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Receptor and Dose-Dependent Effects Exerted *In Vitro* by Methotrexate on Human Immune Cells

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GINA MANDA[1*], MONICA NEAGU[1], ALEXANDRA LIVESCU[1], CAROLINA CONSTANTIN[1], C. CODREANU[2], MARINA NECHIFOR[3]
1. *INCD "Victor Babes", 99-101 Splaiul Independentei 76201 Bucharest, Romania*
e-mail: imunoc@vbabes.ro, fax: 411 51 05
2. The Methodologic Center of Rheumatology "Dr. Ion Stoia", Str. Theodor Massaryk, Bucharest, Romania
3. University of Bucharest, Faculty of Biology, 91-95 Splaiul Independentei 76201 Bucharest, Romania

Abstract

Methotrexate is currently efficacious immunomodulatory drug used in the therapy of rheumatoid arthritis. Our study is focused on the in vitro effects of low concentrations of methotrexate (0.01-1μg mL^{-1}) exerted on several immune functions developed by peripheral leukocytes isolated from rheumatoid arthritis patients. Patients with other osteoarthropathies and normal individuals were considered as controls. Phagocytosis and the respiratory burst developed by granulocytes and lymphocyte proliferation were investigated. Our study points out that methotrexate alters in vitro various functions of both proliferative and non-proliferative immune cells. The effects of methotrexate are dependent on drug concentration, on the receptor that triggers cellular activation and are also differentiated according to the pathology. Functional interferences between methotrexate receptors and CR3 or FcγR were highlighted. Low concentrations of methotrexate exert in vitro both immunosuppressive and immunostimulatory effects on peripheral lymphocytes.

Keywords: methotrexate, rheumatoid arthritis, granulocytes, lymphocytes, phagocytosis, superoxide anion, proliferation.

Introduction

Rheumatoid arthritis (RA) is a systemic chronic inflammatory autoimmune disorder of mysterious etiology [1]. Recruitment of immune cells into the synovial membrane, hyperplasia of synovial lining cells, a shift in the phenotype and function of synovial fibroblasts are accepted as pathological coordinated events in RA [2], culminating in joint injury and life-threatening systemic disturbancies [3]. Due to the complex pathophysiology of the disease, severe RA is a difficult therapeutic target. Owing to a superior efficacy/toxicity ratio, methotrexate (MTX) is at present the most efficacious and best tolerated DMARD, used either as monotherapy or in combination with other DMARDs [4].

MTX is an antifolate drug that enters the cells *via* carriers and receptors, by the same pathway as folic acid and its derivatives [5]. MTX is accumulated within the cell in an active

polyglutamated form [6] that inhibits dihydrofolate reductase (DHFR) and other folate-dependent enzymes [7,8], consequently impairing purine synthesis [9], transmethylation reactions and polyamine synthesis [10]. Clinical and experimental evidence sustain that the low-dose MTX treatment in RA has antiinflammatory effects and a subtle immunomodulatory action [11]. The antiinflammatory effects of MTX are still under debate. MTX induces the shift of the immune response from the Th1 to the Th2 cytokine pattern [12] and inhibits leukocyte recruitment to the inflamed joint cavity [13]. Most of these effects are the result of the MTX-induced increase of adenosine release by connective tissue cells [14].

This study aims to clarify some aspects regarding the *in vitro* action of MTX at low concentrations on peripheral leukocytes, relevant for the potential systemic immunomodulatory or immunotoxic effects of the drug. We report here that the effects exerted *in vitro* by methotrexate are dependent on the drug concentration, on the receptor that triggers cellular activation and are differentiated according to the pathology.

Material and Methods

Patients

We investigated a group of patients with active RA (RA patients), who met the American College of Rheumatology criteria [15]. Patients were treated and monitored at the Methodologic Rheumatology Center "Dr. Ion Stoia", Bucharest. All RA patients presented a rheumatoid factor and an active form of disease characterized by elevated acute phase markers (C reactive protein and erithrocyte sedimentation rate). Patients with other osteoarthropathies (osteoarthritis and bursitis) and normal subjects (healthy volunteers) were considered as controls. At least one week before the investigation patients did not receive glucocorticoid or other immunosuppressive medication.

