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ABSTRACT. Objective. Tacrolimus, a calcineurin inhibitor, is used for treatment of rheumatoid arthritis (RA). It also inhibits functions of P-glycoprotein, which is involved in drug resistance. We examined the mechanisms of early response to 2-week tacrolimus treatment in patients with RA.

Methods. One hundred thirteen patients with refractory RA despite at least 3 antirheumatic agents, including methotrexate, were treated with tacrolimus (1.5–3 mg/day) and the response was assessed at 2 weeks. Expression of the multidrug resistance (MDR-1) gene and P-glycoprotein was assessed in peripheral blood mononuclear cells (PBMC) collected from 113 patients and 40 healthy subjects. The drug exclusion function by the P-glycoprotein was measured by the residual amount of intracellular tritium-labeled dexamethasone cell/medium ratio (C/M ratio).

Results. The disease activity of enrolled patients was 5.8 ± 1.2 (mean ± SD) by DAS28 erythrocyte sedimentation rate. A good response to tacrolimus was noted at 2 weeks in 22 of 113 patients. At baseline, PBMC of patients with RA showed upregulated expression of MDR-1 gene and P-glycoprotein and low C/M ratio. The response to tacrolimus correlated with P-glycoprotein expression and C/M ratio. A significant improvement in C/M ratio was noted after 2 weeks of treatment. The C/M ratio correlated significantly with P-glycoprotein expression on CD4+ lymphocytes.

Conclusion. Early efficacy of tacrolimus treatment depended on its inhibitory effect on the drug exclusion function of P-glycoprotein, leading to restoration of intracellular therapeutic levels of corticosteroids and clinical improvement. Evaluation of P-glycoprotein expression only lymphocytes is potentially useful for predicting the response to RA treatment. (First Release Jan 15 2010; J Rheumatol 2010;37:512–20; doi:10.3899/jrheum.090048)

Key Indexing Terms: P-GLYCOPROTEIN RHEUMATOID ARTHRITIS DRUG RESISTANCE TACROLIMUS CALCINEURIN INHIBITOR
(IL-2), and stimuli from cells injured by long-term exposure to antiinflammatory drugs and corticosteroids, cause translocation of transcription factor YB-1 from the cytoplasm into the nucleus, which in turn triggers the expression of MDR-1. Further, P-glycoprotein is expressed on the surface of lymphocytes, and drugs that are substrates of P-glycoprotein are exported outside the cell.

Similar to cyclosporine, tacrolimus is an effective drug widely used as a calcineurin inhibitor, particularly in transplant surgery\textsuperscript{10,11}. Intracellularly, tacrolimus forms a complex with FK binding protein (FKBP-12). The complex binds to calcineurin, which results in inhibition of its activation. The complex also inhibits the activation of the transcription factor nuclear factor-ATc, thus preventing its entry into the nucleus. Consequently, the transcription of cytokines such as IL-2 and IL-4 is blocked, resulting in suppression of RA disease\textsuperscript{12-15}. We reported previously that results showed that tacrolimus suppresses drug exclusion by cyclosporine, a calcineurin inhibitor, also inhibits P-glycoprotein, and it can thus eliminate drug resistance in systemic lupus erythematosus, which is caused by drug exclusion due to expression of P-glycoprotein\textsuperscript{9,16}.

Tacrolimus also inhibits P-glycoprotein\textsuperscript{17,18}; however, the drug resistance blocking action of tacrolimus is still unexplained, as is the consequence of this on treatment of RA. Studies indicate that the clinical effects of tacrolimus on RA appear after 1–2 months of treatment\textsuperscript{19-25}. In our study, we report rapid improvement in clinical symptoms and laboratory tests after 2-week treatment with tacrolimus. Based on this finding, we then investigated the clinical effects of tacrolimus combined with other drugs including corticosteroids, which are substrates of P-glycoprotein. The results showed that tacrolimus suppresses drug exclusion by inhibiting P-glycoprotein, thus allowing restoration of therapeutic levels of corticosteroids within the cells.

