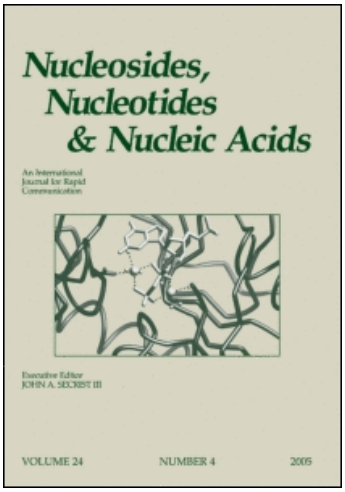

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Two Inhibitors of DNA-Synthesis Lead to Inhibition of Cytokine Production via a Different Mechanism

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ABSTRACT

Methotrexate (MTX) and mycophenolic acid (MPA) are used in the clinic for their immunosuppressive properties. MTX is widely used for the treatment of rheumatoid arthritis (RA). MPA is used to prevent graft rejection and is now experimentally used in systemic lupus erythematosis and RA. It is known that both drugs interfere with DNA synthesis. However, the precise mechanism of action is still debated. We have analysed the effect of the drugs on cytokine production in whole blood during short cultures. The production of T-cell cytokines was inhibited by both drugs. MTX inhibits cytokine production because MTX induces apoptosis in activated T-cells. MPA inhibits cytokine production by preventing T-cells to progress to the S-phase of the cell cycle. Cytokine production by monocytes was slightly decreased by the drugs. The reason for this inhibition is not clear. These results indicate that T-cells are the main target cells of the immunosuppressive drugs MPA and MTX.

Key Words: Methotrexate; Mycophenolic acid; Cytokines; Lymphocytes; Rheumatoid arthritis.

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INTRODUCTION

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

In the inflamed joint the synovial membrane is thickened and invaded with cells (macrophages and T-cells), the synovial fluid is invaded with neutrophils, cartilage is destructed by proteolytic enzymes produced by all these cells, and in progressed stages also bone is destructed. This eventually leads to deformity and disability which is observed in RA patients.

Cytokines are important mediators in the immune system. They are secreted by all immune cells and regulate growth, proliferation, activation, differentiation and they can have chemotactic properties.[2] Various cytokines have similar biological effects, and at the same time a single cytokine can have different effects on different target cells Cytokines act through binding of 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.

To give a complete overview of the cytokine network involved in the pathogenesis of RA is beyond the scope of this article. A brief description of important cytokines in RA will be given. TNFα and IL-1β are considered to be the main cytokines responsible for the pathology in RA but many other cytokines also play a role. These cytokines can de detected in the synovial fluid. TNFα is produced by macrophages, monocytes, and T-cells. Its actions are pro-inflammatory. TNFα is a potent inducer of other cytokines such as IL-1, IL-6, and IL-8. TNFα can also stimulate fibroblasts to express adhesion molecules and perhaps indirectly activates osteoclasts that are responsible for bone degradation (for references see Ref. [3]). A dominant role of TNFα in the pathology of RA is likely since therapeutic blockade of TNFα results in clinical improvement.[4,5]

IL-1β is also produced by macrophages and monocytes. It is implicated in joint destruction, it can stimulate the release of matrix metalloproteinases from chondrocytes and fibroblasts (reviewed in Ref. [6]). Like TFNα, IL-1β can induce arthritis in animal models and intervention with IL-1β signaling can reduce arthritis in collagen-induced arthritis models.[6,7]

MTX

Methotrexate (MTX) is a folate antagonist, it was developed together with several other anti-folates more that 50 years ago for the treatment of malignancies (reviewed in Ref. [8]). MTX enters the cell predominantly via the reduced folate carrier, and can also enter via the folate receptor.[9,10] MTX proved to be an inhibitor of dihydrofolate reductase (DHFR).[11] Like other folate forms MTX is polyglutamated in the cell by folylpolyglutamyl synthetase. Intracellular polyglutamation leads to an increased inhibition of several other enzymes.[12] Inhibition of thymidine synthetase,[13] amino-imidazolcarboxamide ribosyl-5-phosphate (AICAR) transformylase,[14,15] and amido-phosphoribosyltransferase[16] has been described.

