Application of Fluorescence Polarization Immunoassay for Determination of Methotrexate-Polyglutamates in Rheumatoid Arthritis Patients

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Rheumatoid arthritis (RA) is a chronic disease characterized by the painful joints, inflammation, uncontrolled proliferation of synovial tissue and multisystem comorbidities. Weekly low-dose methotrexate (MTX) has been established as effective treatment in RA patients. MTX is converted to γ-glutamyl polyglutamates, an active form of MTX, through the action of folylpolyglutamate synthetase in the cells. MTX-polyglutamates (MTX-PGs) in red blood cells (RBCs) may be useful as a therapeutic marker of RA. However, the previously reported methods for the quantification of MTX and MTX-PGs in RBCs are impractical for clinical use due to time-consuming, laborious and high cost. We attempted to apply a method with the commercially available fluorescence polarization immunoassay (FPIA) kit. We found that anti-MTX monoclonal antibody showed the reactivity to 4-aminoo-10-methylpteroylheptaglutamic acid (MTX-PG₇) as equal to MTX. Good agreement was observed in the concentration-response curves between MTX and MTX-PG₇ spiked samples. Accordingly, the anti-MTX monoclonal antibody for FPIA appeared to show the equal reactivity to MTX and MTX-PGs. The recoveries of MTX and MTX-PG₇ from RBCs were 99.0% and 94.1%, respectively. Furthermore, we determined total MTX-PGs concentrations in RBCs of 71 patients with RA treated with weekly pulse MTX. Total MTX-PGs concentrations in 70% of the patients were found to be more than 50 nM that is the lower limit of MTX-PGs concentration in RBCs for expected therapeutic outcome. The routine measurement of total MTX-PGs concentration in RBCs might be useful for prediction about therapeutic outcome of MTX in RA patients. ———— methotrexate; methotrexate-polyglutamate; rheumatoid arthritis; therapeutic drug monitoring; fluorescence polarization immunoassay.

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Methotrexate (MTX) is an antifolate that is widely used to treat the diseases such as rheumatoid arthritis (RA) and childhood acute lymphoblastic leukemia (Andersen et al. 1985; Weinblatt et al. 1985; Gorlick et al. 1996; Evans et al. 1998; Pui and Evans 1998; Pui et al. 2001). RA is a chronic disease characterized by the painful inflammation of joints, uncontrolled proliferation of synovial tissue and multisystem comorbidities. MTX is generally given orally to RA patients as a single weekly dose. Low-dose MTX is now first-line therapy for the treatment of RA patients of non-responder to non-steroidal anti-inflammatory drugs (American College of Rheumatology Subcommittee on Rheumatoid Arthritis Guidelines 2002).

After its entry into cells, MTX is rapidly converted to γ-glutamyl polyglutamates through the action of folylpolyglutamate synthetase, which sequentially adds up to six glutamyl residues to MTX (Goldman et al. 1968; Shane 1989; Zhao and Goldman 2003). This γ-linked sequential addition of glutamic acid residues enhances the intracellular retention of MTX. Furthermore, this process promotes the sustained inhibition of de novo purine synthesis, thereby promoting the build-up of adenosine, a potent anti-inflammatory agent (Chan and Cronstein 2002). In patients with RA, recent studies suggest a correlation exists between MTX polyglutamates (MTX-PGs) concentrations in red blood cells (RBCs) and disease activity, rather than dose of MTX. MTX-PGs concentrations in RBCs were significantly higher in responders than in non-responders (Angelis-Stoforidis et al. 1999).

Several reports have been made about the measurement of MTX-PGs in RBCs; Kamen et al. (1976, 1986) developed the highly sensitive radiochemical ligand-binding assay. Schroeder and Heinsvig (1985) developed an enzymatic assay. Dervieux et al. (2003) developed HPLC-fluorometry assay in which MTX-PG$_{2-7}$ were quantified individually in a single run. However, these assay methods for the quantification of MTX and MTX-PGs in RBCs are impractical for clinical use due to time-consuming, laborious and high cost.

The fluorescence polarization immunoassay (FPIA) is the method generally used for therapeutic drug monitoring (TDM) of drugs including MTX. This method is so easy and rapid that it is suitable for routine assay at clinical laboratory testing (Stoller et al. 1977; Treon and Chabner 1996). In this study, we applied the MTX-FPIA for monitoring of MTX-PGs in RBCs and found that the MTX-PGs in RBCs in RA patients receiving low-dose weekly pulse MTX can be measurable by MTX-FPIA.