MATERIALS AND METHODS

Patients. The subjects were 113 patients with RA who met the diagnostic criteria of the American College of Rheumatology\textsuperscript{26} and showed resistance to antirheumatic agents. In addition, the study included peripheral blood samples from 40 healthy adults, matched by age and sex to the patients with RA. The control subjects were either staff members of our hospital or subjects who visited our hospital for medical examinations. Patients with RA that was resistant to treatment were defined as patients with a Disease Activity Score (DAS28) erythrocyte sedimentation rate (ESR) score >3.1 for RA activity despite receiving treatment with adequate doses of at least 3 antirheumatic drugs, including methotrexate (MTX), for a minimum of 3 months, and showing no or moderate response, according to the European League Against Rheumatism (EULAR) improvement criteria\textsuperscript{27-29}. Tacrolimus was used for these patients with treatment-resistant RA. Tacrolimus was administered at a daily dose of 3 mg in patients ≥ 65 years of age and 1.5 mg for those aged ≥ 65 years. All other antirheumatic drugs used by the enrolled patients were withheld during our 4-week study, except for nonsteroidal antiinflammatory drugs and corticosteroids. One patient who underwent treatment by biological disease-modifying antirheumatic drugs was not included in our study.

We started tacrolimus at the hospital of the University of Occupational and Environmental Health, Japan. The same person evaluated the clinical efficacy of tacrolimus throughout the observation period. The expression of P-glycoprotein on lymphocytes and polymerase chain reaction (PCR) analysis of the MDR-1 gene were conducted by an individual different from the one who evaluated the clinical effects such as the number of swollen and tender joints, and evaluation by visual analog scale. Data analysis was performed by a third individual blinded to the other 2 investigators.

The Human Ethics Review Committee of the university reviewed and approved our study, including the collection of peripheral blood samples from healthy adults and patients with RA. Each subject provided a signed participation consent form.

Measurements. The background factors investigated were sex, age, duration of RA, dosage of antirheumatic drugs including corticosteroids, history of treatment, use of concomitant drugs such as corticosteroids at the time of initiation of tacrolimus therapy, and the dosage of other drugs. We also evaluated the severity of morning stiffness, number of swollen joints, number of tender joints, and patient-evaluated pain and overall evaluation by a visual analog scale, in addition to evaluation by the attending physician. The response to treatment was evaluated using the EULAR criteria\textsuperscript{28,30,31}. The laboratory tests included C-reactive protein (CRP), ESR, rheumatoid factor (RF), and matrix metalloproteinase-3.

Expression of P-glycoprotein on peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMC) were separated by specific gravity fractionation using Lymphocyte Separation Medium 50494 (Pharmacia Biotech, Uppsala, Sweden). Of the CD antigens that were expressed on PBMC, ≥ 90% were on lymphocytes (CD4, CD8, or CD19+ cells) and ≤ 10% were on mononuclear cells. PBMC were stained and analyzed by flow cytometry using the standard procedure of FACSscan (Becton Dickinson, Mountain View, CA, USA) as described\textsuperscript{32,33}. The cells were divided to a final concentration of 2 × 10\textsuperscript{5} cells/well. Polyclonal γ-globulin (10 µg/ml; Mitsubishi Welpharma Co., Osaka, Japan) was added to the culture to block FC receptors. After culture, MRK-16 (100 µg/ml; Kyowa Medex, Tokyo, Japan), a monoclonal antibody specific for P-glycoprotein\textsuperscript{14}, was added to the culture solution. The cells were further labeled with FITC-conjugated antimouse IgG antibody (5 µg/ml; Fujisawa, Osaka, Japan). For cell staining, cells were treated with antimouse IgG that binds to nonspecific sites before using phycoerythrin-conjugated CD4 mAb or CD19 mAb (1.25 µg/ml; Becton Dickinson). Of these double-stained cells, target cells that were gated based on CD4 or CD19 expression were extracted using FACSscan. Quantification of the cell surface antigens on 1 cell was performed using QIFIKIT beads (Dako, Kyoto, Japan) as reported\textsuperscript{33,35}. The data were used to construct a calibration curve of the mean fluorescence intensity versus antibody-binding capacity. The cell specimen was analyzed on the FACSscan and the antibody-binding capacity calculated by interpolation on the calibration curve. When the green-fluorescence laser detection was set at 500 nm in the FACSscan, the antibody-binding capacity was equal to 202.98 × exp (0.0092 × mean fluorescence intensity) (R\textsuperscript{2} = 0.9995). Subsequently, the specific antibody-binding capacity was obtained after correcting for the background and apparent antibody-binding capacity of the negative control antimouse IgG antibody. The specific antibody-binding capacity represented the mean number of accessible antigenic sites per cell, referred to as antigen density and expressed in sites/cell.