Peripheral immune cells. Fresh blood was collected by venipuncture in heparin-coated vials. Mononuclear cells and granulocytes (PMNs) were isolated from peripheral blood by density gradient centrifugation on Hystopaque [16] and red blood cells were lysed with 0.83% NH_4Cl, 0.084% $NaHCO_3$ [17]. Cellular viability, estimated by the eosin exclusion test, exceeded 96%. Mononuclear cells were suspended in RPMI 1640 culture medium supplemented with 5% fetal calf serum, antibiotic/antimycotic solution, 0.2% $NaHCO_3$ (complete culture medium) and were kept at 4^0C until use. PMNs were suspended in Hank's balanced salt solution supplemented with 2% gelatin (complete HBSS solution) and were kept at room temperature until use.

Phagocytosis. PMNs ($4x10^6$ cells mL^{-1}) were incubated with Z (2 mg mL^{-1}) for 1h at 37^0C in round bottom culture plates, in the absence or presence of various concentrations of MTX. Cells were investigated by optical microscopy. Results were expressed as the percentage of cells that have ingested at least one particle of Z (phagocytosis index).

Superoxide anion release. Superoxide anion release by PMNs was measured by the cytochrome c reduction test [8]. Briefly, test samples contained $1x10^6$ PMNs mL^{-1}, 0.98 mg mL^{-1} cytochrome c, various stimuli and/or MTX, in a final volume of 1ml complete HBSS. A sample containing only 0.98 mg mL^{-1} cytochrome c in complete HBSS was considered as control. Samples were incubated for 30 min at 37°C, under continuous shaking. Reaction was stopped by chilling the samples on ice. The optical densities (OD) of the test sample supernatants were recorded at 535 nm and 550 nm using as reference the control sample. The superoxide anion release was estimated as the difference in optical densities [(OD_{550}-OD_{535}) x1000] for each test sample.

Lymphocyte proliferation. Lymphocyte proliferation was measured by the radioactive method of tritium-labeled uridine incorporation [19]. Briefly, test samples (final volume of 200 μL) containing $2x10^6$ lymphocytes mL^{-1} in complete culture medium were incubated 3

days at 37^0C in 5% CO_2, in absence/presence of mitogens and/or various concentrations of MTX; 18h prior to harvesting, cell cultures were labeled with 1 μCi tritium-labeled uridine per well. At the end of incubation cells were harvested on Skatron glass filters whose radioactivity was further measured with a β-counter. Radioactivity results were expressed as pulses min^{-1} (ppm).

Results. The effect exerted by MTX in the mentioned experimental systems was calculated as

$$\frac{cellular\quad response_{+MTX}}{cellular\quad response_{-MTX}}.$$

Statistics. Experimental results were processed as mean ± standard error of the mean (SEM). The effect exerted by MTX was compared with the unity effect using the t-test: paired two sample for means. The investigated groups were compared by the t-test: two-sample assuming unequal variances. Correlations were performed using the Pearson correlation test.

Results and discussions

Our investigations were focused on the effects exerted *in vitro* by low doses of MTX on some physiologic immune functions developed by peripheral PMNs and lymphocytes isolated from RA patients, considering as controls normal individuals and OA patients. The range of MTX concentrations (0.01-1 μg mL^{-1}) we investigated is therapeutically relevant (7.5-15 mg administered weekly).