**MATERIALS AND METHODS**

**Chemicals**

USP-grade MTX was purchased from Sigma (St. Louis, MO, USA). 4-Amino-10-methylpteroylheptaglutamic acid (MTX-PG$_7$) was purchased from Schircks Laboratories (Jona, Switzerland). All the other chemicals used were of analytical grade.

**Clinical samples**

This study was approved by the Ethics Committee of the Shizuoka Kousei Hospital. Written informed consent was obtained from all patients after a detailed briefing of the study purposes and protocols. This study was carried out with RA outpatients who had been regularly taking orally MTX capsules (RHEUMATREX®) for more than 6 months. Approximately 5 ml of whole venous blood was obtained from 71 unrelated Japanese RA patients using EDTA-2Na Venoject II tubes (Terumo, Tokyo). The RBCs were packed by centrifugation and washed twice with a 4-fold volume of cold saline. The obtained RBCs were stored at –80°C until analysis.

**Spiked samples**

To generate the standard curve for calculation of MTX and MTX-PG$_7$ concentrations, we had prepared spiked samples. A stock solution of MTX and MTX-PG$_7$ were prepared in 0.1 N potassium hydroxide, then they were diluted to 100 μM in water and stored at –80°C. Spiked samples were prepared by adding known amounts of MTX and MTX-PG$_7$ either to a phosphate buffered saline (PBS) or to a pool of RBC lysates isolated from healthy blood donors.

**Extraction of MTX and MTX-PGs from RBC**

The RBCs was lysed by single freeze-thaw cycle. To hydrolyze the proteins in RBCs samples, forty microliter of protease solution (Qiagen protease, Qiagen,
Hilden, Germany) was added and incubated for 10 min at 56°C. After the samples were cooling to room temperature, 60 μl of 1 N NaOH and 500 μl of methylene chloride as a deproteinization agent were added and mixed vigorously for 30 sec. To increase the efficiency of deproteinization, the samples were incubated at −30°C for 1 hr. After incubation, the resultant solution was centrifuged at 15,000 ×g for 5 min. Collected supernatant (200 μl) was neutralized by addition of 4 μl of 10% perchloric acid and then poured into the TDx sample cup and analysis was performed.

**Analysis of MTX and MTX-PGs**

Methotrexate II Reagent Pack for FPIA was purchased from Abbott Laboratories (Abbott Park, IL, USA) and the assay was performed using a TDx analyzer (Abbott Laboratories).

**Calculation of the concentration**

Standard curves were obtained by linear regression using Log C vs Logit P, where C is the final concentration of the added MTX and MTX-PG7 (nM). Logit P was defined by following equation:

\[
\text{Logit } P = \ln \left( \frac{P}{P_{\text{max}}} / (1 - P/P_{\text{max}}) \right)
\]

where P is the net polarization degree of spiked sample obtained by TDx analyzer, and \(P_{\text{max}}\) is the net polarization degree of no added sample. We used MTX standard curve to calculate total MTX-PGs included MTX in RBCs from RA patients with low-dose MTX therapy.

**Statistical analysis**

The slopes and intergroup significant difference of the logit-log plot of MTX compared with that of MTX-PG7 were assessed by multiple linear regression analysis. The statistical analyses were performed using the R project for statistical computing (http://www.r-project.org).

**RESULTS**

**Association between the fluorescence polarization degrees and the concentrations of MTX and MTX-PG7**

Typical sigmoidal curves were obtained from the FPIA data when the polarization degrees were plotted against the logarithm of MTX or MTX-PG7 concentrations in spiked PBS samples (Fig. 1A).

![Fig. 1. A plot of polarization degree vs. MTX and MTX-PG7 concentration in PBS on semi-log axes results in a sigmoidal dose-response curve typical of competitive binding assays (panel A). The samples were spiked MTX (closed symbols) and MTX-PG7 (open symbols) at a final concentration of 20, 70, 300, 600, 1,200 and 2,000 nM. Panel B shows the logit-log plots of the MTX and MTX-PG7 standard curve. The logit-log plots between the MTX and MTX-PG7 were not significantly different by the multiple linear regression analysis. Each value represents the average of triplicates, and the error bar denotes the standard deviation. The error bars are included in the symbols.](image-url)
On the multiple linear regression analysis, there was no significance of difference in the logit-log plots of between MTX and MTX-PG₇ (Fig. 1B, statistical interaction effect: \( p = 0.673 \), intergroup difference: \( p = 0.516 \)). These results indicated that anti-MTX monoclonal antibody showed the reactivity to MTX-PG₇ equal to MTX. The intra- and interday assay variation of the FPIA for MTX was determined at the range of previously reported concentration in RBC lysates (Angelis-Stoforidis et al. 1999). The intraday precision expressed as percentage relative standard deviation (RSD) was measured as 0.2 – 0.5% for 0, 20, 70 and 300 nM MTX in RBCs, with five replicates at each concentration level. The interday precision expressed as RSD was measured as 1.0 – 2.9% for 0, 20, 70 and 300 nM MTX in RBCs, with three replicates at each concentration level.

