Expression of resistance markers to methotrexate predicts clinical improvement in patients with rheumatoid arthritis
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EXPRESSION OF RESISTANCE MARKERS TO METHOTREXATE PREDICTS CLINICAL IMPROVEMENT IN PATIENTS WITH RHEUMATOID ARTHRITIS

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Keywords: Rheumatoid arthritis, methotrexate, multidrug resistance, prediction, clinical response

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ABSTRACT

Objective: MTX is transported into the cell by the reduced folate carrier (RFC) and out of the cell by members of the multidrug resistance protein family (MRP). To determine the potential influence of transport proteins on the therapeutic efficacy of methotrexate (MTX) in patients with rheumatoid arthritis (RA), potential benefit of the presence of RFC and the absence of functional MRP was investigated.

Methods: 163 patients (116 female and 47 male; mean age 59.5 years) on MTX (mean weekly dose 12.2 mg) were enrolled into the study. RFC was determined using reverse transcriptase polymerase chain reaction and MRP function by flow cytometry using a calcein acetoxymethylesther/probenecid assay. Clinical response to MTX was evaluated by the EULAR response criteria and the American College of Rheumatology improvement criteria. The clinical data were obtained at the beginning of MTX therapy and at the time of blood sampling during ongoing therapy. Patients were divided into four groups according to the presence (+) and/or absence (-) of RFC and functional (f) MRP.

Results: fMRP+ RFC+ and fMRP- RFC- patients had significantly more frequent good EULAR response rates (60%; p= 0.014 and 53%; p=0.035, respectively) in comparison to the fMRP- RFC+ group (29%). Also fMRP+ RFC- patients had a low frequency of good responses in disease activity.

Conclusion: The absence of fMRP in conjunction with the presence of RFC did not prove to be related to beneficial effects of MTX, while the lack or the presence of both functional MRP and RFC led to a significantly better therapeutic outcome. Determination of these markers may predict responsiveness to MTX.

INTRODUCTION

At present, methotrexate (MTX) constitutes the gold standard of disease modifying antirheumatic drug (DMARD) therapy of rheumatoid arthritis (RA) 1,2,3,4,5,6. Although more recent insights into its mode of action suggest that MTX exerts its effect via other than folate-mediated mechanisms 6,7, folate inhibition may at least partly be involved, since higher MTX doses appear to be needed to maintain clinical efficacy among patients receiving folate supplementation compared to those without folates 8. The anti-metabolic efficacy of MTX is influenced by molecules that are also involved in the metabolism of folates. In this respect, various proteins and enzymes are involved in the transportation of MTX into and out of the cell and the degree of its intracellular retention. The reduced folate carrier (RFC) induces the influx of MTX into the cell 9, while members of the multi-drug resistance protein family (MRP 1-4) are responsible for an accelerated efflux of MTX 10,11, and folylpolyglutamyl synthetase catalyzing polyglutamylation of MTX 12.
causes a prolonged intracellular retention of MTX and leads to sustained inhibition of dihydrofolate reductase impeding the transformation of dihydrofolate to tetrahydrofolate (Table 1).

Table 1: Resistance Markers for MTX*

<table>
<thead>
<tr>
<th>Resistance marker</th>
<th>Location</th>
<th>Function</th>
<th>Method of determination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced folate carrier = RFC</td>
<td>Cell membrane protein</td>
<td>Influx of reduced folate and MTX</td>
<td>RT - PCR</td>
</tr>
<tr>
<td>Multidrug resistance proteins = MRP 1-4</td>
<td>Cell membrane proteins</td>
<td>Efflux of MTX, cellular detoxification</td>
<td>Flow cytometry</td>
</tr>
<tr>
<td>Folylpolyglutamyl synthetase = FPGS</td>
<td>Cytoplasmatic enzyme</td>
<td>Polyglutamylation of MTX and folate (presumed increase in efficacy)</td>
<td>RT – PCR (not performed here)</td>
</tr>
</tbody>
</table>

*methotrexate (MTX); reverse transcriptase polymerase chain reaction (RT-PCR)

The roles of MRPs and RFC within the folic acid cycle are well established. Other studies have shown the importance of folate receptors in the uptake of folic acid and MTX into activated synovial mononuclear cells. In contrast to patients with malignancies, it was recently shown that the presence of folylpolyglutamyl synthetase in patients with RA did not prove to mediate a better response to MTX.

In a further attempt to determine influences on the response to MTX we investigated if the RFC and function of MRP (fMRP) status of RA patients treated with low dose weekly MTX could be related to the clinical response to MTX.

