Methotrexate selectively modulates TH1/TH2 balance in active rheumatoid arthritis patients

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Abstract

Objective

The mechanism by which low dose methotrexate (MTX, the gold standard treatment for rheumatoid arthritis) exerts its anti-inflammatory effect in rheumatoid arthritis (RA) patients is still debated. Lately, the MTX immunosuppressive effect has been related to apoptosis, especially in active RA patients, with ROS involvement.

Methods

In the present research we investigated MTX oxidative effect and its ability to modulate immune balance in active versus non-active RA patients.

Results

Our results show that MTX induces IL-10 secretion (a TH2 cytokine) and significantly reduces TH1 profile in Peripheral Mononuclear Cells (PMNC) derived from active RA patients (n=28). Additionally, we found that MTX modulates the immune status towards TH2 dominance by decreasing the IL-12R and the CXCR3 receptors typical for the TH1 population. Moreover, MTX was found to inhibit the production of nitric oxide (NO) in these patients, a phenomenon that might contribute to MTX action toward cytokine homeostasis. A significant correlation was found between MTX IL-10 induction and NO inhibition in active RA patients.

Conclusions

Our data suggest that, in active RA patients, apoptosis induction by MTX may be primarily due to IL-10 production via modulation of oxidative stress, which may restore the critically important immune balance. These findings may contribute to determining which group of RA patients may better respond to MTX therapy.

Key words

Methotrexate, rheumatoid arthritis, peripheral mononuclear cells, cytokine balance, reactive oxygen species, nitric oxide**,** TH1/TH2 profile.

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Introduction

Rheumatoid arthritis (RA) is a chronic destructive synovitis affecting the bone and joints. RA has been recognized as a chronic systemic autoimmune inflammatory disease, and immunological effects have been shown to play an important role in its pathogenesis (1-3). Pathologically, RA is characterized by proliferation and activation of lymphocytes that are predominantly of the $CD4^+$/T helper phenotype. The synovial tissue in RA, termed the "pannus", becomes infiltrated with lymphocytes that are predominately of the $CD4^+$/T helper phenotype (2, 4, 5). In this disease, large quantities of proinflammatory cytokines, especially of the TH1 profile, were found in the synovial fluid and membrane (6, 7). These cytokines are produced by activated macrophages or T cells, and strongly contribute to synovial cell proliferation and cartilage destruction in RA (8).

Low dose methotrexate (MTX) has become a standard treatment for patients suffering from active RA (9-12). Several mechanisms have been suggested for MTX action as an anti-inflammatory and immunosuppressive agent at the cellular level. These included the MTX inhibitory effect on folate dependent enzymes via polyglutamated derivatives, inhibiting proliferation (13-15) and MTX mediation of adenosine release, suppressing inflammation (16, 17).

Recently, MTX anti-inflammatory action has been related to the induction of apoptosis. MTX was found to induce apoptosis in activated healthy T cells (2, 15, 18), in lymphocytic cell lines (10) and in $CD4^+$ cells derived from active RA patients (19). However, the actual mechanism of MTX apoptosis induction has not yet been fully elucidated.

One of the possible inducers of apoptosis is intracellular oxidation or oxidative stress by ROS (20, 21). Reactive oxygen species (ROS), including reactive nitrogen species such as nitric oxide (NO), are biologically active oxygen derivatives, which are becoming recognized as important mediators of inflammation, such as in RA (22) and apoptosis due to their redox potential (23, 24). Few attempts have been made to relate MTX immunosuppressive effect in RA patients to ROS production (25-30). We have recently shown an apoptotic effect of MTX induced via an ROS dependent pathway (10).

