

The effects of low-dose methotrexate on thymidylate synthetase activity in human peripheral blood mononuclear cells

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Abstract

Objective

Methotrexate (MTX) in low doses is widely used in the treatment of rheumatoid arthritis (RA) and it is not known whether its effects are due to immunosuppressive and/or anti-inflammatory actions. High concentrations of MTX inhibit the activity of thymidylate synthetase (TS) and dihydrofolate reductase essential for DNA synthesis. This study investigated the effects of low-dose MTX on TS activity and proliferation in human peripheral blood mononuclear cells (PBMC).

Methods

The MTX concentrations in our experiments were chosen according to the plasma concentrations measured in 8 RA patients treated with MTX. The effect of MTX on TS activity and DNA synthesis were measured in stimulated normal PBMC and in PBMC obtained from 6 RA patients treated with oral MTX before and 2 hours after intake of their weekly MTX dose. The effect of MTX on the TS mRNA concentration was also investigated in order to elucidate its effect on TS production.

Results

Low-dose MTX significantly inhibited TS activity and the proliferation of stimulated PBMC independent of the mode of activation. Interestingly, the concentration of TS mRNA in normal PBMC was upregulated by the presence of MTX. Finally, there was no difference between TS activity measured before and after MTX intake in 6 RA patients on long-term MTX treatment.

Conclusion

We show that low concentrations of MTX inhibit TS activity in vitro. An in vivo effect cannot, however, be proven given our study design. The role of these in vitro findings is discussed, particularly in relation to the in vivo effects of MTX.

Key words

Methotrexate, thymidylate synthetase, rheumatoid arthritis.

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Introduction

MTX is well known as an antineoplastic agent in the treatment of various cancers and has strong cytotoxic activity when administered in high doses. Its mechanism of action is mainly due to its inhibitory effect on the dihydrofolate reductase activity and TS activity necessary for DNA synthesis and cell proliferation. In the 1980s treatment with MTX was expanded to include various autoimmune diseases and it soon became the treatment of choice in RA. No other drug used in the treatment of RA, whether administered in high or low doses, is known to inhibit TS activity. Treatment of RA with 5-25 mg of MTX once a week is tolerated relatively well and several long-term studies have demonstrated a sustained clinical response (1, 2).

In spite of the rather successful use of low-dose MTX therapy, its mechanism of action is not completely understood. Thus, it remains a matter of discussion whether the documented beneficial effect of MTX on RA is due to an immunosuppressive/modulatory or rather to an anti-inflammatory action and many conflicting results have been described (2-5). It has been speculated that the known side effects are the results of immunosuppressive actions, whereas the beneficial clinical effects are due to an anti-inflammatory action (3). The latter may be mediated via different mechanisms such as inhibition of the production or activity of inflammatory molecules such as IL-1, IL-6, TNF (6), enhancement of the release of natural cytokine inhibitors (7) or enhancement of adenosine release resulting in the inhibition of neutrophils (3, 8). Other studies have reported the inhibition of polyamine synthesis essential for cell proliferation (9, 10) or of folat-dependent enzymes, such as AICAR, necessary for purine synthesis (11). Recently it was proposed that clonal deletion of activated T cells represents a beneficial immunosuppressive effect of MTX in the treatment of RA (12). Inhibition of TS activity by low-dose MTX would clearly be an immunosuppressive action but it is still not known whether this occurs *in vivo*.

The purpose of the present study was to clarify whether low-dose MTX inhibits TS activity and the proliferative respon-

ses of normal human PBMC stimulated *in vitro*. In order to expose cells to biologically relevant concentrations we measured plasma concentrations in 8 RA patients treated with MTX. Then we determined a concentration range of MTX in our *in vitro* system covering the actual *in vivo* exposition and measured the effect of MTX on TS activity and cell proliferation. We were also interested in the synthesis of TS after MTX exposition and for that reason we measured the effect of MTX on the concentration of TS mRNA in normal human PBMC. Finally we measured TS activity in PBMC isolated from 6 RA patients before and after intake of their weekly dose MTX in order to elucidate any *in vivo* effect.

