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Cytogenetic effects of treatment with methotrexate and infliximab in rheumatoid arthritis patients

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Interaction between two anti-rheumatic drugs, infliximab (monoclonal antibody against tumour necrosis factor α; TNFα) and methotrexate (MTX; disease-modifying anti-rheumatic drug), was analysed in peripheral blood lymphocyte cultures using cytogenetic tests. Genotoxicity of low MTX doses was established in peripheral blood lymphocyte cultures *in vitro* and in peripheral blood lymphocytes from rheumatoid arthritis (RA) patients treated with MTX for short or long periods. In cell culture, blockade of TNFα before cell exposure to MTX significantly decreased the number of baseline and MTX-induced sister chromatid exchanges, showing a possible protective effect of infliximab. However, a significant impairment of cell proliferative abilities was observed in lymphocytes from RA patients after treatment with infliximab and MTX. Our study suggests that some anti-rheumatic drugs can impair the genetic stability and proliferation of immune cells in RA patients. These effects can be responsible for the increased risk of infectious diseases and cancer observed among arthritis patients.

Key words: rheumatoid arthritis, infliximab, methotrexate, sister chromatid exchanges, proliferative abilities

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by a progressive destruction of synovial joints. Joint damage in RA is mediated by inflammatory cytokines, particularly the tumour necrosis factor (TNF) α and interleukin (IL)-1. Inflammatory cytokines induce tissue-degrading enzymes that attack collagen and proteoglycan, leading to destruction of cartilage, nearby bone, and periarticular tissues [1]. The management of RA is based on treatment with nonsteroidal anti-inflammatory drugs (NSAIDs), steroids and disease-modifying anti-rheumatic drugs (DMARDS) administered to patients in the early phase of the disease [2]. Among DMARDs, methotrexate (MTX) is the most commonly used treatment for RA. MTX and other DMARDs control rather than cure RA. Symptoms of the disease are ameliorated without a significant effect on joint destruction [2]. It is thought that MTX causes clinical improvement in RA through suppression of the activity and viability of antigen-activated immune cells [3]. Acute cellular toxicity induced by MTX [3–6] is directly related to unfavorable clinical effects, such as hepatotoxicity, pulmonary toxicity, gastrointestinal events and lymphoproliferative disorders observed during MTX treatment [7].

Critical changes in the management of RA were achieved after discovery of the new treatment strategies that directly neutralize the inflammatory cytokines TNFα and IL-1. A marked improvement in the outcome of active RA is achieved with the usage of those novel biotherapies [8]. Infliximab is a chimeric monoclonal antibody against TNFα currently used for the treatment of active RA. Infliximab directly binds the soluble and membrane TNFα, blocks activity of cytokine, and induces cell cycle arrest or even death of cytokine-producing cells [1, 8]. Multiple clinical trials [9, 10] have achieved excellent results showing a rapid and profound benefit of anti-TNFα therapy for all the clinical response variables measured, including inhibition of structural joint damage. In addition, a combination of infliximab with MTX was shown to be more efficient than treatment with only infusions of infliximab. However, the question of the long-term safety of anti-TNFα agents is yet to be resolved. Increased risk of lymphoma, reactivation of opportunistic infections causing tuberculosis, pneumocystitis and other diseases have been observed in patients treated with anti-TNFα agents [11, 12], indicating the need for a thorough clinical follow-up during and after treatment.

Mortality rates are higher among patients with RA due to the severity of disease and increased rates of infections and malignancies [13]. This raises the need for a more detailed analysis of anti-rheumatic drugs and their combinations at the cellular and molecular level. Recently [14] we have established a marked impairment of proliferative abilities of peripheral blood lymphocytes (PBL) from patients with arthritis, including RA, as compared to healthy controls. Significant associations between the decrease of proliferative abilities of PBL and variables of

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disease, including cytokine production, were established in our study. We addressed the question whether the changes in proliferative abilities of lymphocytes can be related to patient treatment with MTX or MTX and infliximab, and analysed a possible cytotoxic and genotoxic effect of combined drug application in *in vitro* and *in vivo* systems.