Nitric oxide (NO) has been shown to be an important mediator of diverse physiologic and pathologic processes, including arthritis (31, 32). NO, a lipid- and water-soluble gas, is ideally suited as a potent inflammatory mediator because of its strong reactivity with oxygen, superoxide, and iron-containing compounds. Previous work has provided evidence for increased production of systemic NO in rheumatoid arthritis (33-36) and increased expression of inducible NO synthase (NOS2) and NO production (37). It is debated whether NO exacerbates or reduces the inflammatory processes (38). Since RA is T-cell mediated, we wished to assess the potential NO regulation by MTX of T-lymphocytes, and specifically their cytokine expression. MTX effect on NO production and subsequent NO regulation of cytokine balance in specific groups of RA patients has never been reported, however in a single report, MTX at therapeutic concentrations *in vitro* has been shown to inhibit the production of NO in cultured rabbit articular chondrocytes (39). Few attempts have been made to examine the MTX effect on cytokine balance in RA patients. It has been reported that in early stage patients, MTX increases the IL-10 and IL-4 cytokines, which are both characteristic of Th2 cells and reduced TNF-α (40). In a different report, long term RA therapy with MTX in combination with low dose corticosteroids affected the predominance of Type 1 cytokines toward normalization of the cytokine balance in both $CD4^+$ and $CD8^+$ T lymphocytes (41).

The aim of the present study was therefore to determine the MTX effect on oxidative stress and on the normalization of cytokine balance in active and non-active RA patients. A possible distinct effect of MTX in the different RA groups may contribute to better prediction of therapy efficiency for RA patients.

Materials and methods

The present study was conducted in collaboration with the Tel-Hashomer

Medical Center, Israel, in accordance with the Declaration of Helsinki (IRB certification No. 3730/2005). Blood samples were obtained from extensive populations of RA patients, diagnosed according to the ACR criteria. The subjects were asked to give their informed consent to donate 5 ml blood for examination. The blood samples were analyzed at a cell population level using FACS flow cytometry.

Mononuclear cells (MNC) were isolated from peripheral blood leukocytes of 25 RA patients using the Ficoll Paque procedure. The RA patients were classified according to the American College of Rheumatology (ACR) criteria (43). The six criteria for disease activity assessment (duration of morning stiffness, fatigue, joint pain by history, joint tenderness or pain in motion, soft tissue swelling in joints or tendon sheaths, and erythrocyte sedimentation rate) were monitored for each patient. RA patients having signs of remission were defined as *non-active*, and RA patients without signs of remission were defined as *active*, according to the ACR criteria. No patients were included who were taking drugs known to affect the immune system, other than MTX (7.5-15 mg/kg/week). Folate supplementation was being routinely administered (1 to 2 times per week up to 5 mg). Blood was collected within 4-6 days after oral administration of MTX. All patients took MTX therapy for at least 6 months before our *in vitro* examinations.

Following separation, cells were washed twice in saline and once in PBS, then suspended in enriched RPMI medium (with 10% fetal calf serum) at a concentration of $1x10^6$ cells/ml. Before any cytometric analysis, viability was checked by trypan blue exclusion. Each type of experiment was performed in triplicate.

Viability test

Plasma membrane integrity and cell viability were determined by the trypan blue (TB) exclusion test. A viable cell excludes acidic dyes, such as trypan blue, therefore their uptake is indicative of irreversible membrane damage preceding cell death. Cells were loaded with the trypan blue dye on a hematocytometer slide at the ratio 1:1 (v/v) and analyzed by light microscopy. The percentage of dead cells was determined by counting a total of 150-200 cells per independent experiment.

Intracellular measurement of cytokines

Intracellular IL-10 (classical T helper-2 cytokine) and IL-12 (T helper 1 cytokine) were measured in order to classify the RA patients according to their TH1/TH2 cytokine profile, and their *in vitro* response to MTX. $1x10^6$ cells were fixed with 4% paraformaldehide in PBS, followed by permabilization with 0.1% Saponin in HBSS. Staining was performed with anti-human IL-12 (FITC conjugated) and anti-human IL-10 (PE conjugated) antibodies (R&D Systems, Inc., Minneapolis, MN, USA). The cytokine measurements were performed using flow cytometry (FACscan, Becton Dickinson, Mountainview, CA, US).

Cytokine receptor evaluation

MNC were evaluated under free serum conditions for CCR3, CXCR4, IL-12R and IL-4R expression. Flow cytometric staining and analysis of the receptors were performed as described earlier (44). Briefly, the cells were stained in PBS (Ca and Mg-free), supplemented with 5% BCS (Hyclone; Logan, Utah). Primary mAbs were detected with secondary phycoerythrin or fluorescein isothiocyanate (FITC)-conjugated goat antimouse mAbs (Sigma) (1:100) or antirat antibodies. After the final wash, cells were fixed in 1% paraformaldehyde prior to FACS analysis using FACscan (Becton-Dickinson, Mountainview, CA, USA).