Patients and methods

Patients

For measurement of MTX plasma concentrations, heparinized venous blood samples were collected from 8 patients with RA treated with MTX for at least 6 months. The patients were treated with various weekly doses (7.5 mg - 17.5 mg) and venous blood samples (2 ml) were drawn before intake of MTX and 1, 2, 4, 6, 12 and 24 hours afterwards. For the TS activity analysis, another 6 RA patients were included in the study; both groups of RA patients fulfilled the American College of Rheumatology's revised criteria for the classification of RA. The latter group of patients was also treated with different doses of MTX (7.5 mg - 20 mg/week) for at least 6 months and heparinized venous blood samples (10 ml) were obtained before and 2 hours after the intake of MTX. The mean age of the patients was 60 years (range 37-78), the female/male ratio was 2:1, and the mean disease duration was 8 years (range 3-20).

Materials

Phytohaemagglutinin (PHA) reconstituted in RPMI, Phorbol-12-Myristate-13-Acetate (PMA) diluted in ethanol and ionomycin were purchased from Sigma (St Louis, MO). Protein A was purchased from Boehringer Mannheim (Indianapolis, IN), reconstituted with deionized water and kept frozen at -70° C. Recombinant IL-2 (Genzyme, Cambridge, MA) was reconstituted with 10 mM acetic

acid. IL-6 and IL-4 (Genzyme) were reconstituted with RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) and 1% FCS, aliquoted and kept frozen until used. Methotrexate (MTX) (Lederle, Germany) was kept at 5°C in stock solution 2.5 mg/ml. 2'deoxyuridine-5-³H (Sigma) and deoxyuridine-6-³H (NEN, Boston, MA) were kept at 5°C.

Mononuclear cell preparation

PBMC were obtained from healthy donors, from RA patients or from buffy coats prepared from normal healthy blood donors. PBMC were separated from the peripheral blood or buffy coats by layering over Histopaque 1077 (Sigma). The tubes were centrifuged for 30 min at 1800 rpm, and mononuclear cells were collected from the interface. PBMC were subsequently washed three times with RPMI 1640 supplemented with penicillin (10,000 U/l) and streptomycin (10,000 µg/l) (Sigma), counted, and brought to a concentration of 1×10^6 cells/ml if used on the same day. If cells were to be used later they were frozen according to a standard freezing procedure. Cell viability was at least 95% as checked by trypan blue. For measurement of TS activity *ex vivo* blood samples were drawn from 6 RA patients before and 2 hours after the intake of MTX and PBMC were isolated from each sample as described.

Cell cultures

For the *in vitro* experiments measuring TS activity and cell proliferation, PBMC were isolated either from venous blood from healthy donors ($n = 5$) or from buffy coats ($n = 7$) and kept frozen. In each experiment 25×10^6 PBMC (1×10^6 cells/ml) were cultured for 72 hours in 50 ml flasks (Nunc, Denmark) in the presence of either PHA (5 µg/ml), PMA (5 ng/ml) or PMA/ionomycin (1 ng/ml/500 ng/ml). Cells cultured with Protein A (1 µg/ml) and rIL-2 (10 U/ml), rIL-4 (10 ng/ml) and rIL-6 (10 ng/ml) were incubated for 120 hours. Some donors and buffy coats were used for more than one stimulus depending on the number of cells obtained. All cultures were carried out in RPMI 1640, 10% FCS supplemented with penicillin/streptomycin (complete medium) and kept at 37°C in a humidified atmosphere containing 5% CO₂. The above

concentrations and culture lengths induced optimal proliferative responses in preliminary experiments. At the end of the culture period TS activity and cell proliferation were measured.

Plasma MTX concentrations

Tubes were centrifuged at 1800 rpm for 10 min and the plasma was collected and kept frozen at -80°C until analysed for their MTX content. MTX concentrations were measured by a fluorescence polarization immunoassay including a commercially available kit for measuring low concentrations of MTX (Abbott Diagnostics, Abbott Park, IL).