METHODS

Patients and treatment

Patient selection and treatment were conducted at the Rheumatology Department of Vilnius Central University Hospital as part of a larger prospective study [15]. The study was approved by the local Bioethics Committee. For the analysis of MTX effect, 16 patients with seropositive RA were selected. Six of them were diagnosed with early RA and had no prior treatment with MTX. Ten patients had a long-standing disease, with the treatment of MTX lasting more than 6 months. For the analysis of cytogenetic parameters, peripheral blood was collected at the baseline, and for the cases with early RA 4 weeks after initiation of MTX treatment. The mean treatment dose was 10 mg (range 7.5–12.5 mg) of MTX (Ebewe) per week. All patients were females. Five other female patients meeting active RA criteria and not responding to MTX or other DMARD therapy were selected for infliximab treatment. The treatment included three infusions of infliximab (Remicade™, Centocor) at a dose of 3 mg/kg at weeks 0, 2 and 6. All patients received 3 infusions of the drug. The patients continued NSAIDS, corticosteroids (≤10 mg prednisolone/day) and MTX (10 or 12.5 mg/week). For cytogenetic analysis, peripheral blood was collected at the time of enrolment and after the third injection of infliximab. Patients were evaluated for side effects, and clinical-laboratory tests were performed at each visit. At enrolment, all patients answered a questionnaire concerning life-style, occupational and other exposures with a suspected genotoxic effect. None of them were smokers or declared harmful occupational exposure. The main demographic and clinical characteristics of study groups are presented in Table 1.

For the analysis *in vitro*, PBL were collected from 3 healthy donors (all female) with the mean age of 25 years, and 3 patients with RA (1 male, 2 female) with the mean age of 51 years. Purified mouse anti-human TNFα monoclonal antibody (anti-TNFα, PharMingen) in a dose of 2 μg/ml was added at the initiation of cell culture growth in order to completely inhibit soluble or membrane-bound TNFα. MTX (Ebewe) in a dose of 5–100 μg/ml was added into the cell culture at 0, 24 or 64 h of cell growth. A marked inhibition of cell growth was observed in cell cultures exposed to MTX for 72 or 48 h. Also, the concentrations of MTX exceeding 50 μg/ml were toxic for PBL cultures. The exposure of cell cultures for 6 h to 5, 10 and 50 μg/ml of MTX was chosen as the main cell treatment regimen. PBL cultures were exposed to MTX only, or in combination with MTX to 2 μg/ml of anti-TNFα.

Table 1. **Main characteristics of the study groups**

Characteristics	MTX-1 n = 6	MTX-2 n = 10	MTX with infliximab n = 5
Age, years	60.0 ± 3.7 (50–74)	49.1 ± 4.9 (35–75)	48.0 ± 4.5 (35–60)
Disease duration, months	1.5 ± 0.13 (1–2)	14.4 ± 2.6 (2–25)	6.6 ± 1.6 (2–15)
MTX, mg/week	10.4 ± 0.4 (10.0–12.5)	10.8 ± 0.53 (7.5–12.5)	9.5 ± 0.9 (7.5–12.5)

Results are presented as the mean value ± SEM (variation); MTX-1 – the group of patients starting MTX usage, MTX-2 – long-term (>6 months) MTX users, MTX with infliximab – the group treated with infliximab together with MTX.

Cytogenetic analysis

Peripheral blood (7 ml) from patients with RA, ReA, and healthy controls was collected by venipuncture into heparinised tubes. PBL cultures were grown in 10 ml of RPMI 1640 medium with HEPES supplemented with 12% heat-inactivated foetal bovine serum, 50 μg/ml gentamycin and 10 μg/ml 5-bromo-2'-deoxyuridine (BrdU; all reagents from Sigma). For lymphocyte activation, polyclonal mitogen phytohemagglutinin (PHA, 8 μg/ml) was used. Duplicated cultures from each person were grown at 37 °C for 72 hours, and colchicine (0.6 μg/ml) was added for the last 3 hours to collect cells in metaphase. Cells were harvested by hypotonisation in 0.075 M KCl solution and then fixed three times in 3:1 methanol and acetic acid. 2–4 air-dried slides were prepared from each cell culture and stained by the fluorescence plus Giemsa technique [16]. For each person, 50–100 second-division metaphase cells with 44–47 chromosomes were analysed to evaluate the mean SCE number per cell. Cell proliferative abilities were evaluated using the replication index (RI) and mitotic activity (MA) of cells. RI shows the average number of divisions performed by a cell in a cell culture medium containing BrdU. The percentage of cells in mitosis among randomly distributed 1000 cells corresponds to MA.

Statistical analysis

Two-sided Fisher's exact test and Student's t test were used for comparison of categorical and continuous variables, respectively. The value of $P \leq 0.05$ was considered as statistically significant.

