion of the results presented in this thesis. Both MTX and MPA inhibit cytokine production of T-cells, although through different mechanisms. Unlike MTX, MPA has an effect on monocytes. We found a significant inhibition of LPS- induced and SAC-induced cytokine production and a stimulation of LPS-induced IL-1β production by MPA.

In the discussion, we have tried to establish what would be the mechanism of action of MTX when used to treat RA patients. Results of the in vitro tests suggest that the T-cell is the main target of MTX. However, this idea might not be correct since there is no relation between the *in vitro* and *ex vivo* effects of MTX on the T-cell cytokine production of RA patients and their clinical responses. Hence, we need to look for cells and cytokines that may be sensitive to MTX, as well as pharmacological-dynamic parameters that may be related to the variable clinical responses of RA patients.

Chapter 10

Samenvatting

Reumatoïde artritis (RA) is een chronische auto-immuun ziekte die wordt gekenmerkt door ontstekingreacties in de gewrichten. In Nederland komt RA bij 1-2% van de bevolking voor, vaker bij vrouwen dan bij mannen.
In het ontstekingsproces spelen verschillende cellen een rol. De synoviale fibroblasten en macrofagen die zich in het gewricht bevinden zijn waarschijnlijk verantwoordelijk voor de schade aan de gewrichten. Deze cellen kunnen worden aangestuurd door de T-cellen. De cellen van het immuunsysteem communiceren met elkaar door interactie van moleculen op het cel oppervlak en door de productie van cytokinen. Cytokinen zijn kleine eiwitten met veel uiteenlopende eigenschappen; elk cytokine heeft verschillende functies en verschillende cytokinen hebben dezelfde functie. Er zijn cytokinen die cellen aanzetten tot celdeling en tot productie van cytokinen. Andere cytokinen kunnen de celdeling juist afremmen. Bij RA patiënten worden er in het ontstoken gewricht vooral cytokinen aangetroffen die de ontsteking bevorderen.
Een veel gebruikt medicijn voor de behandeling van RA is methotrexaat (MTX). MTX is ontwikkeld als antikanker middel en wordt sinds de jaren tachtig, in lage dosering, gebruikt door RA patiënten. Bij de meeste patiënten verbetert het klinische beeld na behandeling met MTX maar toch moeten veel patiënten stoppen vanwege de bijwerkingen. Ook zijn er patiënten bij wie geen klinische verbetering optreed na behandeling met MTX.
MTX is een folaat antagonist. Het folaat metabolisme is bij veel processen in de cel betrokken, zoals bij purine en pyrimidine synthese, bij de synthese van DNA en RNA, en bij het methionine metabolisme. Het werkingsmechanisme van MTX bij reuma is nog niet helemaal bekend, al zijn er verschillende theorieën. MTX kan adenosine induceren. Adenosine heeft een ontstekingsremmende werking, vooral op macrofagen. Cronstein heeft gepostuleerd dat de inductie van adenosine het belangrijkste mechanisme is voor de ontstekingsremmende werking van MTX. Anderen denken dat de T cel belangrijk is voor de werking van MTX omdat ze zien dat T-cellen in apoptose (celdood) gaan door MTX terwijl weer anderen vonden dat alleen de groei van T-cellen geremd wordt.
Met het onderzoek beschreven in dit proefschrift hebben we geprobeerd meer inzicht te krijgen in werkingsmechanisme van MTX. Dit is noodzakelijk om de behandeling effectiever te maken de bijwerkingen te verminderen.

Na een algemene inleiding (**hoofdstuk 1**) worden in **hoofdstuk 2** de *in vitro* effecten van MTX in volbloed beschreven. MTX remt de productie van alle cytokinen die na stimulatie met anti-CD3/anti-CD28 door de cellen geproduceerd worden, met uitzondering van de productie van interleukine (IL)-8. De remming door MTX is op te heffen door toevoeging van folinezuur. Na stimulatie met bacteriële producten (SAC en LPS) remt MTX de cytokine productie niet. Ook laten we zien dat de cytokine productie van RA patiënten na het innemen van MTX geremd is. Een bewijs dat deze remming door MTX wordt veroorzaakt wordt gegeven doordat de remming van cytokine productie opgeheven wordt als folinezuur aan de celkweek wordt toegevoegd.
In **hoofdstuk 3** wordt bestudeerd wat het mechanisme is van de remming van cytokine productie door MTX. MTX wordt hier vergeleken met mycofenolzuur (MPA) een medicijn dat ook DNA synthese kan remmen. Net als MTX remt ook MPA de cytokine productie in volbloed nadat er gestimuleerd is met anti-CD3/anti-CD28. MPA remt de cytokine productie iets beter dan MTX en in tegenstelling tot MTX remt MPA ook de IL-8 productie. Verder blijkt dat MPA eerder remt dan MTX. De cytokine remming door MTX wordt