Cell medium ratio (CM ratio) of dexamethasone. To assess the function of P-glycoprotein, PBMC were collected from patients with RA and analyzed for the residual amounts of carbon-labeled butanol and tritium-labeled dexamethasone in cells. The CM ratio represented the coefficient of intracellular and extracellular ratio of dexamethasone and was determined using the formula:

\[
C/M\text{ ratio} = \frac{[\text{H}]\text{in cell} + [\text{H}]\text{in medium}}{[\text{C}]\text{in cell} + [\text{C}]\text{in medium}}
\]

Changes in the residual amount of the drug in PBMC of patients with RA were assessed by pretreatment of the cells with tacrolimus 0–50 ng/ml, sulfasalazine (SSZ) 0–50 µg/ml, or MTX 0–400 ng/ml. Carbon-labeled n-butanol (1.61 µCi/mmol; Toho Biomedical, Tokyo,
DAS28 gradually decreased, and the proportion of patients who achieved more than moderate response also increased.

Next, we investigated the mechanism of the marked response to 2-week tacrolimus treatment. To examine the possibility that the early efficacy was due to the inhibitory effect of tacrolimus on P-glycoprotein, we analyzed P-glycoprotein expression on lymphocytes and drug exclusion by P-glycoprotein using the C/M ratio. P-glycoprotein was overexpressed in CD4 and CD19 lymphocytes of patients with RA, compared with the healthy controls (Figure 2A). The MDR-1 gene was not expressed in the lymphocytes of healthy controls. In contrast, even with no stimulation, the MDR-1 gene was clearly expressed (Figure 2B) in lymphocytes of all of 5 representative patients with RA (patients 1–3 had good response and 4 and 5 had moderate response).

We also measured intracellular concentrations of tritium-labeled dexamethasone in vitro. In healthy adult lymphocytes that do not express P-glycoprotein, the C/M ratio increased in a time-dependent manner. In contrast, there was no increase in C/M ratio in P-glycoprotein-expressing lymphocytes of patients with RA (Figure 3A). The addition of tacrolimus at 0–50 ng/ml, which has an inhibitory effect on P-glycoprotein, increased the C/M ratio in a dose-dependent manner (Figure 3B). However, the addition of MTX or SSZ as the control drug did not result in any significant change in the C/M ratio (Figure 3B).

Next, we analyzed the relationships among various background factors and the response to tacrolimus treatment (Table 1). The response to such treatment was not influenced by factors related to RA disease activity, including number of swollen joints, number of tender joints, visual analog scale, CRP, and ESR. Further, the response to tacrolimus treatment did not correlate with age, sex, duration of illness, mean corticosteroid dose, and tacrolimus dose. On the other hand, the rate of concomitant use of corticosteroids was significantly different among nonresponders (32/53; 60.4%), moderate responders (18/38; 47.4%), and good responders (22/22; 100%), as shown in Table 1. The percentage of patients using corticosteroids who did not achieve good response to tacrolimus (53.9%) was significantly lower than that of patients who did.

We also analyzed the correlation between response to treatment and P-glycoprotein expression and C/M ratio (Table 1). In this analysis, P-glycoprotein expression on CD4+ and CD19+ lymphocytes was stratified by the response to treatment. The expression levels of P-glycoprotein in both CD4+ and CD19+ lymphocytes were signifi-
Figure 1. Changes in disease activity and treatment response at baseline (before induction of tacrolimus treatment) and at 2 and 12 weeks of treatment in patients with treatment-resistant RA. RA disease activity was assessed by DAS28-ESR. Treatment response was assessed using EULAR criteria. A. Disease activity. Data for individual patients and mean ± SD values for baseline and posttreatment for the groups. *p < 0.05, Wilcoxon test. B. Treatment response at 2 and 12 weeks after commencement of tacrolimus treatment.