RESULTS

Cytogenetic effect of MTX and anti-TNFα *in vitro*

To evaluate the genotoxicity and cytotoxicity of MTX, PBL cultures from healthy controls (n = 3) and RA patients (n = 2) were incubated with 5, 10 and 50 μg/ml of MTX (Table 2). At the baseline, i. e. without exposure to MTX, statistically significantly ($p < 0.05$) higher rates of SCE/cell were observed in lymphocytes from RA patients as compared to healthy donors, while the measures of cell proliferative abilities were similar in all cases. The lowest dose of MTX (5 μg/ml) caused an increase of SCE/cell in cell cultures from RA patients; the effect was at the statistical borderline of significance ($p = 0.05$; Table 2). No significant increase in frequencies of SCE was observed in cell cultures of healthy donors incubated with 5–50 μg/ml of MTX. A statistically significant ($p < 0.05$) decrease of RI was observed in cell cultures from healthy donors exposed to MTX. MTX exposure also caused a marked impairment of cell mitotic activity in cell cultures from healthy donors and from RA patients (Table 2).

The effect of pre-treatment with monoclonal antibody against TNFα (anti-TNFα, 2 μg/ml) was studied in PBL cultures from 2 healthy donors and 2 RA patients exposed to MTX. A statistically significant decrease in the baseline level of SCM/cell (9.06 ± 0.51 vs. 7.70 ± 0.41, $p < 0.05$) was observed in cell cultures from a healthy donor exposed to anti-TNFα (data not shown). In addition, TNFα blockade statistically significantly (Figure) decreased the number of SCE/cell in PBL from a RA patient exposed to 5 μg/ml of MTX (10.30 ± 0.51 vs. 8.54 ± 0.38; $p < 0.01$). TNFα blockade did not change the proliferation rates of PHA-activated cells as measured by RI. A statistically significant ($p < 0.05$) increase of MA was observed in cell cultures from healthy donors and a RA patient pre-treated with anti-TNFα and then exposed to MTX (10 and 50 μg/ml) as compared with cultures exposed to MTX only (Figure).

Table 2. **Frequency of sister chromatid exchanges (SCE) and proliferative abilities (RI and MA) in cultured peripheral blood lymphocytes from patients with arthritis and healthy controls exposed to methotrexate (MTX) *in vitro***

MTX, μg/ml	SCE/cell	RI	MA
Healthy controls			
0	8.25 ± 0.30	2.84 ± 0.04	2.82 ± 0.29
5	8.64 ± 0.30	2.68 ± 0.04[a]	2.15 ± 0.26
10	7.82 ± 0.30	2.67 ± 0.04[a]	1.11 ± 0.19[a]
50	8.52 ± 0.29	2.68 ± 0.04[a]	1.35 ± 0.20[a]
Arthritis patients			
0	9.50 ± 0.30[b]	2.84 ± 0.03	2.21 ± 0.23
5	10.47 ± 0.40[a,b]	2.90 ± 0.05	1.39 ± 0.26[a]
10	8.70 ± 0.37	2.97 ± 0.05[a]	1.21 ± 0.24[a]
50	8.49 ± 0.36	2.92 ± 0.05	0.44 ± 0.14[a]

Results from independent experiments in cell cultures from healthy controls (n = 3) or rheumatoid arthritis patients (n = 2) were pooled and expressed as the mean value ± SEM. Statistically significantly ($p \leq 0.05$) different [a]as compared to MTX non-exposed control, [b]as compared to healthy controls exposed to an equal dose of MTX.

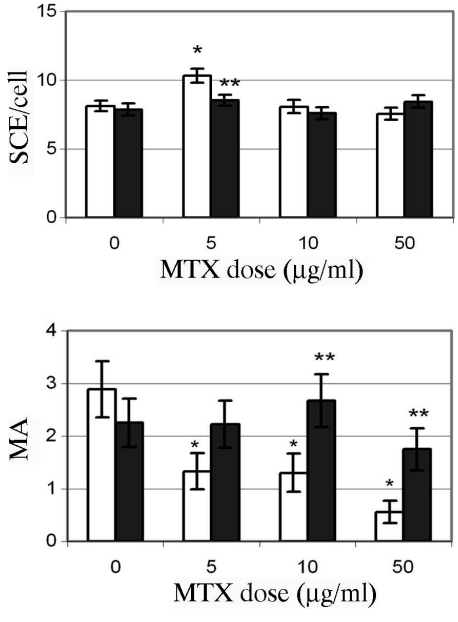

Figure. Frequency of sister chromatid exchanges (SCE) and mitotic activity (MA) in cell cultures from peripheral blood lymphocytes of rheumatoid arthritis patient pre-treated with anti-TNFα (2 μg/ml) and exposed to different doses of MTX. White columns – MTX exposure, black columns – MTX exposure after pre-treatment with anti-TNFα. Statistically significantly ($p \leq 0.05$) different * when compared with non-exposed control, ** when compared with cultures not pre-treated with anti-TNFα.