**PATIENTS AND METHODS**

**Patients**
163 patients (116 female, 47 male, mean age 59.5 years) with RA classified according to the ACR criteria were enrolled into the study. Rheumatoid factor (RF) was present in 102 of these patients (62.5%), while 61 patients were RF
negative (37.5%). 96 patients were on low dose corticosteroids (59%). Mean disease duration was 7.6 ± 7.2 years (range 7 months to 25 years). 139 patients were on monotherapy with MTX, 19 were also treated with chloroquin, 3 with sulfasalazine and 2 with ciclosporine. 59 also received folic acid supplementation (Table 2). Additionally, most patients were treated with NSAIDs, usually diclofenac, ibuprofen or coxibs, and none of the patients was on indomethacin. All patients gave written informed consent and the local Ethical Committee had approved this investigation.

Blood samples for determination of RFC and fMRPs were taken cross-sectionally during ongoing MTX therapy but at least 12 weeks after its initiation. The mean weekly dose of MTX was 12.2 ± 3.8 mg (range 7.5 to 22.5 mg) with a mean treatment duration of 30 ± 25.1 months mean (range 4 to 121 months).

All clinical and serologic variables were evaluated prospectively in the course of routine clinical care and blinded to the results of RFC and fMRP testing. This evaluation included joint counts, patient and physician global assessment as well as patient pain assessment by visual analogue scale (VAS) and acute phase reactants. Therapeutic response was evaluated using the EULAR response criteria which employ the Disease Activity Score (DAS28) 20,21,22. The DAS was prospectively determined at the initiation of MTX therapy and at the time of the present investigation. A DAS28 of more than 5.1 indicates high disease activity, a DAS28 of more than 3.2 up to 5.1 is considered a moderate disease activity, while 3.2 or below suggests low disease activity. Furthermore, a DAS28 decrease of >1.2 represents a good EULAR response (unless DAS is in the high disease activity range at the end of observation) and a decrease of > 0.6 but ≤ 1.2 is considered a moderate response, while a change of ≤ 0.6 (or high disease activity under whatever change of the DAS) is regarded as lack of clinical response 20,21,22. In addition, the American College of Rheumatology 20% response was evaluated, determined by a 20 percent decrease in numbers of swollen and painful joints and at least three of the following : patient pain assessment, patient and physician global assessment, erythrocyte sedimentation rate (ESR) or C-reactive Protein (CRP) and HAQ score 23.

**Determination of RFC mRNA expression and MRP function**

In Table 1, the role of the individual resistance factors and their method of determination are shown. RFC mRNA expression was determined using reverse transcriptase polymerase chain reaction (RT-PCR). Briefly peripheral blood mononuclear cells were isolated by density gradient centrifugation. Total RNA was isolated using an RNA isolation kit (RNEasy, Qiagen; Valencia, CA). cDNA was obtained using Superscript II reverse transcriptase (Life Technologies; Rockville, MD). RT-PCR was then performed. 35 PCR cycles were performed (denaturation, annealing, polymerisation) followed by a final elongation step. For RFC, forward primer RFC-617 (5´-CCAAGCGCAGCCTCTTCTTCAACC-3´, bases 617-640) and reverse primer
RFC-949 (5´-CCAGCAGCGTGGAGGCAGCATCTGCC-3´, bases 924-949) were used leading to a product of 333 bp. RT-PCR products were separated by 2% agarose gel electrophoresis with 0.5 µg / ml ethidium bromide for DNA visualisation.

β2 microglobulin was used as house keeping gene, RFC+ samples were defined by visual detectability of RFC-PCR products after electrophoresis; RFC-samples yielded no visible bands of PCR products (low RFC mRNA copy number). As there was a large variation in expression of RFC (from not visible to very intense bands) and two distinct patterns (not visible, i.e. negative or positive) were found, further densitometric analyses of the bands were not performed. Human erythroleukemia K 562 cells were included as a positive control in all assays.

MRP function was measured by flow cytometry of total mononuclear blood cells using a calcein acetoxydimethylster/probenecid assay with a final probenecid concentration of 1 mM. In brief, cells were pre-incubated with warm RPMI 1640 medium after several washing steps to remove any remaining serum components followed by a 15 minute incubation with calcein acetoxydimethylster. After 90 minutes incubation in medium with or without probenecid as modulator of MRP activity, fluorescence was measured with a FACScan cytometer (Becton Dickinson; Franklin Lakes, NJ). Functional MRP (fMRP) was defined by transport of MTX and successful barring by probenecid. fMRP+ means there is a transport of MTX through the cell membrane which can be inhibited by probenecid. fMRP- stands for no efflux due to the absence of any transport mechanism or transport of MTX by another mechanism not inhabitable by probenecid, such as P- glycoprotein. In both cases there is no functional MRP present.