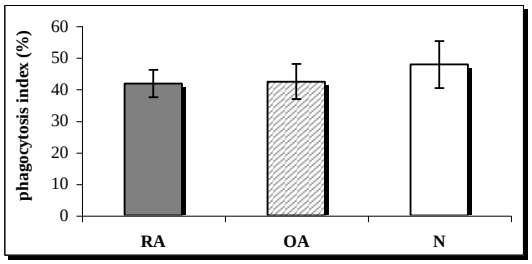
The in vitro *effect of MTX on human peripheral PMNs*

Recruited in the inflamed joint cavity, PMNs play an important role in the pathogenesis of RA by evoking joint damage *via* the release of degrading lysosomal enzymes and reactive oxygen and nitrogen species [21]. We investigated the CR3-mediated phagocytosis and the respiratory burst (superoxide anion release) developed by peripheral PMNs, as mechanisms of non-specific immune defense triggered *in vitro* by receptors with major role in the immune response, such as CR3 and FcγR. CR3 is a β_2-integrin involved in cellular locomotion, mediates PMNs adhesion to the cellular matrix [22], captures iC3b-coated pathogens [23] and triggers microbicidal cellular functions. In our experiments we activated CR3 with zymosan particles (Z) that interact with the receptor *via* surface β-glucans, thus mimicking the challenge of PMNs with bacteria and yeast [23]. FcγR [24] bridges the innate and adaptive immune responses and has a critical role in antibody-mediated inflammatory responses. We stimulated FcγR with human heat aggregated IgG (IgGa) that mimicks insoluble immune complexes and triggers cellular functions by coactivating FcγRII and III [25].

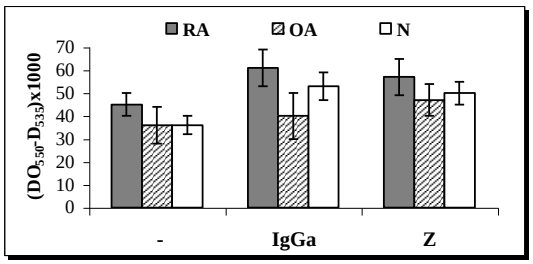
For the investigated groups of patients and normal subjects, our experimental data **(Figure 1)** show no statistically significant differences at the level of peripheral PMNs functionality (phagocytosis, superoxide anion release) in all the experimental models of cellular activation (Z, IgGa), suggesting that the local joint inflammation has no echo in the peripheral immune compartment.

In vitro exposure of PMNs to 0.01-1 μg mL^{-1} MTX have no major effect on the CR3-mediated phagocytosis in the case of normal subjects and RA/OA patients **(Figure 2)**. We emphasize though that MTX tends to intensify phagocytosis in the case of PMNs from normal subjects and RA patients, but distinct drug concentrations are biologically active for each group: 1 μg mL^{-1} MTX for normals ($p_{one\ tail}<0.06$) and 0.01 μg mL^{-1} MTX for RA patients ($p_{one\ tail}<0.05$). In the case of OA patients **(Figure 2)** 0.01 μg mL^{-1} and 1 μg mL^{-1} MTX have a slight inhibitory action on phagocytosis ($p_{one\ tail}<0.08$). These opposite effects exerted by

MTX on the peripheral PMNs from the OA patients and the normal/RA groups are statistically significant ($p_{one\ tail}<0.05$ and $p_{one\ tail}<0.06$, respectively).



a. CR3-mediated phagocytosis



b. The superoxide anion released by peripheral PMNs stimulated with 100μg mL^{-1} IgGa or 1mg mL^{-1} Z.

Figure 1. Functional parameters of peripheral PMNs isolated from normal subjects (N) (n=8), RA (n=8) and OA (n=5) patients.