TS activity and proliferative responses

TS activity was measured in intact cells from normal donors, buffy coats and from 6 RA patients as previously described (13). Briefly, the cells were centrifuged at 600 g for 10 min and the supernatant was removed and discarded. The cells were then gently resuspended in pre-warmed (37°C) complete medium containing different concentrations of MTX and 1 uCi of 2'deoxyuridine-5-³H per assay. The cells were then incubated at 37°C for 1 hour at 5% CO₂ and subsequently pelleted by centrifugation and the supernatant was transferred to another centrifuge tube. In order to precipitate the 2' deoxyuridine-5-³H, 500 µl of charcoal (100 mg/ml) was added to the supernatant and mixed vigorously. The charcoal was removed by centrifugation at 1000 g for 10 min and the radioactivity in the supernatant was measured in a liquid scintillation beta counter. Background was measured in a control tube without cells. All experiments were performed in duplicate and the coefficient of variation was less than 15%.

Subsequently we measured the proliferative responses of PBMC obtained from donors and buffy coats. After removing the supernatant containing the isotope, the cells were resuspended in complete medium and brought to a concentration of 1×10^6 cells/ml. The cells were then transferred to a round bottomed microtiter plate (100 µl/well) in triplicate and labelled with 0.25 µCi of 2' deoxyuridine-6-³H. Following a 2-hour incubation, they were harvested by an automatic cell harvester (Skatron, Norway)

and the radioactivity of the filters containing cell bound 6-[³H]-dUMP was counted in a liquid scintillation beta counter.

Isolation of RNA

Total RNA was isolated from PBMC obtained from 8 healthy controls who donated 60 ml of EDTA stabilised venous blood. The PBMC were stimulated with PHA (5 µg/ml) for 3 days and MTX was added in 3 different concentrations for either 24 hours or 1 hour at the end of the culture period. RNA was then isolated from each condition by using a commercially available kit (Qiagen, USA) according to the manufacturer's instructions. Following isolation, the concentration and purity of the RNA preparations were analysed by optical density at wavelengths of 260 and 280 nm (Gene Quant, Pharmacia) and the RNA was kept frozen at -80°C until the RT-PCR was performed (14).

RT-PCR

TS mRNA was measured by an RT-PCR assay which was validated and previously described in detail by Ehrnrooth *et al.* (15). Briefly, it is a single tube RT-PCR assay with an internal RNA standard where a calibrator curve is used to determine the amount of TS mRNA in the samples. The calibrator had been prepared from RNA isolated from a normal control and the concentrations of the calibrator stock solution had been determined previously in our group by a traditional quantitative RT-PCR assay (15). In the present experiments reverse transcription reactions were performed and a calibration curve was generated by serial dilutions of the calibrator (6 concentrations) and by the addition of a fixed amount of the internal standard (5.6×10^{-20} mole) to each of the calibrator dilutions and to the unknown samples. The TS specific primers have been described previously (16). The calibrator curve was obtained by plotting the ratio of amplified target/amplified standard against the amount of TS present in the calibrators and the concentration of TS mRNA in the unknown samples was determined by use of the calibration curve. All samples were performed in duplicate and the results are presented as 10^{-20} mole/µg RNA.

Statistical analysis

A normality test was performed in order to evaluate whether the data was gaussian distributed. The significance of the difference between mean values was analysed by Student's paired t-test. All results were expressed as the mean \pm 1 SD. Differences were considered significant if $p < 0.05$.

Results

MTX plasma concentrations

A pharmacokinetic curve with the plasma concentration versus time was cre-

ated for 8 RA patients (Fig. 1). The highest concentration was measured 1 hour after MTX intake in 6 patients and after 2 hours in 2 patients. Maximum *in vivo* MTX exposure was then calculated based on the concentrations measured 1 and 2 hrs after intake of the drug and were found to range from 0.050 $\mu\text{g/ml}$ to 0.686 $\mu\text{g/ml}$ (mean 0.265 $\mu\text{g/ml}$, median 0.207 $\mu\text{g/ml}$) in that particular time period. Subsequently 0.01 $\mu\text{g/ml}$ was chosen as the lowest concentration and 1.0 $\mu\text{g/ml}$ as the highest concentration for the *in vitro* studies.