Measurement of intracellular ROS

The production of ROS was fluorometrically estimated using a fluorescent probe dihydro-rhodamine which is oxidized to a fluorescent intercalator rhodamine by cellular oxidants, particularly superoxide radicals. Cells were first incubated in the presence of 10μM DHR1239 (from Calbiochem, Darmstadt, Germany) for 15 min in incomplete RPMI 1640 medium (without FCS), containing 25 mM Hepes buffer solution, at 37° C. At the end of probe loading, cells were washed with PBS. The kinetics of RH123 fluorescence intensity (Excitation: 488nm, Emission: 530nm) resulting from oxidation of DHR123 were measured using the Tecan spectrofluorimeter.

Measurement of intracellular NO

The production of NO was fluorometrically estimated using a fluorescent probe DAF (from Calbiochem, Germany). All cells were first incubated in the presence of 1μM DAF, and at the end of probe loading, washed with PBS. The kinetics of DAF fluorescence intensity resulting from its oxidation were measured with the Tecan spectrofluorimeter.

Data analysis

Data are expressed as mean ± SD, and were analyzed by the two-tailed paired Student t-test. Differences were considered significant at $p \leq 0.05$. Correlations were assessed using linear correlation coefficients, and in cases when the data were not normally distributed we used non-parametric correlation coefficients (Kendall's tau-b and Spearman's correlation coefficients).

Results

In the present study, we assessed the connection between the previously demonstrated immunosuppressive effect of MTX (*in vitro*) and its hypothesized ability to regulate the immune status of RA patients toward homeostasis. All our experiments were done in both non-active and active RA patients, as these two groups were previously found to differ with respect to their immune cell status (19).

First, we examined the cytokine balance (TH1/TH2 profile) in non-active RA patients clinically treated or untreated with MTX. Three untreated and 6 clinically MTX treated (7.5-12.5mg/week) non-active patients, exhibited different profiles (Fig. 1A). The untreated subjects showed 9% CXCR3 positive cells and 6% CCR4 positive cells, implying TH1 dominance. Non-active RA patients clinically treated with MTX exhibited a slight difference, with 7% CXCR3 and 6.5% CCR4 positive cells. Next, we tested the cytokine production typical for the $CD4^+$ subpopulations:

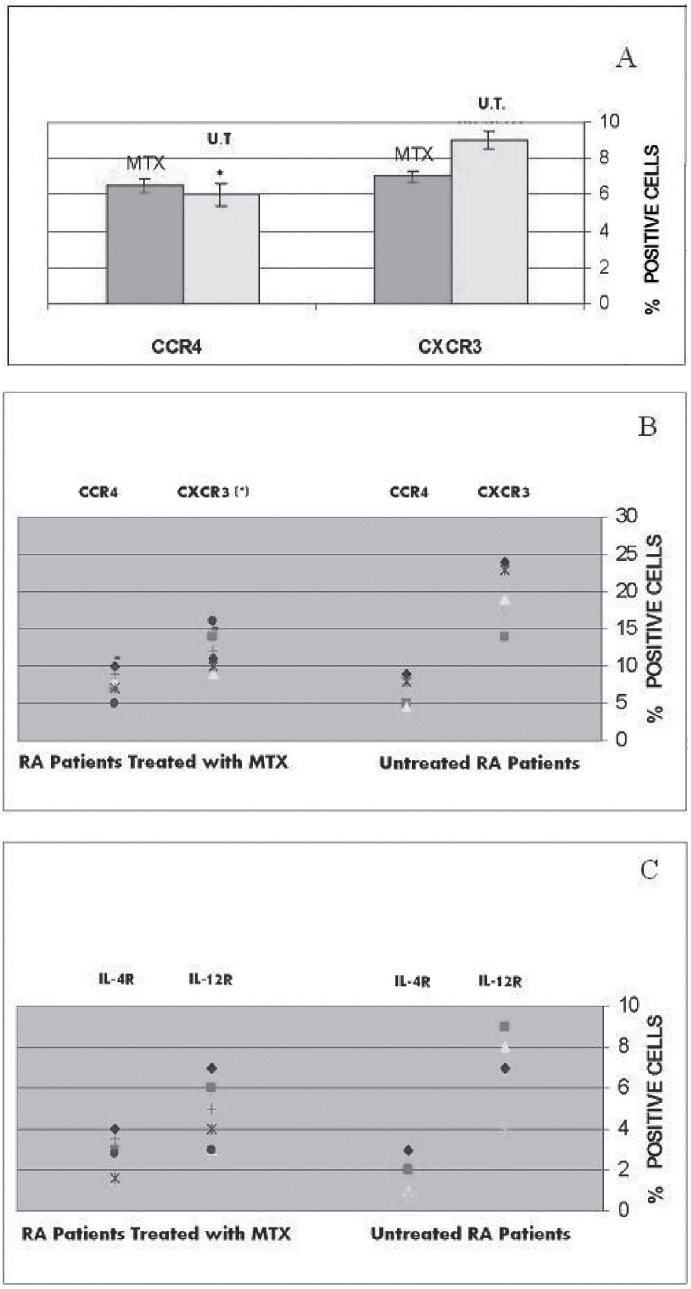

Fig. 1. (**A**) CCR4 and CXCR3 expression by $CD4^+$ cells derived from non-active RA patients clinically treated or not with MTX. *= $p \leq 0.05$. UT: untreated patients. (**B**) CXCR3 and CCR4 expression by MNC derived from active RA patients treated or untreated with MTX. (**C**) IL-12R and IL-4R expression by MNC derived from active RA patients treated or untreated with MTX.

IL-10, a TH2 cytokine, and IL-12, a TH1cytokine. In addition, we assessed the cellular production of nitric oxide and ROS, which are known to modulate the immune response. Three untreated non-active RA patients and 8 non-active patients clinically treated with MTX showed different behaviors of cytokine production. A significant difference (p=0.04) in IL-10 production was observed between MTX-treated patients and non-active patients untreated with MTX. Untreated patients exhibited an average of 6.2±0.28% positive cells while the treated subjects showed 5.1±0.32% (Fig. 2A). IL-12 production did not differ between the 2 groups and was ~ 9±1.7% on average.

Interestingly, when NO generation was measured in the same patients, an opposite effect was observed. The MTX-treated patients showed a significant increase in NO generation (from 5600±330 to 7700±550, p<0.05) as compared to the untreated patients (Fig. 2C). In order to better understand the effect of MTX on oxidative stress, ROS production was measured. A non-significant difference in ROS production was observed in the two groups (Fig. 2C).

Our next experiment tested the involvement of MTX therapy in cytokine balance and its possible connection to immunosuppression in active RA patients. Similarly to non-active patients, we first analyzed the lymphocyte subpopulation TH1/TH2 in active RA patients treated (n=7) or untreated (n=5) with MTX. As it is seen in Fig. 1B, MTX (*in vivo*) significantly reduces CXCR3 expression (a typical TH1 receptor, from an average of 19.4% CXCR3 positive cells in untreated RA patients to 12.5% in MTX treated patients, p = 0.04) and enhances CCR4 expression (a typical TH2 receptor, from an average of 6.7% to 8.25% positive cells).

To further validate our results, we tested two other distinctive receptors typical for the TH1/TH2 subpopulations. IL-12R was measured as a TH1 indicator and IL-4R as a TH2 indicator. A pattern of TH1 dominance was observed in the untreated group (n=4, an average of 7% of IL-12R+ cells), whereas in the treated group (n=7) the average of IL-12R+ cells was 4.6%, p<0.05 (Fig. 1C).

Then we checked the lymphocyte production of IL-10, IL-12 and NO in active RA patients (n=12). No significant differences in IL-12 production were observed (data not shown). However, notably, in active RA patients, an opposite pattern was seen compared to non-active patients. Untreated active patients exhibited a low amount of IL-10 positive cells (Fig. 2B). However, MTX-treated patients demonstrated a significant increase in IL-10 levels (an increase from 4.3%±0.86% to 7.3%±0.73, p=0.05). This may indicate a shift toward TH2 dominance. Concomitantly, MTX-treated patients showed lower levels of NO production as compared to untreated patients (Fig. 2D).

