Reduced folate carrier polymorphism determines methotrexate uptake by B cells and CD4+ T cells

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Objective. To examine if polymorphism 80G → A in the Reduced Folate Carrier (RFC) affects uptake of MTX in B- and CD4+ T-cells.

Methods. Mononuclear cells were isolated from peripheral blood of healthy persons. Real-time PCR was used to detect the RFC80 variants. FITC-labelled MTX was added to cells stimulated with Candida albicans or tetanus toxoid, and the uptake of MTX was measured by flow cytometry. A FITC-conjugated monoclonal antibody against RFC was used to detect the cellular RFC expression.

Results. Antigen-stimulated CD4+ T cells and B cells from individuals with the GG variant (n = 9) exhibited lower uptake of MTX than individuals expressing the AA variant (n = 8), or the GA variant (n = 8). No difference could be demonstrated between the three groups with respect to the expression of RFC by CD4+ T cells and B cells, and CD4+ T cells from individuals homozygous for the G allele exhibited lower uptake of MTX per receptor than CD4+ T cells from individuals homozygous for the A allele.

Conclusion. MTX is taken up more efficiently via the A allele than via the G allele. This difference between the variant forms of RFC suggests that genotyping could be relevant for determining the relevant dosage of MTX in the treatment of neoplastic and autoimmune disease.

Key words: Methotrexate, Genetic polymorphism, Reduced folate carrier.

MTX is among the most widely used drugs in the treatment of inflammatory rheumatic diseases. Patients with RA receive MTX for longer periods than other DMARDs [1]. However, the response to MTX varies greatly, and 10–30% of the patients discontinue MTX therapy due to toxicity [2]. Therefore, methods that can predict efficacy and side-effects are needed. Measurement of the plasma levels of MTX as a predictor of efficacy or toxicity has little value, as no MTX can be detected in the blood 24 h after administration [3]. Intracellular levels of MTX polyglutamates in erythrocytes and polymorphonuclear cells have been shown to correlate with the efficacy in RA patients, but analysis for MTX polyglutamate levels is not used in the daily clinic [4].

MTX enters the cell via the RFC primarily [5]. An RFC 80G → A polymorphism (histidine-to-arginine substitution at codon 27) has been described, but it has been a matter of controversy whether this polymorphism affects the cellular uptake of MTX [6]. In a study of children with acute lymphoblastic leukemia, patients with the AA variant displayed higher MTX plasma levels and a worse outcome than patients with GG or AG [7]. In contrast, RA patients with AA variant revealed a better clinical response to MTX than patients with different variants [8].

In the present study, we have measured the direct uptake of FITC-labelled MTX by CD4+ T cells and B cells, and demonstrate a decreased uptake capacity of B- and CD4+ T cells from individuals expressing the GG variant.

Materials and methods

Subjects

Venous blood was harvested from 25 healthy blood donors using sodium citrate as anticoagulant. The whole blood cells were washed twice in phosphate buffered saline (PBS) before isolation of peripheral blood mononuclear cells (PBMCs) by density centrifugation in lymphocyte separation medium (Axis-Shield, Oslo, Norway). The local ethics committee approved the study. Informed patient consent was obtained before inclusion into this study.

Genotyping procedures

Genomic DNA was extracted from 5 ml EDTA-stabilized blood and purified by NaCl-precipitation. Allelic discrimination was performed to detect the RFC80 G → A polymorphism using fluorogenic 3'-minor groove-binding probes in a real-time PCR assay. Each 5-μl of reaction contained 160 nM of each probe (GG: 5'-FAM-CAC GAG GCG CCG C-MGB-3' and AA: 5'-VIC-CAC ACG AAG TGC CGC-MGB-3'), 720 nM each of forward primer 5'-CCG AGC TCC GCT G-3' and reverse primer 5'-CAT GAA GCC TTC TTG A-3', 3 μl 2X Taqman Universal PCR Master Mix (Applied Biosystems, Europe) and 10–50 ng DNA. PCR cycling conditions consisted of one 2-min cycle at 50°C, one 10-min cycle at 95°C, followed by 40 cycles of 92°C for 15s and 60°C for 1 min on an ABI 7500 Fast platform (Applied Biosystems).