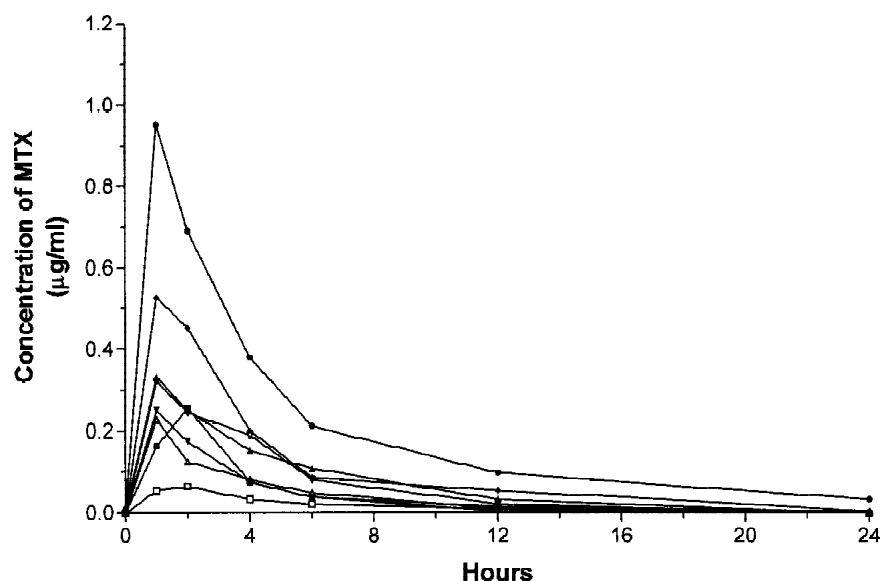


Fig. 1. MTX plasma concentrations measured in 8 patients with RA who were treated with MTX (7.5 mg - 17.5 mg). Heparinized venous blood was drawn at 0, 1, 2, 4, 6, 12 and 24 hours after intake of the weekly MTX dose.

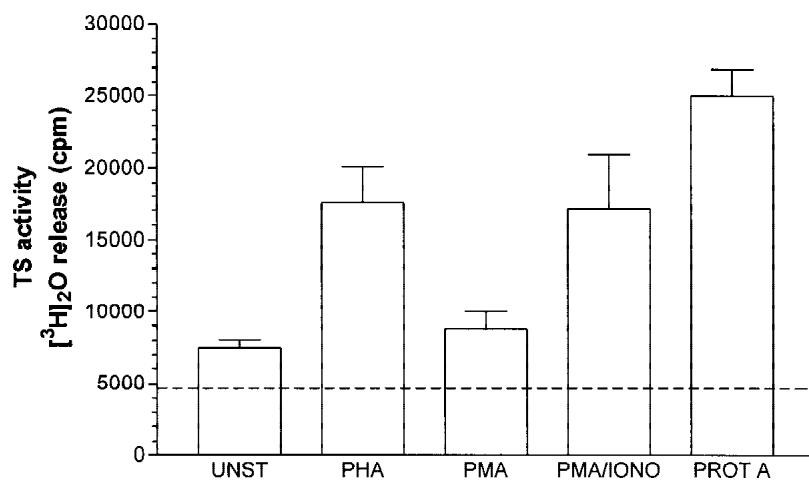


Fig. 2. TS activity measured in PBMC cultured without (UNST) or with various stimuli: PHA, PMA, PMA/ionomycin for 3 days or Protein A for 5 days. The columns represent the means \pm 1 SD of 5 experiments. The dashed line denotes the mean background level (4571 ± 553). All experiments were performed in duplicate.

TS activity and proliferative responses and the effect of MTX

TS activity of PBMC cultured with or without PHA (5 $\mu\text{g/ml}$), PMA (5 ng/ml), PMA/ionomycin (1 ng/ml/500 ng/ml) or Protein A (0.5 $\mu\text{g/ml}$) supplemented with rIL-2/rIL-4/rIL-6 is shown in Figure 2. The background activity is shown by the dashed line. TS activity increased significantly after stimulation with either stimuli compared to the unstimulated control, except when the cells were cultured in the presence of PMA, which did not reach the level of statistical significance. Subsequently the effect of MTX on TS activity was investigated. MTX was added for 1 hour in 4 different concentrations and the effect of MTX on TS activity in the stimulated PBMC was measured. The results are shown in Figure 3. The background activity has been subtracted from the results. It shows that MTX inhibited TS activity independently of the mode of activation, and significant inhibition was observed at the concentration of 0.01 $\mu\text{g/ml}$ MTX except when the cells were stimulated with PMA-ionomycin. In this case significant inhibition was observed with 0.1 $\mu\text{g/ml}$ MTX.