Figure 2. P-glycoprotein expression on lymphocytes of patients with RA treated with tacrolimus and healthy controls. A. Mononuclear cells were isolated from peripheral blood of patients with treatment-resistant RA and controls and stained with CD4 and CD19 antibodies and P-glycoprotein antibodies. Expression of P-glycoprotein on lymphocyte was measured. Data for individual subjects and mean ± SD values of the 2 groups are shown. **p < 0.01, Wilcoxon test. B. Multidrug resistance gene (MDR-1) was assessed by RT-PCR. Note the expression of MDR-1 in patients with treatment-resistant RA but not in healthy controls. Representative results of 5 experiments with similar results. Electrophoresis was performed on the amplifier together with Marker 4 in 3% agarose gel.
significantly higher in the good response group compared with the other groups (no response and moderate response; Table 1, Figure 4A). Moreover, the C/M ratio was significantly lower in the good response group compared with the moderate or no response group. Interestingly, the C/M ratio before the initiation of tacrolimus treatment was significantly lower in the good response group compared with the moderate and no response groups (Figure 4B). However, at 2 weeks after the initiation of tacrolimus treatment, the value improved significantly in the good response group compared with the moderate and no response groups (Figure 4C). In addition, P-glycoprotein expression at 2 weeks after initiation of tacrolimus therapy was similar to that at baseline (before therapy; Figure 4D).

Finally, multivariate logistic regression analysis was used to determine those factors that can explain the response to tacrolimus treatment. The analysis included the C/M ratio, P-glycoprotein expression on CD4+ lymphocytes, CRP, and morning stiffness. Multivariate logistic regression analysis identified the C/M ratio and P-glycoprotein expression on CD4+ lymphocytes as significant and independent determinants of a good response to tacrolimus treatment (Table 2).

DISCUSSION

The safety and efficacy of tacrolimus in the treatment of RA is currently under investigation. The response of patients with RA to tacrolimus treatment is normally investigated about 1–2 months after initiation of such treatment\textsuperscript{19-25}. In our study, however, we noted that some patients responded
markedly well to tacrolimus treatment after only 2 weeks. In order to explain this early response to treatment, we investigated the inhibitory effect of tacrolimus on P-glycoprotein, both in vitro and in vivo. PBMC of patients with RA showed expression of the MDR-1 gene, and overexpression of P-glycoprotein on the lymphocyte surface. These features were not identified in PBMC from healthy adults. Further, the C/M ratio in patients with RA was lower than that of the healthy adults, indicating enhanced drug exclusion function of P-glycoprotein. However, in in vitro experiments, the addition of tacrolimus to PBMC improved the C/M ratio to a level similar to that of healthy adults, and the response depended on the dose of tacrolimus added to the cell culture. We also tested the response of PBMC to 0–50 g/ml SSZ and 0–400 ng/ml MTX. These doses represent the C\text{max} values used clinically. At these concentrations, SSZ and MTX, which do not inhibit P-glycoprotein, did not improve the C/M ratio. These findings indicate that the improvement in C/M ratio is not due to a secondary inhibition of P-glycoprotein through inhibition of lymphocyte activation. The results of in vitro experiments were confirmed in vivo; tacrolimus produced a significant increase in the C/M ratio of patients who showed a good response to 2-week tacrolimus therapy (based on the improvement criteria of EULAR). Multivariate logistic analysis indicated that after 2 weeks of tacrolimus treatment, overexpression of P-glycoprotein on lymphocytes in the good response group correlated significantly with a low C/M ratio before treatment. Since all patients were also treated with corticosteroids throughout the study, the above findings suggest that the early response to tacrolimus therapy was not due to tacrolimus alone but also to inhibition of extracellular exclusion of corticosteroids, mediated by the inhibitory effect of tacrolimus on P-glycoprotein expression. In addition, there were no differences in P-glycoprotein expression between initiation and 2 weeks after initiation of tacrolimus (Figure 4D). Therefore, the main effect of tacrolimus was due, not to a decrease in P-glycoprotein molecules in lymphocytes, but to functional competitive inhibition of drug exclusion.