Cells

PBMCs were stained with PKH-26 (PKH26 Red Fluorescent Cell Linker Kit, Sigma, Brondby, Denmark) as described [9], suspended in RPMI containing 30% (v/v) serum, and distributed in 96-well flat-welled microtitre plates (Nunc, Roskilde, Denmark). In order to stimulate B- and T cells to proliferate, the cells were grown in the presence of 10 μg/ml tetanus toxoid, a kind gift from Claus Koch (State Serum Institute, Copenhagen, Denmark) or 10 μg/ml Candida albicans (Candida) whole cell extract, kindly donated by Else Svegaard (Department of Dermatology, Bispebjerg Hospital, Copenhagen, Denmark). FITC-conjugated MTX (Wyeth, Taplow, UK) was added to a final concentration of 1 μg/ml (calculated to equalize a dose of 5 mg MTX in vivo) to some cultures at day 6. We chose to use 1 μg/ml as these doses worked equally well at 10 μg/ml for induction of apoptosis in a previous study [9]. As a control for unspecific uptake, 5-FAM-lysine (MW: 619, CPC scientific, http://www.cpscscientific.com was added instead of FITC-MTX (MW: 451).
incubated antigen-stimulated PBMCs from three individuals with different RFC80 variants was specific for MTX, we previously reported [9]. In order to examine the influence of RFC polymorphisms on the cellular uptake of MTX, we therefore estimated the uptake per receptor for each individual with FITC-labelled 5-FAM-lysine, a compound with a similar molecular weight to FITC–MTX. Indeed, the MTX uptake efficiency of RFC was assessed as the CD4+ T cell content of FITC–MTX (expressed as MFI) divided by the corresponding RFC expression (in MFI). All data are shown as mean ± S.E.M.

**Flow cytometry**

Following staining with anti-CD4-Peridinin–chlorophyll–protein complex (PerCP; DAKO, Copenhagen, Denmark) and anti-CD19-allophycocyanin (APC; Becton-Dickinson, Brondby, Denmark), respectively, the uptake of FITC-MTX or 5-FAM-lysine by CD4+ T cells and CD19+ B cells was assessed by flow cytometry using a FACScalibur (BD Bioscience, Copenhagen, Denmark) flow cytometer (Becton-Dickinson) with an argon-ion laser excitation at 488 nm. Samples from some antigen-stimulated cultures were incubated with human IgG (1 mg/ml, Baxter, IL, USA) for 1 h to block Fc-receptors, before incubation for 1/2 h with an anti-RFC antibody (Alpha Diagnostic, San Antonio, TX, USA; used at 3 μg/ml), conjugated in our lab to a FITC antibody ratio of 6.2 which corresponds to 2.0 soluble fluorocine equivalents per antibody [10]. Using the Quantum FITC MESF kit (no. 824 Alimnunodiagnostic OY, Hämeenlinna, Finland) the cellular RFC expression could be calculated from the mean fluorescence intensity (MFI) of cell-bound antibodies, assuming binding of one antibody per RFC [10].

**Statistics**

Unpaired or paired t-tests were used, the null-hypothesis being no different between the data sets compared; P < 0.05 was considered statistically significant.

**Results**

The uptake of MTX and 5-FAM-lysine by antigen-stimulated T helper cells and B cells

MTX is taken up by CD4+ T helper cells undergoing division, as previously reported [9]. In order to examine the influence of RFC polymorphisms on the cellular uptake of MTX, we therefore stimulated PBMC for proliferation with Candida or tetanus toxoid, and added MTX to the cultures at day 6 where a high degree of uptake occurs [9]. During the following 24 h, the CD4+ T cell uptake of MTX in GG variants was lower than in GA variants when Candida was used as the stimulating agent (Fig. 1A), and lower than in AA variants when the cells were stimulated with tetanus toxoid.

Following stimulation with Candida, B cells from the GG variants took up less MTX than B cells from the GA and AA variants, while no significant differences were observed upon stimulation with tetanus toxoid (Fig. 1B).

To test whether the differences in uptake capacity between cells with different RFC80 variants was specific for MTX, we incubated antigen-stimulated PBMCs from three individuals of each genotype with FITC-labelled 5-FAM-lysine, a compound with a similar molecular weight to FITC–MTX. Indeed, the uptake of 5-FAM-lysine by dividing CD4+ T cells and B cells did not differ between the three variant groups (Fig. 1C).

RFC expression by antigen-stimulated and unstimulated CD4+ T cells

We further examined whether the decreased uptake of MTX by CD4+ T cells homozygous for the RFC80 G allele was associated with a decreased expression of the receptor, or with a reduced capacity to take up MTX on receptor-to-receptor basis. To this end, we labelled a monoclonal anti-RFC antibody with FITC and used it for quantification of the cellular RFC expression by means of flow cytometry. The expression of RFC increased upon stimulation of CD4+ T cells with antigen. The numbers of RFC molecules per stimulated CD4+ T cell ranged from 992 to 1735 and did not differ between three groups of variants (Fig. 2A).