After TS activity had been measured, the cells were collected for the measurement of DNA synthesis (Fig. 4). The proliferative responses were also inhibited significantly by MTX, but generally higher concentrations of MTX were necessary to obtain significant inhibition as compared to TS activity (PMA/ionomycin: 0.01 $\mu\text{g/ml}$, PHA and PMA: 0.1 $\mu\text{g/ml}$ and Protein A: 0.5 $\mu\text{g/ml}$).

TS activity measured ex vivo

Thereafter TS activity was measured in PBMC from 6 RA patients and blood samples were drawn from each patient before and 2 hours after intake of their weekly MTX dose. There was no difference in the levels of TS activity in PBMC obtained before and 2 hours after MTX intake ($902 \text{ cpm} \pm 152$ versus $1112 \text{ cpm} \pm 319$ after subtraction of the background, $p > 0.05$). Results are presented as TS activity in cpm per 1×10^6 cells.

Effect of MTX on TS mRNA in normal human mononuclear cells

TS mRNA levels in PBMC from 8 heal-

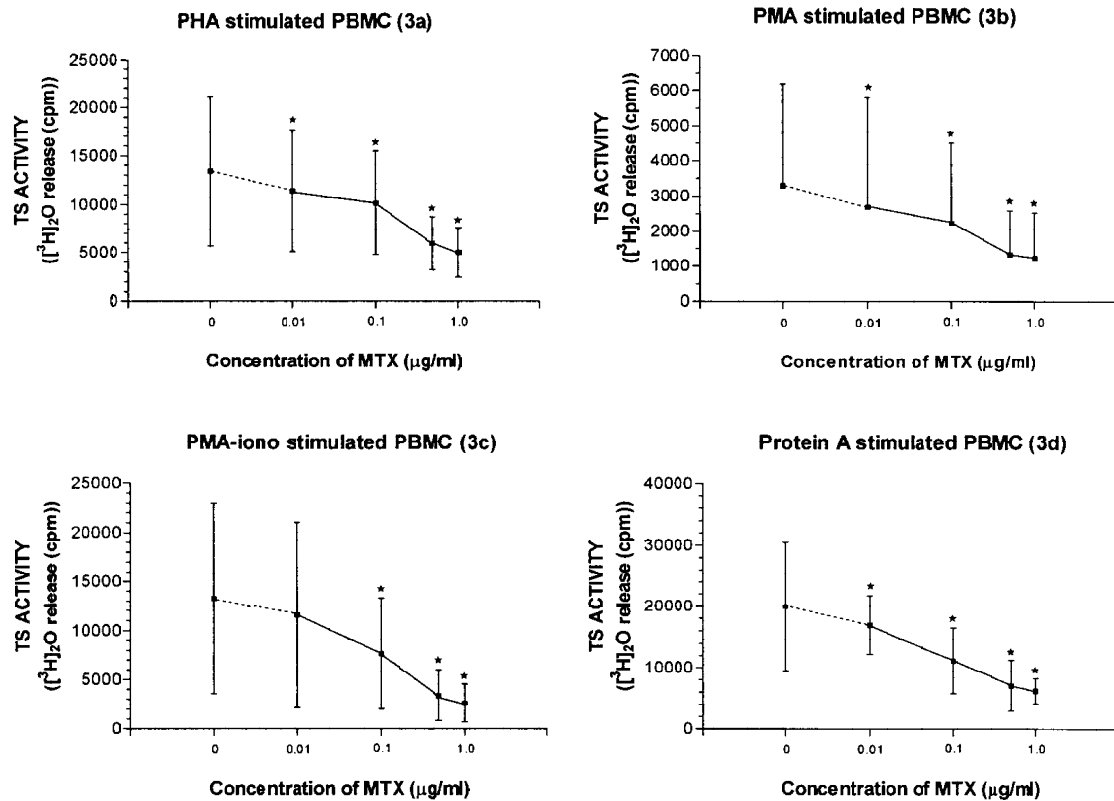