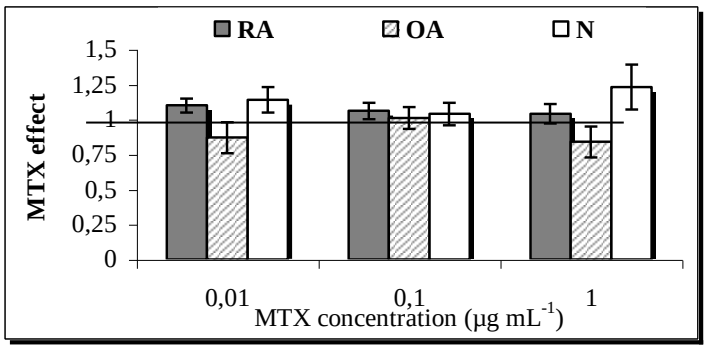


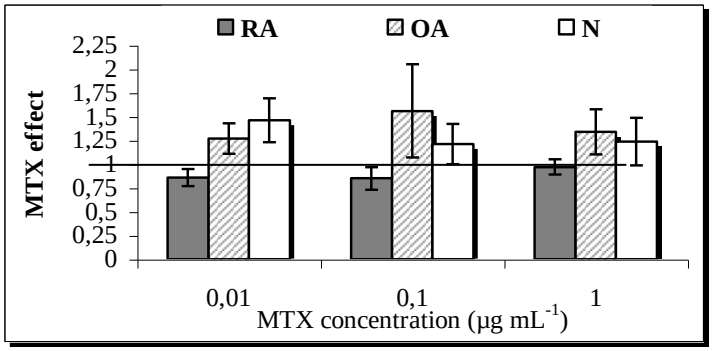
Figure 2. The effect exerted *in vitro* by MTX on the CR3-mediated phagocytosis developed by peripheral PMNs isolated from normal subjects (N) (n=8), RA (n=8) and OA (n=5) patients.

0.01 μg mL^{-1} MTX tends to stimulate the basal respiratory burst of normal cells ($p_{one\ tail}<0.05$), while slightly inhibiting ($p_{one\ tail} < 0.08$) the functionality of PMNs from RA patients **(Figure 3a)**. MTX has no statistical effect on the basal respiratory burst of PMNs isolated from OA patients. The differences between the RA and the normal or OA groups are relevant at 0.01 μg mL^{-1} MTX ($p_{one\ tail}<0.04$).

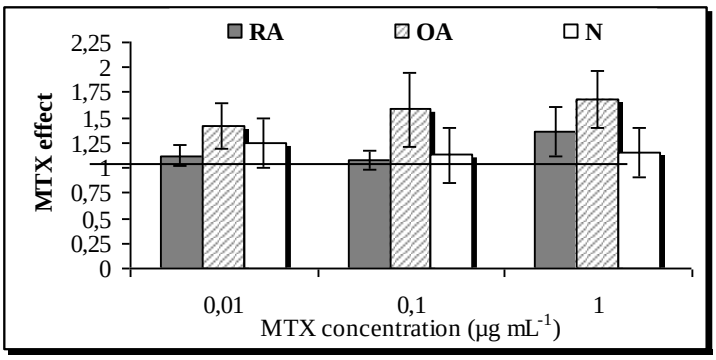
Higher doses of MTX (1 μg mL^{-1}) tend to stimulate the superoxide anion release triggered by IgGa *via* FcγR in the case of PMNs from RA and OA patients ($p_{one\ tail}<0.09$, $p_{one\ tail}<0.04$) **(Figure 3b)**, while having no effect on normal IgGa-activated PMNs.

When PMNs are activated *via* CR3 with Z, MTX has a different pattern of action **(Figure 3c)**. Superoxide anion release by normal PMNs is stimulated by MTX especially at higher doses of drug (0.1 and 1 μg mL^{-1}, $p_{two\ tail}<0.03$), while PMNs isolated from RA and OA patients are not affected. Statistically significant differences between the normal and the RA/OA groups ($p_{one\ tail}<0.03$) were observed. A negative correlation between the effect exerted by MTX and the magnitude of the superoxide anion release by normal Z-activated PMNs (Pearson correlation= - 0.71) indicates that high-responsive cells are less sensitive to MTX. No correlation between MTX effect on the CR3-mediated phagocytosis and the corresponding respiratory burst were noticed, suggesting that the signals that trigger these cellular functions are not connected. It seems that, in the case of normal PMNs, MTX