Humoral factors such as TNF-\(\alpha\), IL-6, and IL-1 that are locally produced in joints, or upregulation of P-glycoprotein expression through induction of transcription of MDR-1 gene following activation of YB-1 by certain drugs that are added to the treatment for RA such as antirheumatic drugs and corticosteroids, are involved in the development of resistance to treatment in patients with RA. Often, the same drug must be used continuously over a long period to control RA disease activity. During this period, expression of the multidrug resistance genes may be induced due to increased disease activity, resulting in drug resistance. For example, increased disease activity and low response rate following longterm use have been reported during treatment with SSZ. In fact, upregulation of P-glycoprotein expression was reported in low responders to SSZ. With SSZ, which is used in the treatment of RA, stimulation of cells that enhance P-glycoprotein expression causes simultaneous extracellular exclusion of the drug. Thus, the low response to SSZ is probably due to increased extracellular exclusion of the drug through P-glycoprotein. The above finding of the inhibitory effect of tacrolimus on P-glycoprotein expression suggests that tacrolimus could potentially improve the resistance to other drugs in addition to corticosteroids.

We demonstrated that tacrolimus inhibits drug exclusion mechanisms operating through P-glycoprotein in patients with good response after 2 weeks of treatment. As a drug used for management of treatment-resistant RA, tacrolimus

### Table 1. Baseline clinical characteristics of patients with RA treated with tacrolimus. P value represents differences between good response and moderate and no response groups.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>No Response (n = 53)</th>
<th>Moderate Response (n = 38)</th>
<th>Good Response (n = 22)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>65.1 ± 12.7</td>
<td>66.2 ± 15.3</td>
<td>68.0 ± 12.2</td>
<td>NS</td>
</tr>
<tr>
<td>Disease duration (mo)</td>
<td>166 ± 143</td>
<td>115 ± 108</td>
<td>171 ± 157</td>
<td>NS</td>
</tr>
<tr>
<td>Morning stiffness (min)</td>
<td>278 ± 369</td>
<td>171 ± 323</td>
<td>195 ± 413</td>
<td>NS</td>
</tr>
<tr>
<td>No. of tender joints</td>
<td>10.2 ± 6.1</td>
<td>8.6 ± 4.6</td>
<td>9.9 ± 8.2</td>
<td>NS</td>
</tr>
<tr>
<td>No. of swollen joints</td>
<td>9.0 ± 4.3</td>
<td>7.3 ± 4.3</td>
<td>7.0 ± 3.9</td>
<td>NS</td>
</tr>
<tr>
<td>Visual analog scale (mm)</td>
<td>56.5 ± 18.6</td>
<td>66.5 ± 21.0</td>
<td>54.6 ± 24.5</td>
<td>NS</td>
</tr>
<tr>
<td>C-reactive protein (mg/dl)</td>
<td>2.8 ± 2.6</td>
<td>1.5 ± 1.6</td>
<td>2.2 ± 2.6</td>
<td>NS</td>
</tr>
<tr>
<td>Erythrocyte sedimentation rate (ESR, mm/h)</td>
<td>60 ± 29</td>
<td>48 ± 26</td>
<td>42 ± 32</td>
<td>NS</td>
</tr>
<tr>
<td>Rheumatoid factor (IU/I)</td>
<td>275 ± 341</td>
<td>423 ± 1103</td>
<td>68 ± 63</td>
<td>NS</td>
</tr>
<tr>
<td>Matrix metalloproteinase (mg)</td>
<td>306 ± 302</td>
<td>302 ± 371</td>
<td>192 ± 139</td>
<td>NS</td>
</tr>
<tr>
<td>Disease activity 28 ESR</td>
<td>5.8 ± 0.7</td>
<td>5.7 ± 0.8</td>
<td>5.3 ± 0.9</td>
<td>NS</td>
</tr>
<tr>
<td>Prednisone (mg)</td>
<td>1.3 ± 2.6</td>
<td>1.5 ± 3.8</td>
<td>2.9 ± 2.7</td>
<td>NS</td>
</tr>
<tr>
<td>Patients on prednisone (%)</td>
<td>60.4 (32/53)</td>
<td>47.4 (18/38)</td>
<td>100 (22/22)</td>
<td>0.05</td>
</tr>
<tr>
<td>Tacrolimus (mg)</td>
<td>2.1 ± 0.8</td>
<td>2.0 ± 0.8</td>
<td>1.9 ± 0.8</td>
<td>NS</td>
</tr>
<tr>
<td>P-glycoprotein expression on CD4 (molecule/cell)</td>
<td>140 ± 58</td>
<td>159 ± 65</td>
<td>248 ± 110</td>
<td>0.05</td>
</tr>
<tr>
<td>P-glycoprotein expression on CD19 (molecule/cell)</td>
<td>374 ± 171</td>
<td>388 ± 173</td>
<td>586 ± 193</td>
<td>0.05</td>
</tr>
<tr>
<td>C/M ratio</td>
<td>0.49 ± 0.12</td>
<td>0.49 ± 0.17</td>
<td>0.36 ± 0.12</td>
<td>0.01</td>
</tr>
</tbody>
</table>