Cytogenetic effect of MTX and infliximab on cells from treated patients

To evaluate the cytogenetic effect of MTX therapy, two groups of patients with RA were analysed: patients with early RA (PBL collected before the initiation of MTX treatment and 4 weeks after) and long-term users of MTX (>6 months). Data according to a cumulative dose of MTX in the study groups are presented in Table 3. The number of SCE/cell increased with an increase in the cumulative dose of MTX. The effect did not reach statistical significance when PBL from RA patients treated with MTX were compared with PBL from RA patients, collected before MTX treatment. When comparing our data with the data of healthy controls of the above-mentioned larger study [14], a significant increase in SCE/cell in PBL from short-term (8.59 ± 0.48 vs. 7.38 ± 0.95, $p < 0.01$) and long-term (8.81 ± 0.56 vs. 7.38 ± 0.95, $p \leq 0.001$) users of MTX was observed. The usage of MTX did not cause any marked change in the measures of cell proliferative abilities (Table 3) when compared with PBL from RA patients before MTX treatment or with the healthy controls.

Five long-term MTX users with severe RA were selected for infliximab treatment and received three infusions of infliximab, while MTX treatment was also continued. A slightly lower frequency of SCE was observed in this group of patients as compared to long-term MTX users, and the number of SCE diminished even more after the treatment with infliximab (Table 3). An evident decrease (3.35 ± 0.45 vs. 0.85 ± 0.21, $p \leq 0.001$) of MA was observed in PBL grown in cell cultures after three infusions of infliximab, while the rate of proliferation was the same as before the treatment.

Table 3. **Frequency of sister chromatid exchanges (SCE) and proliferative abilities (RI and MA) in peripheral blood lymphocytes from rheumatoid arthritis patients treated with infliximab and/or methotrexate (MTX)**

Treatment	n	SCE/cell	RI	MA
Healthy controls[a]				
No treatment	30	7.38 ± 0.95	2.59±0.14	3.82±1.66
MTX				
Before therapy	5	8.10 ± 0.54	2.57 ± 0.06	3,21 ± 0.51
4 × 10 mg	6	8.59 ± 0.48[b]	2.58 ± 0.07	3.62 ± 0.21
>24 × 10 mg	10	8.81 ± 0.56[b]	2.59 ± 0.07	3.48 ± 0.35
MTX with infliximab				
Before therapy	5	7.94 ± 0.20	2.41 ± 0.10	3.35 ± 0.45
After therapy	5	7.42 ± 0.11	2.38 ± 0.10	0.85 ± 0.21[c]

Results are presented as the mean value ± SEM. [a]Data published in [14]. Statistically significantly ($p \leq 0.01$) different [b]when compared to healthy controls or [c]when compared to RA patients before treatment or to RA patients treated with MTX only.

DISCUSSION

Twenty-one patients with RA treated with MTX or with the combination of infliximab and MTX were analysed in our study by cytogenetic tests. In addition, the interaction of MTX and monoclonal antibody to TNFα was analysed in PBL cultures *in vitro*. The genotoxicity of low doses of MTX was established in PBL *in vitro* and in PBL from patients treated with MTX. The *in vitro* blockade of TNFα before cell exposure to MTX significantly decreased the number of baseline and MTX-induced SCE and improved the proliferative abilities of cells. However, a significant impairment of cell proliferative abilities was observed in lymphocytes from RA patients after treatment with infliximab and MTX.

Cytogenetic parameters such as chromosomal aberrations, sister chromatid exchanges or micronuclei, are the markers widely used to evaluate the genetic integrity of cells exposed to harmful agents of environmental or internal origin [17]. SCE are symmetrical exchanges between sister chromatids in the chromosome, which form during the processes of DNA replication and reparation. In several studies [7, 18–22], cytogenetic alterations have been observed in PBL and epithelial cells of patients with RA and ankylosing spondylitis. Some of the studies [4, 7, 19] revealed the genotoxic effect of several anti-rheumatic drugs. Also, direct associations between the increase of genetic damage and inflammation-induced oxidative stress have been observed in PBL from RA patients [20, 23]. Multiple chromosomal aberrations are also detectable in synovial cells of rheumatic patients as a consequence of chronic oxidative stress [21]. In our study, patients with RA possessed a higher number of SCE than did healthy donors, and the number of genetic alterations was statistically significantly higher in PBL from RA patients treated with MTX for short (4 weeks) or long (>6 months) periods as compared to non-treated healthy controls. The genotoxic effect of 50 μg/ml of MTX, the concentration of the drug obtained in the serum of a patient after a low dose MTX treatment [3] was also detected in the *in vitro* analysis. The genotoxicity of MTX has been shown in multiple studies using cell culture models, laboratory animals, and assessing patients treated with MTX [4, 5, 7, 13, 22, 24]. In our study, a marked reduction of cell proliferative abilities, especially the mitotic activity, was observed in a cell culture exposed to MTX. The suppressive effect of MTX therapy was less evident. Similar to our observation, anti-proliferative activity of MTX was shown in different cell cultures [22, 25, 26] with a milder effect seen in PBL cells from treated patients [6].