Fig. 2 shows the correlation of the concentrations of the spiked MTX-PG₇ and the measured MTX-PGs in RBC lysates using the calibration curve for MTX. A good correlation was found between the spiked concentrations of MTX-PG₇ and the measured concentrations of MTX-PGs \( (r^2 = 0.986) \). From these results, MTX-PGs can be determined by commercially available MTX-FPIA methods.

**Application for measurement of MTX-PGs in RBC in patients with RA receiving low dose MTX**

The total MTX concentrations in the RBC lysates from 71 RA patients receiving the low-dose weekly pulse MTX therapy are presented in Table 1. The average weekly dose for MTX was 6.1 mg for RA patients. The total MTX-PGs were defined as the sum of MTX + MTX-PGs. The disease status of patients was evaluated by C-reactive protein (CRP), that is a marker of inflammation. Seventy-one RA patients were classified into three groups according to the serum CRP levels. Total MTX-PGs concentrations in 70% of the patients were found to be more than 50 nM that is the lower limit of MTX-PGs concentration in RBCs for expected therapeutic outcome. The patients with RBCs concentration of total MTX-PGs more than 50 nM were 64%, 68% and 92% of the patients in the no or slight inflammation group (CRP < 0.60 mg/dl), mild inflammation group (0.60 ≤ CRP < 2.00 mg/dl) and severe inflammation group (CRP ≥ 2.00 mg/dl), respectively. These patients can be defined as responders, partial-responders and non-responders, respectively, as shown in Table 1. From these results, the total MTX-PGs in RBCs in RA patients receiving low dose MTX were measurable by our established method, and MTX-PGs may be a promising marker for estimation of
Determination of MTX-PGs in RBCs by FPIA in RA Patients

DISCUSSIONS

MTX is one of the most widely used anti-cancer and anti-inflammatory agents. Low-dose MTX, orally administered in a weekly pulse, has been extensively used in the treatment of RA (Andersen et al. 1985; Weinblatt et al. 1985). MTX is converted to MTX-PGs in the cells, then MTX-PGs bind to and inhibit several important enzymes (Ranganathan et al. 2003). MTX-treated patients often show the side effects such as liver dysfunction with raised aminotransferases, hematological abnormalities and central nervous system toxicity (Allegra and Boarman 1990). TDM of MTX is clinically employed for avoiding side effects in MTX-treated patients. Serum MTX concentrations are monitored routinely during and after MTX administration. As for low-dose MTX-treated patients, serum MTX level would be under detection limit after 24 hrs of MTX administration. Instead of serum MTX, MTX-PGs in RBCs may be applicable as a marker for efficacy and side effects of MTX (Angelis-Stoforidis et al. 1999).

Several investigators reported the methods for the quantification of MTX-PGs in RBCs. Kamen et al. (1976, 1986) reported the quantification of RBC MTX-PGs by radiochemical ligand-binding assay. They extracted MTX-PGs from RBC by heat-treatment and fractionated by HPLC then quantified. Schroeder and Heinsvig (1985) reported the DHFR inhibition enzymatic assay, in which MTX-PGs were quantified by measurement of the decrease in absorbance accompanied by NADPH consumption during enzymatic conversion of dihydrofolate to tetrahydrofolate. As Dervieux et al. (2003) point out, its application to the quantification of MTX-PGs in RBCs seems to be difficult because of possible competition between MTX-PGs and endogenous folate for DHFR binding (Matherly et al. 1983; Allegra and Boarman 1990). Dervieux et al. (2003) reported that HPLC-fluorometry assay, in which MTX (MTX-PG₁) and MTX-PG species (MTX-PG₂₋₇) were individually quantified in a single run. HPLC methods may be time-consuming, laborious and high cost. Thus, more rapid, easy and practical methods will be expected for measurement of MTX-PGs in RBCs at the bedside.

In this study, we tried to apply FPIA-MTX for the quantification of MTX-PGs in RBCs. The data are presented as mean values with standard deviations. * Inflammatory status was evaluated by serum CRP.