Considerable correlations were found between NO and IL-10 production in active MTX-untreated RA patients (r=0.6, p=0.05, n=8) and in active RA patients clinically treated with MTX only (r=0.78, p=0.04, n=13).

We further checked the possible involvement of MTX in ROS generation. MTX significantly increased the overall ROS production in active RA patients by 27% (Fig. 2D). Notably, MTX decreased the NO generation in these patients.

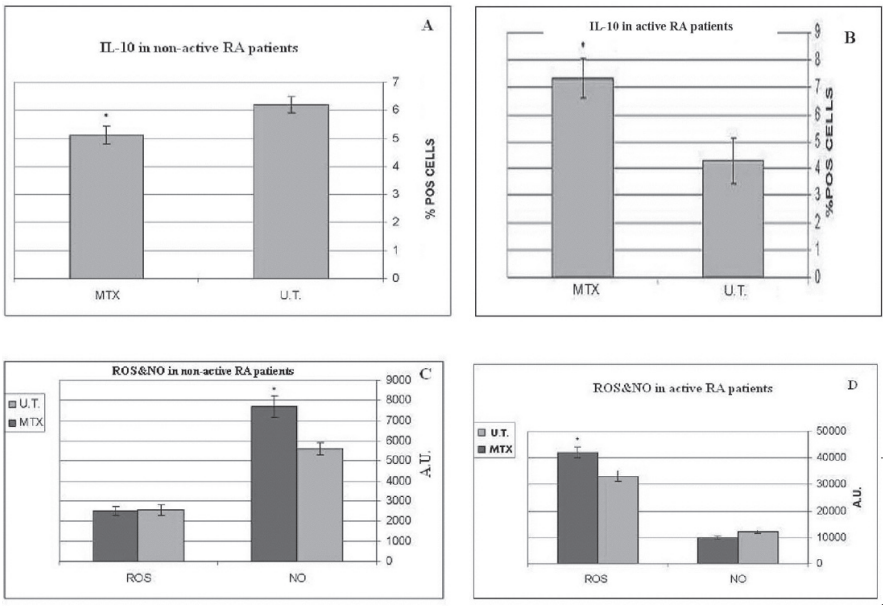

Fig. 2. (**A**) IL-10 production by MNC derived from non-active RA patients clinically treated or not with MTX. (**B**) IL-10 production by MNC derived from active RA patients, treated or untreated with MTX. (**C**) NO and ROS induction in non-active RA patients, treated or untreated with MTX. (**D**) NO and ROS production in Active RA patients, treated or untreated with MTX. Correlations were found between NO and IL-10 production in active MTX-untreated RA patients (r=0.6, p=0.05, n=8) and in active RA patients clinically treated with MTX only (r=0.78, p=0.04, n=13).

Table I. MNC derived from non-active RA patients, clinically treated with MTX and in addition *in vitro* treated with 0.1 μM MTX (n. of patients = 3). $^*p< 0.05$ (when comparing *in vitro* treated with non-treated MNC).

	% CCR4 Positive Cells	% CXCR3 Positive cells	% IL-10 Positive cells	NO production (AU)	ROS production (AU)
MTX *in vivo* treatment only	6.5% ± 0.4	7.1% ± 0.8	5% ± 0.6	7300 ± 450	2200 ± 180
MTX in *vivo* + *in vitro* treatment	6.4.% ± 0.8	6.2% ± 0.5	4.4% ± 0.3	8100 ± 400	3500 ± 250*

Table II. MNC derived from active RA patients, clinically treated with MTX and in addition *in vitro* treated with 0.1 μM MTX (n. of patients = 5). $^*p< 0.05$ (when comparing *in vitro* treated with non-treated MNC).

	% CCR4 Positive Cells	% CXCR3 Positive cells	% IL-10 Positive cells	NO production (AU)	ROS production (AU)
MTX *in vivo* treatment only	8.3% ± 0.5	12.5% ± 0.9	7 ± 0.8%	11000 ± 750	33000 ± 4000
MTX *in vivo* + *in vitro* treatment	9.5% ± 0.6	9.5% ± 0.7*	9.1 ± 0.8%*	8500 ± 900*	42000 ± 3400*

Thus, our results show that MTX induces IL-10 production and inhibits nitric oxide secretion and CXCR3 and IL-12 receptors in mononuclear cells derived from active rheumatoid arthritis patients only.