veroorzaakt doordat MTX apoptose induceert in de cellen die prolifereren. MPA induceert geen apoptose, maar stopt de voortgang van de celcyclus, waardoor de cellen niet tot proliferatie (en cytokine productie) komen.
Naar aanleiding van de bovenstaande resultaten denken we dat MTX prolifererende T-cellen doodt en daardoor immunosuppressief werkt. Om te onderzoeken of MTX werkt via verhoging van adenosine laten we in **hoofdstuk 4** zien dat MTX, *in vitro*, anders werkt dan een adenosine analoog (cAdo). In tegenstelling tot MTX remt cAdo namelijk wel de IL-8 productie. IL-8 wordt niet door T-cellen gemaakt, maar door monocyten en waarschijnlijk ook door granulocyten. Een ander verschil met MTX is dat cAdo ook de cytokine productie na LPS stimulatie remt. Hiermee tonen we aan dat de remming van de cytokine productie door MTX anders verloopt dan bij adenosine en dat adenosine en MTX, in vitro, een verschillende werking hebben.
Tijdens onze vergelijkende studies van MTX en MPA stelden we vast dat MPA een effect heeft op monokine productie. Dit is verder onderzocht in **hoofdstuk 5**. MPA remt de productie van monokines IL-6, IL-8 en TNFα maar stimuleert IL-1β productie. Deze stimulatie wordt niet veroorzaakt door een hogere expressie van IL-1β mRNA in aanwezigheid van MPA. Omdat proIL-1β uit de cellen verdwijnt onder invloed van MPA, lijkt het er op dat MPA het knippen van proIL-1β naar IL-1β stimuleert. Of het induceren van IL-1β in vivo gebeurt en of dit klinische consequenties heeft moet nog onderzocht worden.
Om MTX in serum van patiënten te kunnen meten beschrijven we in **hoofdstuk 6** een nieuwe methode voor detectie van MTX in serum. Een nieuw aspect van deze methode is dat bioactief MTX gemeten kan worden. De methode is gevoeliger dan de meest gebruikte methoden en makkelijk uit te voeren in immunologische /reuma-tologische laboratoria, zonder speciale apparatuur.
Voor de behandeling van RA is het belangrijk er zo vroeg mogelijk achter te komen of het voorgeschreven medicijn werkt of niet. Daarom we hebben in **hoofdstuk 7** gekeken of in vitro effecten van MTX te correleren zijn aan de klinische respons van 34 RA patiënten. Direct vóór (t=0) en 2 uur (t=2) nadat de patiënt voor het eerst MTX heeft ingenomen is er bloed afgenomen. In dit bloed is de concentratie MTX gemeten. Bovendien is in het t=0 monster de *in vitro* gevoeligheid voor MTX bepaald. Tenslotte is in beide monsters de cytokine productie na T cel activatie gemeten. De cytokine productie na inname van MTX is significant lager dan ervoor. Helaas bleken *in vitro* gevoeligheid, cytokine productie voor en na MTX inname en de plasma MTX concentratie niet voorspellend voor de klinische respons op de behandeling. Omdat de klinische respons al na 12 weken is gemeten, is de verbetering bij de meeste patiënten nog erg klein. Er is wel een negatieve relatie gevonden tussen de mate van inhibitie van cytokine productie na 2 uur en de vermindering van gezwollen gewrichten (SJC). Dat wil zeggen dat de patiënten die weinig remming van cytokine productie vertonen op t=2, een grotere afname van gezwollen gewrichten hebben. De gevonden relatie is echter zwak en mogelijk afhankelijk van één patiënt met een goede klinische respons en een verhoogde cytokine productie op t=2. Meer patiënten zouden getest moeten worden om te verifiëren of deze relatie zal blijven bestaan. Als dat zo is dan kunnen we de gevonden relatie uitleggen doordat MTX ook anti-inflammatoire cytokinen, zoals IL-10, kan remmen.

In de algemene discussie (**hoofdstuk 8**) van dit proefschrift wordt ingegaan op de behaalde resultaten. Zowel MTX als MPA remt de cytokine productie van T-cellen, al gebeurt dit via verschillende mechanismen. In tegenstelling tot MTX heeft MPA wel effect op monocyten. We vonden een significante remming van LPS- en SAC-geïnduceerde cytokine productie en een stimulatie van de LPS-geïnduceerde IL-1β productie.
Verder gaat de discussie in op de vraag wat het werkingsmechanisme van MTX is bij RA patiënten. De resultaten van de *in vitro* proeven geven aan dat de T cel het belangrijkste doelwit zou zijn van MTX. Maar dit idee is misschien niet correct. Er blijkt namelijk geen relatie te bestaan tussen de *in vitro* en *ex vivo* effecten van MTX op T cel cytokine productie van RA patiënten en hun klinische respons. Daarom dient er verder gezocht te worden naar de rol van andere cytokinen en andere cellen die mogelijk gevoelig zijn voor MTX en naar farmacodynamische parameters die betrokken kunnen zijn bij de variabele klinische respons in RA patiënten.

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Sacha

Curriculum vitae

De auteur van dit proefschrift werd geboren op 5 januari 1975 te Amsterdam. Na het behalen van het VWO diploma heeft zij een half jaar in Spanje gewoond. In 1994 is zij begonnen met de studie Medische Biologie aan de Universiteit van Amsterdam. Tijdens de specialisatie fase verrichte zij onderzoek onder leiding van Dr. E. Wierenga naar de regulatie van de IL-12 receptor β2 keten op de afdeling celbiologie en histologie van het AMC in Amsterdam. Tijdens haar tweede stage deed ze onderzoek naar de 'Fas signal transduction pathways' op het CLB onderleiding van Dr. K. Tesselaar en prof. R van Lier. Na het behalen van het doctoraal examen werd in 1999 het onderzoek gestart dat resulteerde in het proefschrift dat nu voor u ligt onder begeleiding van prof. L Aarden bij de afdeling immunopathologie van Sanquin Research (voorheen het CLB). Sinds enkele maanden is zij in opleiding tot klinisch chemicus in het Reinier de Graaf gasthuis in Delft.


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