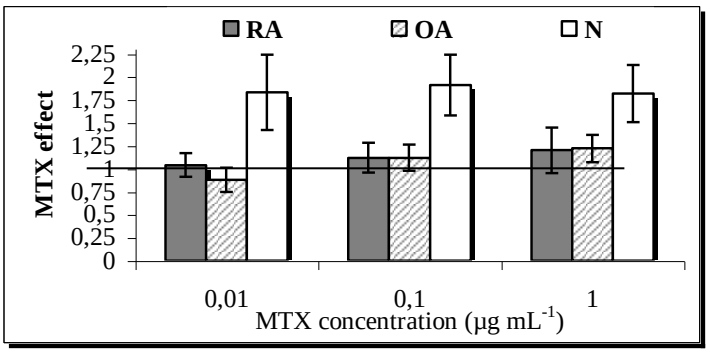
stimulates the translocation of NADPH-oxidase components to the plasma membrane, thus favoring superoxide anion leakage outside the cells despite the phagosome formation.



a. unstimulated PMNs



b. PMNs stimulated with 100μg mL^{-1} IgGa



c. PMNs stimulated with 1 mg mL^{-1} Z

Figure 3. The effect exerted *in vitro* by MTX on the superoxide anion release of peripheral PMNs isolated from normal subjects (N) (n=8), RA (n=8) and OA (n=5) patients. PMNs were *in vitro* activated with 100 μg mL^{-1} IgGa or 1mg mL^{-1}.

Our results indicate that MTX interferes with the functionality of non-proliferating cells and, considering the short time of cells exposure to MTX (20 min), drug action is probably not associated with DHFR inhibition. Considering that MTX action is highly dependent on the receptors that trigger cellular activation, the observed rapid effects exerted by the drug on granulocyte respiratory burst might be mediated by MTX-specific receptors [5]. Accordingly, functional interferences between MTX receptors and CR3 or FcγR were highlighted.

The* in vitro *effect of MTX on human peripheral lymphoytes

In vivo lymphocyte proliferation during the antigen-specific immune response is generally mimicked *in vitro* by polyclonal activation of mononuclear cells with lectin mitogens [26], such as phytohemmagglutinin (PHA) which is specific for CD4+ and CD8+ T lymhocytes [27] and pokeweed mitogen (PWM) which is specific for B lymphocytes and also for a subpopulation of T lymphocytes that helps B lymphocytes [27]. We measured the mitogen-induced lymphocyte proliferation by the radioisotopic method using tritium-labeled uridine in order to avoid the artifacts generated by the use of thymidine due to the MTX-induced decrease of the intracellular thymidine pool [28].

In all the experimental models of cellular activation (PHA, PWM), our experimental data show no statistically significant differences of the proliferation potential of peripheral lymphocytes from RA patients and normal subjects **(Figure 4)**. OA patients show a slightly enhanced proliferative capacity, especially when stimulated with PHA ($p_{one\ tail}<0,08$) **(Figure 4)**.

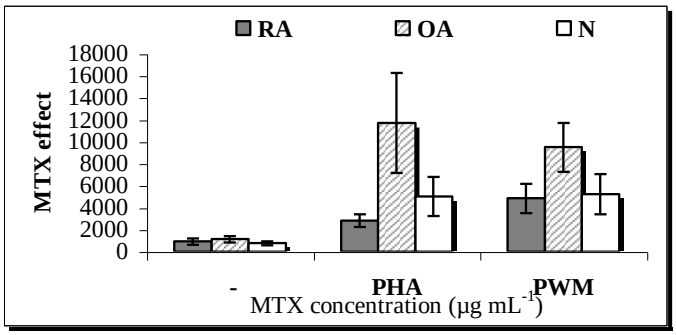
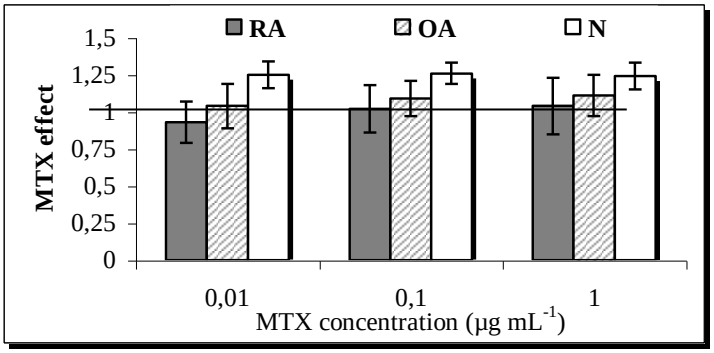