MTX was developed more than 50 years ago for the treatment of malignancies.[8] In 1951 the related drug aminoptherin was found to inhibit cell proliferation in psoriasis and RA.[17] But it was not until the 1980s that MTX has proved its efficacy in several placebo controlled trials.[18–21] In RA, MTX is administered weekly in low doses.

The mechanism of action of the low dose administration in RA is debated. Cronstein et al. argue that MTX works via the increase of extracellular adenosine which is secreted by lymphocytes as a result from MTX-induced accumulation of purine intermediates (reviewed in Ref. [22]). In mouse models for arthritis this hypothesis is confirmed by the fact that MTX can inhibit the leukocyte infiltration at inflamed sites[23] and the combination of two non selective adenosine receptor inhibitors theophilline and caffeine reversed the therapeutic effect of MTX.[24] Indeed adenosine has anti-inflammatory properties. In human whole blood it inhibits neutrophil degranulation[25] and in synovial fluid from RA patients high concentrations of adenosine correlated with poor apoptosis induction in neutrophils.[26] Induction of IL-10 production and inhibition of TNFα production are also reported.[27,28] IL-10 is considered an anti-inflammatory cytokine since it can inhibit the production of cytokines such as IL-1β and TNFα.

There are also indications that adenosine does not mediate the effects of MTX in RA. Adenosine receptor antagonists enhanced the beneficial effects of MTX in an antigen induced arthritis model.[29] In addition, plasma levels of adenosine were not increased in RA patients up to 7 days after treatment with MTX, whereas uridine, hypoxanthine, and uric acid were decreased.[30]

In contrast to the adenosine hypothesis, Genestier et al. observed apoptosis induction by MTX in activated lymphocytes in vitro.[31] Adenosine did not induce apoptosis in these experiments, nor did adenosine deaminase (ADA) inhibit MTX induced apoptosis.[31] In the mouse in vivo as well as in vitro activation-dependent apoptosis by MTX was also observed.[32,33] We observed that MTX is a potent inhibitor of TNFα production in T-cells. Probably this inhibition is a result of apoptosis that is induced by MTX.[34,35]

MPA

Mycophenolic acid (MPA) is an inhibitor of inosine monophosphate dehydrogenase (IMPDH) a rate limiting enzyme in the guanosine synthesis.[36] MPA has proven to be an effective drug to prevent transplant rejection after renal transplantation[37,38] and is now experimentally used for the treatment of RA.[39,40] In the mouse, in vitro as well as in vivo, MPA inhibited TNFα and IFNγ production but it did not affect IL-6 production.[41] Furthermore, no effect on IL-2 and IL-10 mRNA expression was seen after ConA stimulation of mouse cells.[42] In human T-cells, MPA inhibits superantigen-induced cytokine production whereas it had no effect on LPS- or mitogen-induced cytokine production.[43] However MPA inhibited the production of all cytokines after T-cell receptor stimulation.[35] It has been reported that MPA blocked lymphocyte proliferation at the G1/S transition[44,45] but IL-2 production is not changed.[46,47]

EFFECTS OF MTX AND MPA ON CYTOKINE PRODUCTION IN WHOLE BLOOD

Considering the importance of the cytokine network in RA, our aim was to investigate the effects of MTX and MPA on cytokine production. We started our experiments using a whole blood (WB) culture system. The reason to choose this culture system is that all blood cells are present, and it is therefore a more

physiological system than isolated cell cultures and cell lines. In view of the sensitivity to de novo purine synthesis inhibition, lymphocytes are likely candidates to be affected by the drugs. Therefore we have studied the effects of both drugs on the cytokine production after T-cell stimulation. In most healthy donors high amounts (> 1ng/ml) of IL-2, IL-4, IL-8, IL-13, IFNγ, TNFα and GM-CSF are produced in WB after stimulation with anti-CD3 and anti-CD28 (for T-cell receptor signaling and co-stimulation respectively). IL-1β, IL-6 and IL-12(p40) are produced in low amounts. At clinically relevant concentrations both MTX and MPA inhibit the production of IL-4, IL-13, IFNγ, TNFα and GM-CSF. Production of IL-8, which is chemotactic for neutrophils, is inhibited by MPA but not by MTX (Table 1). Inhibition of cytokine production by MPA was more profound than the inhibition by MTX. Furthermore MPA inhibits cytokine production as early as day 2, whereas inhibition by MTX starts at day 3. Inhibition of cytokine production by MTX could be prevented by addition of hypoxanthine and thymidine or addition of folinic acid to the cell cultures (Fig. 1).