MTX is a folate antagonist exerting its activities through inhibition of several enzymes involved in purine and pyrimidine synthesis. A deficiency in nucleotide synthesis can cause genetic damage to the cell, reduce the efficiency of damage repair and decrease the ability of cells to proliferate and even induce their death [3]. The usage of folinic acid was shown as a protective means that decreases the genotoxic and cytotoxic effects of MTX [4, 5]. In our study, a significant decrease of spontaneous and MTX-induced SCE was observed upon cell pretreatment with anti-TNFα. Our results suggested that, within a special treatment schedule, TNFα blockade can decrease the risk of genetic damage caused by MTX and chronic inflammation. TNFα itself is known as a genotoxic agent exerting its effect through induction of reactive compounds such as nitric oxide [27, 28]. In RA, the TNFα production is significantly increased as compared to healthy controls [15], thus the genotoxic alterations can be caused by TNFα itself. Therefore, the protective effect of monoclonal antibodies against TNFα can be caused by a reduction in the amounts of TNFα and cytokine-induced reactive compounds. Further studies are needed for a detailed exploration of a possible gene-protective effect of TNFα blockade in conditions of chronic inflammation.

Despite the protective activities of anti-TNFα observed *in vitro,* the treatment of patients with infliximab and MTX significantly impaired the proliferative abilities of PBL. The effect was not seen in *in vitro* experiments, possibly because of the difference in the sequence of exposure to the drugs or a lower stability of the monoclonal antibody against TNFα in *in vivo* conditions. Despite this discrepancy, our study suggests a synergistic suppressive effect of infliximab and MTX on the proliferative abilities of PBL from RA patients. The strategy of intensive immunosuppression is widely used in the treatment of RA and other autoimmune diseases. Recent analysis of the molecular mechanisms of the anti-inflammatory effect of MTX [6, 26] and infliximab [29–31], supports the possibility of a synergistic interaction between infliximab and MTX. The anti-inflammatory effect of MTX is mainly based on induction of apoptosis in mitogen-activated cells through a depletion of the nucleotide pool and generation of reactive oxygen species [6, 25], while infliximab can induce cell death through signal transduction from the membrane-bound TNFα [28, 29].

This and our previous [14] studies suggest that the disease-related conditions and cytotoxicity of some anti-rheumatic drugs may impair the genetic stability and proliferation of immune cells in RA patients. Because the blockade of TNFα, the cytokine responsible for cellular defense against infection and cancer can increase the risk of infectious diseases and malignancies [9, 11, 12], special attention should be drawn to the characteristics of the immune system in patients proposed for treatment with anti-TNFα agents.

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METOTREKSATO IR INFLIKSIMABO CITOGENETINIO EFEKTO ĮVERTINIMAS SERGANTIESIEMS REUMATOIDINIU ARTRITU

S a n t r a u k a

Infliksimabas – tai monokloninis antikūnas prieš navikų nekrozės veiksnį (TNFα), metoteksatas (MTX) – ligą modifikuojatis priešreumatinis vaistas. Naudodami citogenetinius testus, įvertinome šių dviejų vaistų nuo reumatoidinio artrito sąveiką periferinio kraujo limfocitų (PKL) kultūrose. MTX *in vitro* paveiktose PKL kultūrose nustatytas mažų vaisto dozių genotoksiškumas. Gydymas MTX pasižymėjo genotoksiniu poveikiu tiek vaistą naudojant ilgai, tiek ir trumpai. Atrankaus TNFα inhibitoriaus įvedimas į PKL kultūras prieš MTX naudojimą sumažino pastarojo genotoksiškumą. Tiriant infliksimabo kartu su MTX terapijos poveikį, buvo nustatytas ženklus ligonių limfocitų proliferacinių charakteristikų sumažėjimas, palyginus su tik MTX gydytais ligoniais. Mūsų tyrimas rodo, kad kai kurie vaistai nuo reumato sumažina leukocitų genetinį stabilumą bei slopina jų sugebėjimą dalytis. Šios priešreumatinių vaistų savybės gali lemti didesnę infekcinių ir onkologinių ligų riziką, dažną tarp sergančiųjų reumatoidiniu artritu.