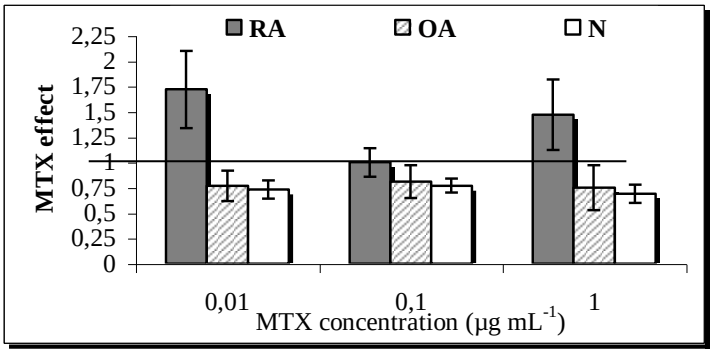
Figure 4. The proliferation capacity of peripheral lymphocytes isolated from normal subjects (N) (n=8), RA (n=8) and OA (n=5) patients, activated *in vitro* with lectin mitogens (PHA or PWM).

MTX exerts *in vitro* no statistically relevant effect on the basal proliferation of lymphocytes isolated either from normal subjects or patients **(Figure 5a)**. The investigated low-doses of MTX might be insufficient to inhibit DHFR or enzyme inhibition is accompanied by activation of nucleotide salvage pathway and consequently the cell cycle is not affected by the drug [29]. Another promising hypothesis, is that MTX binding to DHFR expressed by slow proliferating cells possibly blocks the normal degradation of the enzyme [30].

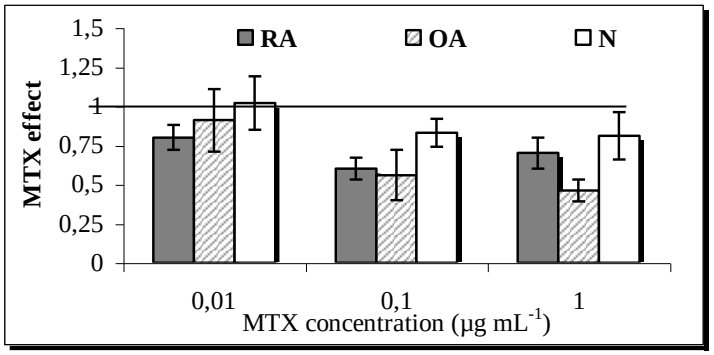
All the tested doses of MTX have an immunosuppressive action on normal T lymphocytes activated with PHA ($p_{two\ tail}<0.04$) **(Figure 5b)**. A good positive correlation was observed at 0.1 μg mL^{-1} (Pearson correlation=0.84), indicating that low proliferation rates are associated with a more intense inhibitory effect of MTX. Surprisingly, in the case of RA patients, low doses of MTX (0.01 μg mL^{-1}) tend to activate the cellular response of PHA-activated T lymphocytes ($p_{one\ tail}<0.08$), while higher drug concentrations have no significant effects **(Figure 5b)**. This stimulatory action is negatively correlated with the intensity of proliferation (Pearson correlation=-0.93), indicating that low proliferative responses are predisposed to MTX stimulation. The drug exerts no statistically significant effect on T lymphocytes isolated from OA patients **(Figure 5b)**.



a. unstimulated lymphocytes



b. lymphocytes stimulated with PHA



c. lymphocytes stimulated with PWM

Figure 5. The effect exerted *in vitro* by MTX on the proliferation of peripheral lymphocytes isolated from normal subjects (N) (n=6), RA (n=6) and OA (n=5) patients, stimulated with lectin mitogens (PHA or PWM).