To understand why cytokine production was inhibited we looked for apoptosis in our cultures. Detection of apoptosis by staining of annexin-V to the flip-flopped PS residues on the cell membrane is not possible in WB. Inhibition of cytokine production by the drugs was similar in mononuclear cells (MNC) and in WB. Hence isolated MNC were used for the following experiments. When MNC were stimulated with aCD3/aCD28 and analyzed for the induction of apoptosis by MTX or MPA, we observed that MTX indeed induced apoptosis after 3 days (Fig. 2) as described before.[31,33] The number of apoptotic cells increased after a longer incubation. To our surprise MPA did not induce apoptosis at all (Fig. 2). Cells stimulated in the presence of MPA did not show any sign of proliferation, visible on the FACS forward and side scatter patterns (not shown). Induction of apoptosis by MPA has been reported in cell lines that are continuously proliferating.[48] Indeed when we added MPA 3 days after the stimulus, we also found induction of apoptosis by MPA (not shown).

To further dissect the inhibition of activation by MPA we have determined the expression of activation markers on the cell membrane. CD25 (IL-2 receptor α-chain) and CD69 are activation markers that appear on the cell surface several hours after

Table 1. Cytokine production in pg/ml (SEM).

Cytokine	No drug	MTX	MPA
IL-4	1463 (187)	506 (82)	98 (25)
IL-13	6908 (660)	1017 (155)	46 (8)
IL-8	202585 (23781)	164311 (22082)#	17681 (6394)
GM-CSF	73785 (8186)	4281 (806)	274 (45)
IFN-γ	203267 (25492)	17272 (4228)	963 (202)
TNF-α	3317 (867)	451 (180)	76 (38)

Effect of MTX and MPA on cytokine production by T-cells. 1:10 diluted whole blood (WB) of 8 healthy donors was stimulated with a-CD3/aCD28 with or without MTX or MPA. After 3 days supernatants were harvested and tested for the presence of cytokines by ELISA. Data are presented as mean of 8 donors with SEM. Wilcoxon signed rank test was performed on paired data to test whether differences were statistically significant ($P < 0.05$). MTX and MPA significantly inhibited cytokine production, except for the condition indicated with #.

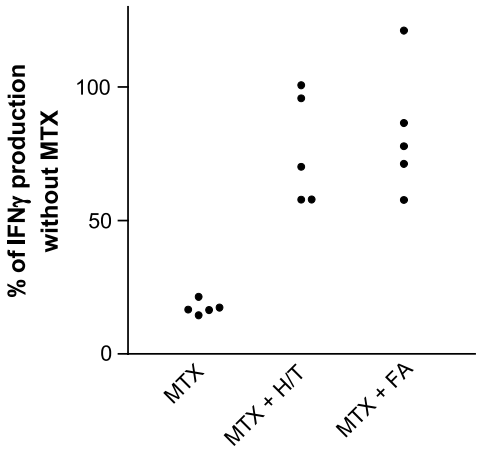

Figure 1. Hypoxanthine and thymidine of folinic acid can prevent MTX-induced cytokine inhibition. WB of 5 healthy donors was stimulated as before. MTX was added in combination with hypoxanthine and thymidine (H/T) or folinic acid (FA). At day 3 supernatants were harvested and tested for the presence of cytokine production by ELISA. The results are presented as the percentage IFNγ production in the absence of MTX. Each dot represents a donor.

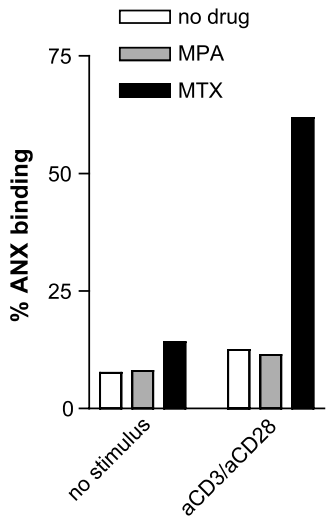

Figure 2. Induction of apoptosis. Freshly isolated mononuclear cells (MNC) were stimulated in the presence or absence of MTX or MPA. At day 4 cells are stained for annexin-V. Staining was analysed on a fluorescence activated cell sorter (FACS). The percentage annexin-V positive cells within the T-cell population (CD2 positive) is depiced on the y-axis. Similar results are obtained in 3 separate experiments. (Published in Ref. [35].)