Fig. 3. The effect of different concentrations of MTX on TS activity in PBMC stimulated with: (a) PHA (n = 9); (b) PMA (n = 6); (c) PMA/ionomycin (n = 8); and (d) Protein A (n = 4). The concentrations of MTX (except 0 µg/ml) are shown on a logarithmic scale. Results are presented as the means \pm 1 SD (* denotes significant inhibition of TS activity). Each experiment was performed in duplicate.

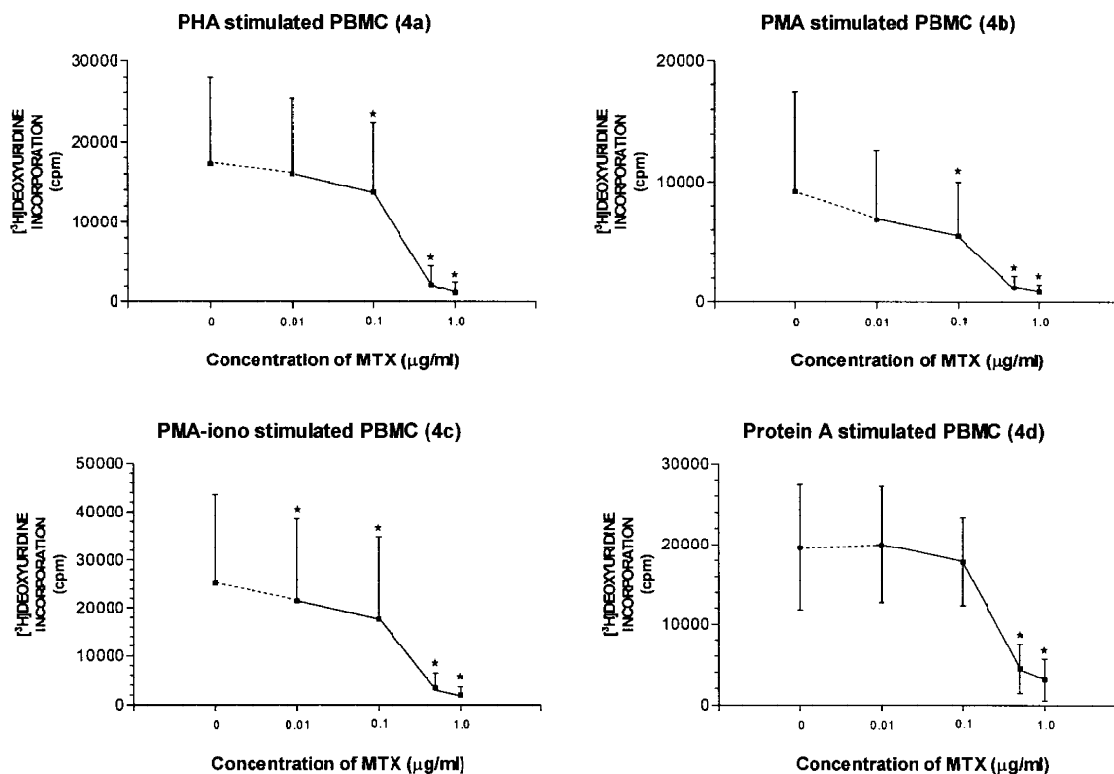


Fig. 4. The effect of different concentrations of MTX on proliferative responses in PBMC stimulated with: (a) PHA (n = 7); (b) PMA (n = 6); (c) PMA/ionomycin (n = 6); and (d) Protein A (n = 4). The concentrations of MTX (except 0 µg/ml) are shown on a logarithmic scale. Results are presented as the means \pm 1 SD (* denotes significant inhibition of proliferation). Each experiment was performed in triplicate.

thy volunteers and the effect of MTX is shown in Figure 5. Stimulation with PHA clearly enhanced the levels of TS mRNA as compared to the unstimulated control. It was found that the addition of all concentrations of MTX for 24 hours and 0.4 µg/ml for 1 hour significantly upregulated the TS mRNA concentration ($p < 0.05$).