The mean fluorescence index for fMRP+ was 1.12 (95% confidence interval 1.07 – 1.32), for fMRP- 0.93 (95% confidence interval 0.9 – 0.97). The cut-off-level for MRP functionality was defined as > 1.00. Probenecid may not be equally effective on all subgroups of MRP, but at the time of testing it has been the best specific modulator available, therefore function of MRP stands for a group of multidrug resistance proteins including all yet known MRP subgroups in contrast to P-glycoprotein.

**Analyses**

In analogy with their role in malignant diseases the presence of RFC and the absence of fMRP was hypothesised to be associated with a better therapeutic response to MTX and thus to have prognostic value. Therefore, patients were divided into four groups according to the presence and/or absence of RFC and fMRP. The frequency of EULAR and ACR 20 responders was determined for each group. In addition, mean change (± SD) of DAS was determined for each
group and the results were compared to those of the MRP-RFC+ group assumed
to obtain the greatest clinical benefit.

Statistical evaluation was performed using Student’s t-test comparing the mean
change of DAS28 in each group to the fMRP-RFC+ group. The frequencies of
good and good plus moderate as well as ACR 20 responders were compared by
Chi-square testing.

RESULTS

Seventy of the 163 patients fell into the fMRP-RFC+ group (43.0%). The
therapeutic characteristics of this group of patients were not different
statistically from those of the other groups (Table 2).

### Table 2: Mean MTX dose and other DMARDs

<table>
<thead>
<tr>
<th></th>
<th>Mean MTX dose</th>
<th>Folic acid supplementation</th>
<th>dual therapy of MTX with chloroquine</th>
<th>sulfasalazine</th>
<th>ciclosporin</th>
</tr>
</thead>
<tbody>
<tr>
<td>fMRP- RFC+ (n=70)</td>
<td>12.1 ± 3.9 mg</td>
<td>29 (41%)</td>
<td>11 (15.7%)</td>
<td>1 (1.4%)</td>
<td>1 (1.4%)</td>
</tr>
<tr>
<td>fMRP- RFC- (n=32)</td>
<td>12.1 ± 3.7 mg</td>
<td>9 (28%)</td>
<td>3 (9.3%)</td>
<td>1 (3.1%)</td>
<td>1 (3.1%)</td>
</tr>
<tr>
<td>fMRP+ RFC+ (n=33)</td>
<td>12.7 ± 3.7 mg</td>
<td>12 (36%)</td>
<td>1 (3.0%)</td>
<td>1 (3.0%)</td>
<td>0</td>
</tr>
<tr>
<td>fMRP+ RFC- (n=28)</td>
<td>12.0 ± 3.9 mg</td>
<td>9 (32%)</td>
<td>4 (14.2%)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

In contrast to our expectations, this group had the lowest mean DAS
improvement (mean reduction of 0.71) (Table 3).
Table 3: Mean DAS28 and Reduction of DAS28

<table>
<thead>
<tr>
<th>Groups according to resistance markers</th>
<th>Change of DAS28 (mean ± SD)</th>
<th>Mean DAS28 at start of therapy (mean ± SD)</th>
<th>Mean DAS28 at time of blood sampling (mean ± SD)</th>
<th>P value compared to fMRP-RFC+</th>
</tr>
</thead>
<tbody>
<tr>
<td>fMRP- RFC+ (n=70)</td>
<td>0.71 (± 1.27)</td>
<td>3.08 (± 1.02)</td>
<td>2.37 (± 1.00)</td>
<td></td>
</tr>
<tr>
<td>fMRP- RFC- (n=32)</td>
<td>1.23 (± 1.44)</td>
<td>3.22 (± 1.30)</td>
<td>1.99 (± 0.93)</td>
<td>0.035</td>
</tr>
<tr>
<td>fMRP+ RFC+ (n=33)</td>
<td>1.32 (± 1.36)</td>
<td>3.38 (± 1.10)</td>
<td>2.05 (± 1.14)</td>
<td>0.014</td>
</tr>
<tr>
<td>fMRP+ RFC- (n=28)</td>
<td>1.09 (± 1.29)</td>
<td>3.39 (± 0.95)</td>
<td>2.30 (± 0.98)</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

In fact, only 29% of these patients had a good DAS28 response (Figure 1), and only 36% fulfilled the ACR 20 criteria. Interestingly, both the fMRP- RFC- and the fMRP+ RFC+ groups had significantly higher mean improvements of DAS compared to the fMRP- RFC+ group (1.23 and 1.32 respectively, p=0.035 and p=0.014) (Table 3). Moreover in these two groups good EULAR responses were seen in 53% and 60% of the patients, respectively, and 57% and 55% respectively, achieved ACR 20% response (0.1>p>0.05 and p< 0.05, respectively, compared to fMRP-RFC+ patients). In the fMRP+ RFC- group intermediate results were obtained: 46% had a good DAS28 response, while 43% of the patients achieved ACR 20. Thus, concordance for either the presence or the absence of fMRPs and RFC conferred a high likelihood for a good response to MTX. In contrast, the fMRP- RFC+ group was associated with highest risk of a lacking response (Figure 1).