While the decreased CD4+ T cell uptake of MTX in the GG variant group (Fig. 1A) thus was not caused by low copy numbers of RFC molecules, we examined whether it could be accounted for by a decreased transport efficacy by RFC encoded by the G allele. We therefore estimated the uptake per receptor for each individual by relating the MTX uptake by Candida-stimulated CD4+ T cells to the number of RFC molecules expressed by that individual's CD4+ T cells (Fig. 2B). Indeed, antigen-stimulated CD4+ T cells from individuals with the GG variant took up significantly less MTX per receptor than cells from individuals with the AA variant.
RFC and MTX uptake in B- and CD4+ T-cells

Discussion

It is well established that the response of patients with autoimmune diseases to MTX therapy varies greatly. Neither the exact function of MTX, when used for the treatment of autoimmune diseases, nor the pharmacogenetics underlying the response variability are fully understood [5], but it is assumed that the drug must enter the cells to exert its effect. Whether genetic variations of the RFC contribute to interindividual cellular uptake of MTX has been a matter of controversy [6]. In the present study we have measured the uptake of FITC-conjugated MTX in CD4+ T cells and in B cells, both of which play important roles in the pathogenesis of RA. We show that homozygosity for the RFC80 G allele predisposes to decreased MTX uptake. Thus, CD4+ T cells of GG variants took up significantly less MTX than heterozygote cells upon stimulation with Candida, and AA cells on stimulation with tetanus toxoid. These differences were supported by similar tendencies in the reciprocal situations, and by a significantly lower MTX uptake by B cells homozygous for RFC80 A allele than by B cells from the two other variants following stimulation with Candida. Moreover, when the MTX uptake was related to the numeric expression of RFC per cell, molecules encoded by the RFC80 A allele took up twice as much MTX as molecules encoded by the G allele. The cellular expression of RFC increased upon antigenic stimulation of the cells and did not differ between the three groups. Neither did the groups differ with respect to the uptake of our control, 5-FAM-llysine. We regard it as improbable that 5-FAM-llysine was taken up via RFC80. In fact, the alternative route used by 5-FAM-llysine may also have been used for some unspecific uptake of MTX, leading to underestimation of the difference in MTX transport capacity between the RFC80 variants. The possibility remains that unspecific uptake accounts for the entire uptake observed in GG variants, due to non-functional gene products.

Our data are in agreement with a previous study in which MTX polyglutamates were used as markers for MTX uptake. Dervieux et al. [8] demonstrated that RA patients with AA variant were much more likely to have higher erythrocyte MTX polyglutamate levels than patients with the GG and GA variants. In a recent clinical study, it was found that the probability of remission was more than 3 times higher in individuals with the AA variant than in individuals with the GG variant [11]. The ACR 20 response criteria was used to measure efficacy. In a clinical study by Wessels et al. [12] genetic variants in RFC, methylenetetrahydrofolate reductase and dihydrofolate reductase were examined for toxicity and effect on the outcome of RA patients. However, no associations could be demonstrated regarding the RFC variants. There is no clear explanation for the differing results from these studies. The frequency of each variant was almost equal in the studies, ~50% GA, 30% GG and 20% AA. The usage of different methods to evaluate the outcome (ACR response vs disease activity score) does not seem to explain the opposing results. In a study where MTX uptake was measured in an in vitro model using an erythroblastic cell line, no difference among the RFC variants with respect to MTX uptake could be demonstrated [13]. However, this finding may be cell line specific.

Clearly, more studies are needed to establish whether polymorphism in the RFC gene affects the clinical efficacy of MTX. It may be relevant to examine both polymorphisms in RFC and in the multidrug resistance protein, the proteins responsible for the efflux of MTX from the cells. Wolf et al. [14] studied the potential beneficial effect of the presence of RFC and the absence of functional MRF. RFC polymorphism was not examined in the study, which focused on the presence or absence of RFC and MRF on PBMCs. The RA patients who expressed the two proteins at the same time, or did not express any of the proteins, had good response rates. On the other hand, absence of the MRF and presence of RFC did not show beneficial effects of MTX [14]. Addition of genetic polymorphisms to such a study would be of interest. Furthermore, more needs to be known about the regulation of the RFC. The protein is ubiquitously expressed, but loss of it, or decreased activity connected with certain genetic variants as demonstrated here, may be associated with MTX resistance [15].

Rheumatology key message

- Methotrexate enters the cell via RFC. Polymorphism exist for this protein, and the presence of one of these variants seems to be associated with less uptake of MTX in T and B lymphocytes.

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References