Discussion

Since the introduction of MTX for the treatment of autoimmune diseases such as psoriasis and RA there has been a continuous search to clarify its mechanism of action. Immunosuppressive actions have been studied using tritiated thymidine in proliferative assays and have shown either an MTX induced enhancement of the proliferative responses or no effect in spite of a clear inhibitory action on cell cycle progression (17). In contrast, a strong inhibitory effect is observed when tritiated deoxyuridine is used and this is now more accepted in proliferative assays studying MTX (18-20).

In the present study, tritiated deoxyuridine was used to investigate the effect of MTX on TS activity and proliferative responses. It is shown that stimulation of PBMC enhances TS activity as com-

pared to the unstimulated control. MTX significantly inhibits both TS activity and DNA synthesis independent of the mode of activation, indicating that MTX does not interfere with the cell activation pathways prior to the action of TS. It is very likely that the observed decrease in DNA synthesis is due mainly to the observed inhibitory effect on TS activity in the same cells, which is indicated by comparison of the results from the two assays (50% inhibition was achieved with MTX concentrations between 0.1 and 0.5 µg/ml in both assays). Similarly, in another *in vitro* study MTX inhibited TS activity in PHA-stimulated PBMC obtained from patients with RA (4). In this study, however, MTX was present for 6 days in culture, whereas in our culture system MTX was present for 1 hour. Our results are compatible with the hypothesis that low-dose MTX inhibits TS activity *in vivo*, suggesting an immunosuppressive action not previously described for MTX in low-dose treatment regimens.

We were not, however, able to detect any difference in TS activity measured *ex vivo* in 6 RA patients before and 2 hours after intake of MTX. This is in agreement with a previous study showing no difference in TS activity measured in RA

patients (5 days after MTX intake) and in normal controls (4). In this study a cell cycle analysis was also performed which detected no difference in the percentage of cells in the S phase as compared to normal controls. This is in accordance with another study where an increased percentage of cells in the S phase was found prior to MTX therapy, a percentage which rapidly declined in response to MTX therapy (21).

Additional signs of cell activation in patients with RA are the increased expression of surface activation antigens on synovial fluid lymphocytes (22) and increased levels of cell activation markers such as sIL-2R in serum (23). Studies comparing peripheral blood with the synovium have found persisting or proliferating T cell infiltrates in the joint in spite of peripheral lymphopenia (24) and other studies comparing synovial fluid mononuclear cells with PBMC have reported the increased expression of activation markers on synovial fluid mononuclear cells compared to PBMC (25, 26). In our assay the cells were stimulated *in vitro*, thereby increasing the number of cells in the S phase. A possible explanation for the discrepancy between our *in vitro* and *ex vivo* findings may well be that PBMC isolated from