<table>
<thead>
<tr>
<th>Inflammatory status*</th>
<th>Total MTX-PGs in RBCs</th>
<th>Mean CRP (mg/dl)</th>
<th>Mean total MTX-PGs in RBCs (nM)</th>
<th>MTX therapeutic outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>No or slight inflammation (CRP &lt; 0.60 (mg/dl), n = 39)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (&lt; 50 nM)</td>
<td>14 (36)</td>
<td>0.19 ± 0.21</td>
<td>37.1 ± 9.2</td>
<td>Stable</td>
</tr>
<tr>
<td>High (≥ 50 nM)</td>
<td>25 (64)</td>
<td>0.21 ± 0.13</td>
<td>80.6 ± 31.8</td>
<td>Responsive</td>
</tr>
<tr>
<td>Mild inflammation (0.60 ≤ CRP &lt; 2.00 (mg/dl), n = 19)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (&lt; 50 nM)</td>
<td>6 (32)</td>
<td>1.40 ± 0.36</td>
<td>40.6 ± 8.9</td>
<td>Expected under therapeutic concentration</td>
</tr>
<tr>
<td>High (≥ 50 nM)</td>
<td>13 (68)</td>
<td>1.02 ± 0.39</td>
<td>73.2 ± 24.6</td>
<td>Partial-responsive</td>
</tr>
<tr>
<td>Severe inflammation (2.00 ≤ CRP (mg/dl), n = 13)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low (&lt; 50 nM)</td>
<td>1 (8)</td>
<td>3.11</td>
<td>42.2</td>
<td>Expected under therapeutic concentration</td>
</tr>
<tr>
<td>High (≥ 50 nM)</td>
<td>12 (92)</td>
<td>4.08 ± 2.08</td>
<td>83.3 ± 25.1</td>
<td>Resistant</td>
</tr>
</tbody>
</table>
for measuring MTX-PGs in the RBCs. FPIA is based on the change of fluorescence polarization induced by antigen (drug) and antibody (anti-drug monoclonal antibody) interaction. A plot of polarization degree vs MTX and MTX-PG concentrations in the PBS on semi-log axes results in a sigmoidal dose-response curve (Fig. 1A) typical of competitive binding assays. Since there was no significance of difference in the logit-log plots between MTX and MTX-PG (n = 2–7), anti-MTX monoclonal antibody in the FPIA kit showed the reactivity to MTX-PG, as equal to MTX. Anti-MTX antibody may, thus, recognize the structure other than the glutamine residue in MTX. These results implicate that MTX-PG (n = 2–7) can be quantified by anti-MTX monoclonal antibody with FPIA as equal to MTX (MTX-PG).

Our method was applied to measure MTX-PGs in RBCs of RA patients receiving weekly low-dose MTX therapy. We determined total MTX-PGs in RBCs in 71 RA patients treated weekly pulse MTX. Total MTX-PGs concentrations in 70% of the patients were more than 50 nM. Angelis-Stoforidis et al. (1999) reported that patients with RBC MTX-PGs of > 50 nM were more likely to have a good therapeutic response. MTX-resistant patients and partial-responsive patients who had expected under therapeutic concentration of MTX-PGs in RBCs (< 50 nM) would be improved inflammation by dose-up of MTX. However, though 12 patients with severe inflammation had sufficient concentration of MTX-PGs in RBCs, their CRPs were still high. They might be non-responders against MTX. In these cases, change to therapy with infliximab, etanercept or tacrolimus at an early stage would be recommended.

According to manufacturer’s instructions, anti-MTX monoclonal antibody shows no cross-reactivity to some metabolites of MTX and folic acid (e.g., 7-hydroxymethotrexate, dihydrofolate, tetrahydrofolate and 5-methyltetrahydrofolate). We showed that anti-MTX monoclonal antibody reacts with MTX-PGs as well as with MTX (Fig. 1). From our results and above observation, MTX and MTX-PGs in clinical specimen can be determined by our established method. Whether MTX-PGs concentration in RBCs can predict MTX toxicity remains unknown. Furthermore, the individual variation of MTX-PGs in RBCs may be accounted by folate pathway single nucleotide polymorphisms (Dervieux et al. 2004). Evaluation of the usefulness of MTX-PGs as a marker for efficacy or toxicity of MTX is now in progress using a large number of RA patients.

**CONCLUSIONS**

In this study, we applied the FPIA method for quantification of MTX and MTX-PGs in RBCs. We found that anti-MTX monoclonal antibody in the commercially available FPIA kit showed the reactivity to MTX-PG, as equal to MTX. We confirmed that MTX and MTX-PGs in RBCs in clinical specimen could be quantitatively measured by our method. This method can therefore be applied to monitor MTX and MTX-PGs in RA patients who were receiving low-dose weekly pulse MTX.

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**References**


polyglutamate levels in patients with rheumatoid arthritis. *Pharmacogenetics, 14,* 733-739.