C/M: cell/medium. NS: nonsignificant.
inhibits disease activity by blocking the calcineurin pathway and improves the low response by inhibiting drug exclusion of concomitantly used drugs through P-glycoprotein. Thus, among the existing antirheumatic drugs, tacrolimus is a potentially useful drug, although its mechanism of action is unknown. Patients on combination treatment have high expression levels of P-glycoprotein and low C/M ratio before the initiation of tacrolimus treatment because the concomitantly used drug, such as a corticosteroid, becomes a substrate for P-glycoprotein. Thus, in such patients, treatment with tacrolimus is expected to result in an increase in intracellular concentration of corticosteroids through the inhibitory effect of tacrolimus on P-glycoprotein, leading to a rapid remanifestation of the clinical efficacy of corticosteroid and thus early efficacy. In other words, it is likely that tacrolimus, through its inhibitory action on P-glycoprotein, can lower treatment resistance due to extracellular exclusion of drugs such as corticosteroids and SSZ, leading to improved response to treatment. Our results suggest that initiation of tacrolimus treatment for such patients is highly useful clinically. In addition, the determination of P-glycoprotein expression level on lymphocytes, which correlates with clinical response, is useful for predicting the response to treatment as well as helping with the choice of therapy.

Figure 4. Responses to 2-week treatment with tacrolimus. Data represent values of individual patients and group mean ± SD values. A. Expression of P-glycoprotein on CD4 and CD19 lymphocytes of patients with treatment-resistant RA. B. C/M ratio of peripheral blood mononuclear cells (PBMC) from patients with treatment-resistant RA. C. C/M ratio measured in PBMC of patients with RA before and after 2-week tacrolimus treatment. D. Expression of P-glycoprotein on CD4 lymphocytes of patients with RA before and after 2-week tacrolimus treatment. *p < 0.05, **p < 0.01, Wilcoxon test; ns: nonsignificant.
Table 2. Multivariate logistic analysis of factors involved in response of patients with RA to tacrolimus treatment.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Estimate</th>
<th>SE</th>
<th>Chi-square</th>
<th>Pr &gt; z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>0.1069</td>
<td>2.2912</td>
<td>0.00</td>
<td>0.9628</td>
</tr>
<tr>
<td>C/M ratio</td>
<td>9.0576</td>
<td>4.4412</td>
<td>4.16</td>
<td>0.0414</td>
</tr>
<tr>
<td>P-glycoprotein expression on CD4+ lymphocyte</td>
<td>−0.0133</td>
<td>0.00609</td>
<td>4.79</td>
<td>0.0286</td>
</tr>
<tr>
<td>CRP</td>
<td>−0.1996</td>
<td>0.24054</td>
<td>0.69</td>
<td>0.4066</td>
</tr>
<tr>
<td>MS</td>
<td>0.00149</td>
<td>0.001593</td>
<td>0.88</td>
<td>0.3490</td>
</tr>
</tbody>
</table>

C/M: cell/medium; CRP: C-reactive protein; MS: morning stiffness.
REFERENCES