In the case of PWM-activated B lymphocytes **(Figure 5c)** 0.1 and 1 μg mL^{-1} MTX exert an antiproliferative action on lymphocytes isolated from RA ($p_{two\ tail}<0.04$) and OA ($p_{two\ tail}<0.001$) patients, while having no effect on normal cells. The difference between patients and normal subjects is significant, especially at 0.1 μg mL^{-1} ($p_{two\ tail}<0.05$) and is not dependent on the intensity of cell proliferation. Thus, PWM-stimulated lymphocytes from OA and RA patients show similar reactivity to MTX, although OA lymphocytes develop a more intense proliferative response (stimulation index induced by PWM: 8.9 ± 2.5 for OA patients compared with 3.1 ± 0.9 for RA patients, $p_{one\ tail}<0.05$).

The intriguing fact that, in certain lymphocyte activation conditions, MTX exerts no effect or even stimulates cellular proliferation might be explained by differences at the level of DHFR sensitivity to MTX, by enhanced nucleotide synthesis *via* the salvage pathway or by different requirements for purine nucleotides in the polyclonal expansion of lymphocytes

[29]. Anyway, our proliferation results have to be further verified by an alternative experimental method for detecting DNA synthesis, in order to avoid the artifacts related to MTX-induced deficiency of purines synthesis.

Our results revealed a high interindividual variability with respect to MTX effects in all the investigated experimental models. The correlation between dose and the clinical effect of MTX is not clear, but a marked interindividual variability is reported, mainly regarding the beneficial effects versus toxicity of MTX [31].

Conclusion

Our study points out the fact that low concentrations of MTX alter *in vitro* various functions of both proliferative (lymphocytes) and non-proliferative (granulocytes) peripheral immune cells, even after short *in vitro* exposure of cells to the drug. The statistical effects exerted *in vitro* by MTX are dependent on the drug concentration and on the receptor that triggers cellular activation. The action of MTX is also differentiated according to the pathology (normal individuals, RA or OA patients), although the investigated groups presented statistically similar cellular functional parameters.

The dependence of the drug action on the receptors that trigger PMNs activation (CR3, FcR) suggests a possible interaction between these receptors and the folate receptors expressed by PMNs. Possibly, at low doses of MTX, receptor-mediated intake of the drug is outweighing the carrier-mediated transport.

Our results point out the efect that low concentrations of MTX exert both inhibitory and stimulatory effects on polyclonal lymphocyte proliferation. The investigated low doses of MTX might be insufficient to inhibit DHFR or different requirements for *de novo* purine synthesis characterize lymphocyte polyclonal expansion.

According to our experimental data obtained *in vitro*, the mechanisms underlying the reversible susceptibility to infections induced by MTX treatment in RA are not associated with deficient CR3-mediated phagocytosis or superoxide anion generation. MTX does not exert toxic effects by amplifying the oxidative stress developed by activated PMNs in RA peripheral blood. The high interindividual variability onserved by us indicates that MTX therapy has to be individually adjusted.

This experimental model might be physiologically relevant for the systemic effects of MTX and its immunomodulatory or immunotoxic action. The studies focused on the effects exerted *in vitro* by MTX are important for the background and further development of new therapeutical approaches combining MTX with other DMARDs or biologic agents, aiming to limit inflammation and to prevent long-term tissue damage at acceptable drug toxicity.

Acknowledgements

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