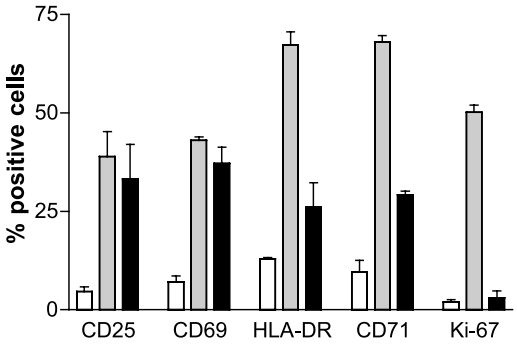

Figure 3. The effect of MPA on activation markers. MNC were not stimulate (white bars), stimulated with anti-CD3/CD28 in the absence (gray bars) or presence of MPA (black bars). Cells were harvested, stained with the appropriate antibodies and analysed on FACS. Expression of CD25 and CD69 was measured after 24 hours. Expression of HLA-DR, CD71 and Ki-67 was measured after 72 hours. The percentage of positive cells is depicted on the y-axis. Similar results are obtained in 3 separate experiments. (Published in Ref. [35].)

CD3/CD28 stimulation. CD71 (tranferrin receptor), Ki-67 and HLA-DR are detectable after more than 24 hours. Expression of CD69 and CD25 was not inhibited by MPA but expression of CD71, HLA-DR and Ki-67 was decreased by MPA (Fig. 3). Furthermore the progression of the cell cycle was analyzed by staining of the DNA content of the cells. Cells stimulated in the presence of MPA did not enter the S-phase of the cell cycle and remained in the G0/1 phase like in stimulated cells whereas stimulated cells did enter the S and subsequent G2/M phase (Fig. 4). It has been described that the effects of MPA can be prevented by addition of guanosine to the cultures. In our experiments the addition of a combination of guanosine and adenosine was required to prevent MPA from working. Because MPA inhibited T-cell activation and did not induce apoptosis we tested whether the actions of MPA were reversible. MNC were stimulated in the presence of MPA for four days. After four days adenosine

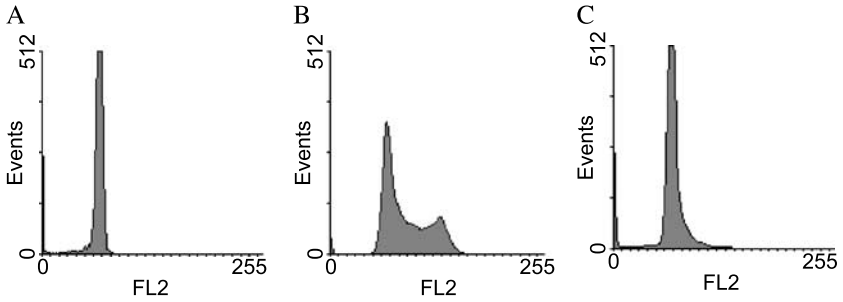

Figure 4. MPA prevents progression to S-phase of the cell cycle. MNC were cultured with nothing (A), anti-CD3/CD28 (B), and anti-CD3/CD28 + MPA (C) for 3 days. Cells were permeabilized and incubated with propidium iodide to stain DNA in the cells. DNA content was analysed on a FACS and is depicted on the x-axis (FL2). Similar results are obtained in 4 separate experiments.