To further validate our results, the *in vitro* effects of MTX at the optimal concentration of 0.1 μM (2) were measured on MNC derived from active and non-active patients clinically treated with MTX. The results, shown in Tables I and II, indicate the same patterns of change, yet less pronounced. Especially significant changes in most of the parameters were noted in active patients; whereas in non-active patients, only the ROS increase was significant.

Discussion

The objective of the present study was to investigate *in vitro* the oxidative status and cytokine balance in RA patients with a specific disease status: active or non-active, with the aims of clarifying the MTX immunosuppressive/anti-inflammatory mechanism and predicting MTX efficiency in the different groups of RA patients.

RA is a chronic autoimmune disease characterized by TH1 dominance (6, 7). In addition it has been found that free radicals play a fundamental role in RA pathogenesis (45, 46). It is therefore of great importance to increase the TH2 secretion in order to restore the cytokine balance, and to reduce oxidative stress. Low dose MTX, the standard clinical treatment for RA, has been shown to exert an immunosuppressive effect in RA patients (15, 18, 19), although its mechanism of action is not yet fully understood. Recently, it has been related to induction of apoptosis through ROS involvement (10, 20, 21). MTX has been reported to induce cytokine balance (40, 41) and enhance anti-inflammatory cytokines (47, 48).

Our results demonstrate two distinct/opposite patterns of MTX action in active and non-active RA patients. In active RA patients only, MTX has been shown to selectively reduce TH1 profile (decreased CXCR3 and IL-12 receptors) and increase TH2 profile (moderately raised CCR4 and IL-4R) toward cytokine balance, whereas in non-active patients this phenomenon was absent. Moreover, MTX reduced NO secretion and enhanced ROS generation in active RA patients only, in addition to enhancing IL-10 secretion, a typical TH2 cytokine. These results are reinforced by our previous studies. When investigating the MTX apoptotic effect on $CD4^+CD28^+$ and $CD4^+CD28^-$ subpopulations, we found that the apoptotic effect was more pronounced in $CD4^+$ cells derived from active RA patients in contrast to non-active (19).

There is a controversy over the mechanism by which NO regulates cytokine expression in RA patients, and whether it is selective for certain cytokines. Thus one group hypothesized that NO might promote TH2 responses in mice and

humans (49, 50). Contradictory studies reported that NO inhibits proliferation of cloned mouse TH1 and TH2 cells equally (51). We therefore assessed both the effect of MTX on NO regulation and on cytokine normalization in active and non-active RA patients.

NO has been earlier reported to selectively modulate the lymphocytic TH1/TH2 responses. In mouse stimulated macrophages it has been demonstrated to regulate TH1 cell development through the inhibition of IL-12 synthesis (52, 53). Here we show that in active RA patients, there is a significant decrease in NO due to MTX. This decrease may be responsible for the change in cytokine balance in active RA patients. This connection between NO decrease and TH2 enhancement has been shown in various models (42, 54, 55). While a significant decrease in NO production was observed, concomitantly, a significant increase in ROS generation was found in active RA patients, which is an established phenomenon (56). We assume that ROS increase contributes to the apoptotic effect. The relation between high production of ROS and enhanced apoptosis has been demonstrated by many researchers: in spermatozoa (57), in brain tumors (21), and cell mitochondria (20, 21).

Thus, under the treatment by MTX, while NO decrease may contribute to cytokine homeostasis, ROS generation may be responsible for the apoptotic effect, leading to MTX beneficial action in active RA patients. Additionally, there was found a large difference in ROS base line production between active and non-active patients, in patients untreated by MTX (Fig. 2C and D). This difference could contribute to a better distinction between the different activity statuses of the disease. ROS and NO generation could serve as a part of a battery of *in vitro* tests to better characterize the immune status of MNC in individual patients (before or after MTX therapy), in addition to the differential TH1/TH2 balance and modulation by MTX found in active and non-active patients. Thus, these findings may contribute to a better understanding of MTX efficiency in different RA groups, and thus to optimizing and personalizing therapy for RA patients.

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