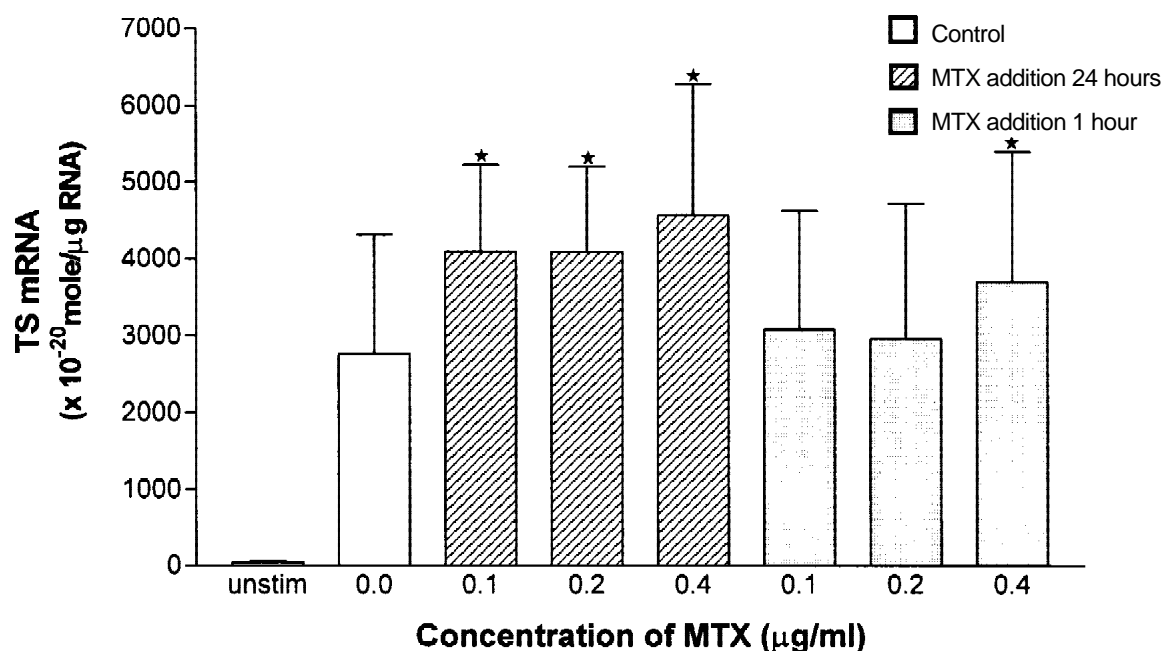


Fig. 5. The concentration of TS mRNA in PBMC cultured with (blank column) or without (unstim.) PHA for 3 days and the effect of different concentrations of MTX. The pre-stimulated cells were incubated with MTX for either 1 or 24 hours. Each column represents the mean \pm 1 SD of 8 experiments (* denotes significant inhibition of TS mRNA concentration). Each experiment was performed in duplicate.

RA patients are mainly resting and that a possible inhibition of TS activity cannot be demonstrated if only a small fraction of the mononuclear cells contains TS activity as compared to cells stimulated *in vitro*.

It is, however, quite possible that MTX exerts an inhibitory effect on TS activity *in vivo* on proliferating T cells in the synovium of patients with RA. It is also possible that the inhibition of TS activity is responsible for the decrease in the percentage of S phase cells observed in RA patients starting MTX therapy (21), and that we were not able to detect this effect since the patients included in our study had been treated with MTX for more than 6 months. It is well known that MTX is converted to polyglutamate derivatives intra-cellularly and that MTX polyglutamates are much stronger inhibitors of TS activity than the parent drug (11). The question is whether a sustained inhibition of TS activity by MTX polyglutamates in lymphocytes is partly responsible for the clinical effect.

Finally, the TS mRNA concentration in PBMC was significantly upregulated by MTX depending on MTX exposure and there are no previous reports on this matter. Chu *et al.* have suggested that TS mRNA translation is regulated by the cellular level of TS protein since they found that high levels of TS inhibited TS mRNA translation (27) by binding with high affinity to its own mRNA (28). Thus, it is tempting to speculate that the increase in TS mRNA observed in our study was the result of a positive feedback mechanism due to a lack of TS activity followed by a thymidine deficient state created by MTX. Recently it has been shown that TS mRNA levels are indicators of cellular stimulation (15) and are possibly more sensitive than TS activity or cell proliferation. Future studies are needed to explore the above finding and to establish a possible role for TS mRNA measurements in PBMC from patients treated with MTX.

In conclusion, this study presents data showing the effects of MTX in low doses not previously described. We show that low doses of MTX inhibit TS activity in stimulated PBMC *in vitro*, independent of the mode of activation, and that subsequent cell proliferation is closely cor-

related to the level of TS activity. This is in agreement with the known antiproliferative effect of high dose MTX, where the inhibition of TS activity is a rate-limiting step in DNA synthesis, thus supporting the hypothesis that low-dose MTX may exert immunosuppressive actions by inhibiting TS activity. We were not, however, able to demonstrate an inhibitory effect of MTX *ex vivo*, most likely because this was performed on a PBMC suspension consisting mainly of resting cells. In order to elucidate this issue it will be necessary to investigate the effect of MTX on TS activity in cell populations activated *in vivo*. This may possibly be achieved by the isolation of activated cell subpopulations from peripheral blood or by obtaining synovium material containing proliferating mononuclear cells.

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