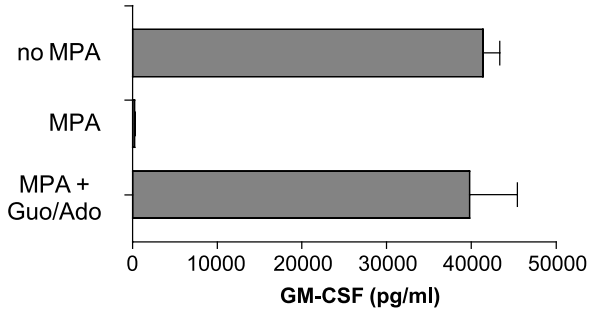

Figure 5. Guanosine and adenosine can reverse the effects of MPA. MNC were stimulated (aCD3/aCD28) for 4 days in the presence or absence of MPA, after 4 days guanosine and adenosine (guo/ado) were added to the cultures containing MPA. 4 days supernatants are harvested and tested for cytokine production. GM-CSF production is depicted on the x-axis. This is a representative of 4 experiments performed. Error bars indicate the SEM of triplicate cultures.

and guanosine were added to relieve the G1 to S block. Again four days later cytokine production was measured (Fig. 5) and proliferation was determined (not shown). After 4 days of incubation with MPA, inhibition of cytokine production and proliferation was reversed by the addition of guanosine and adenosine. This is a confirmation that MPA did not induce apoptosis.

In conclusion, T-cell cytokine production is inhibited by MPA and MTX. However, there are differences between the two drugs. MTX does not inhibit IL-8 production and inhibition by MTX starts at a later time point than MPA does. Inhibition of cytokine production by MTX is caused by the induction of apoptosis in T-cells that are activated by the stimulus. MPA reversibly inhibits activation of the cells by inducing a block before cells enter the S-phase of the cell cyclus.

MONOCYTES

Besides the effects of MTX and MPA on T-cells we investigated the effects on monocyte-derived cytokine production. for that purpose WB was stimulated with bacterial products. Lipopolysaccharide (LPS) from *N. meningitidis* was used for stimulation via Toll like receptor (TLR) 4 and Staphylococcus aureus cells (SAC) were used for stimulation via TLR2.[49] Cytokines produced after 24 hours of stimulation with LPS or SAC are TNFα, IL-1β, IL-6 and IL-8 and to a lesser extent IL-12 p40. The effects of MTX and MPA on the monocyte cytokine production were not as clear as on T-cell cytokine production. However, inhibition by MPA was significant for most cytokines. The results are depicted in Table 2. Interestingly, MPA significantly increased IL-1β production. IL-1β is a cytokine with an abnormal secretion route. It is synthesised as an inactive precursor of 35 kD and it is cleaved by interleukin 1 converting enzyme (ICE or caspase-1) and concomitantly secreted as 17kD active cytokine.[50–52] When monocytes or macrophages are stimulated with LPS a secondary stimulation with ATP is required for optimal processing and secretion[53] of active IL-1β. The purinergic receptor $P2X_7$ is responsible for ATP stimulation of IL-1β

Table 2. Cytokine production in pg/ml (SEM).

	SAC			LPS		
Cytokine	No drug	MTX	MPA	No drug	MTX	MPA
IL-6	3456 (496)	3313 (490)	1938 (423)*	2802 (266)	2435 (230)*	1869 (278)*
TNF	2360 (369)	2084 (256)*	1495 (232)*	543 (88)	431 (29)	308 (40)*
IL-8	28250 (5310)	25440 (4619)	17040 (4299)*	10050 (2372)	8170 (2782)*	5441 (2155)*
IL-1β	4140 (567)	4040 (536)	3917 (484)	1270 (195)	1215 (209)	3299 (365)*

Effect of MPA and MTX on cytokine production by monocytes. 1:10 diluted WB of 8 donors was stimulated with SAC or LPS in presence or absence of MTX or MPA. Supernatants were harvested after 24 hours and tested for cytokine production. The mean cytokine production is depicted. Wilcoxon signed rank test was performed on paired data to test whether differences were statistically significant ($P < 0.05$). *Indicates a significant difference between the sample with and without a drug.

processing and secretion.[54,55] Analysis of IL-1β mRNA expression and analysis of intracellular and extracellular IL-1β protein suggest that MPA activates cleavage of the precursor form, resulting in more (active) IL-1β in the supernatant (manuscript in preparation).

Although the effects of MTX and MPA on cytokine production by monocytes are intriguing we don't know how they are achieved. Monocytes do not proliferate in culture. Therefore the perturbation of DNA synthesis by MTX or MPA is not likely to be as important as in proliferating T-cells. Possibly, other mechanisms are involved. In conclusion our results indicate that T-cells are the main target cells of the immunosuppressive drugs MPA and MTX.

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