The mean MTX doses were similar among the different groups (Table 2). Thus differences in response to therapy were not due to different doses of MTX.
DISCUSSION

For many years and in many clinical and therapeutic settings attempts have been made to predict responsiveness of RA patients to DMARD therapy. However, these attempts were not successful hitherto. In the present study we reveal that patients who are concordant for either the presence or the lack of fMRPs and RFC have better responses to MTX than patients who are not concordant for these proteins. Interestingly and in contrast to our expectations, a particularly low clinical response rate was found among patients with a fMRP- RFC+ status.

The data obtained suggest a predictability of response to MTX. In addition, they may allow some insights into mechanisms of MTX unresponsiveness. First, functional MRP might not be the cause of resistance to MTX in RA, since very good clinical responses were obtained in MRP+ patients. Nevertheless, it cannot be excluded that MRP family members (e.g. MRP4) other than investigated here (MRP 1-3) may be involved in MTX resistance in RA. Also, some patients may have increased FPGS activity preventing MTX polyglutamates from being effluxed by MRPs, still allowing MTX responsiveness regardless of the presence of MRP; however, we did not find the MTX response to be associated with presence of absence of FPGS. Second, since the presence of RFC and the absence of MRP confer the highest intracellular MTX levels, the weak clinical response to MTX in this group of patients suggests that MTX effects in RA and folate-mediated MTX effects are based on different intracellular pathways. This is further supported by the lack of association of clinical response with the presence or absence of FPGS. Thus, it may be important in future studies to have a combined look at the mechanisms studied here as well as on the inhibition of AICAR-transformylase, which plays and essential role in the mechanisms of action of MTX in RA.

The observation that MTX had only weak efficacy among fMRP- RFC+ patients indicates not only that the mere influx of MTX into the cell (as mediated by RFC) may be insufficient to allow MTX to exert its effect as a DMARD within the cell. Other pathways for MTX into the cell, which were not examined in this study, under the precondition of their existence and proper function might be more efficient in this respect, but also the influence of MRP on MTX may be more complex than just transporting the drug out of the cell. Moreover, given the result in this subset of fMRP- RFC+ patients, it is unlikely that MTX upregulates MRPs as has been described for other agents like cisplatinum compounds or the glucocorticoid dexamethasone. Interestingly, Ma et al have reported that in folate depleted breast cancer cells, exposure to low-dose MTX under certain conditions resulted in decreases in RFC-1 expression and the
initial rate of MTX uptake over time decreased to 22% of the baseline value\textsuperscript{38}. Since the current investigation was performed at 12 weeks after initiation of MTX, such effects, if present in vivo, could also be responsible for some of our observations.

Among RA patients treated with MTX, 30 – 50% do not fulfil response criteria. These data, obtained in clinical trials, are confirmed here in a cohort of patients followed during routine clinical care. However, the highest frequency of non responders (57%) was observed among the fMRP- RFC+ patient population, while the lowest frequency of DAS28 non-responders (37%) was seen among fMRP-RFC- patients.

The results obtained also suggest an impact on clinical decision making: they could provide rheumatologists with a tool to predict responsiveness to MTX therapy. Since a mean of 57% of the fMRP+ RFC+ and fMRP- RFC- patients had a good clinical response compared to only half that frequency in fMRP- RFC+ patients, determination of these proteins may be helpful to predict the probability of responsiveness to MTX.

At present, neither responsiveness to traditional DMARDs nor to biological agents can be predicted on clinical or laboratory grounds. Here a potential tool for detecting likely MTX responders from likely non-responders is revealed. Without doubt, further studies in other patient populations will be needed to confirm the results obtained. However, once confirmed, this finding could constitute a first major breakthrough in predicting responsiveness to therapy in RA.

This study was supported by the “Jubiläumsfonds der Oesterreichischen Nationalbank” (Project number 8783).

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Figure 1: EULAR RESPONSE (fMRP,RFC)

- fMRP- RFC+
- fMRP- RFC-
- fMRP+ RFC+
- fMRP+ RFC-

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>fMRP- RFC+</td>
<td>70</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>fMRP- RFC-</td>
<td>32</td>
<td>&gt;0.6</td>
</tr>
<tr>
<td>fMRP+ RFC+</td>
<td>33</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>fMRP+ RFC-</td>
<td>28</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Legend:
- none (<0.6)
- moderate (0.6 - 1.2)
- good (>1.2)
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