Myasthenia gravis (MG) is an autoimmune disorder generally mediated by antibodies against the acetylcholine receptors (ACh-R) of skeletal muscles. Production of these antibodies in B cells depends upon ACh-R specific T cells. In general, four therapeutic strategies are available for MG: enhancement of neuromuscular transmission by means of anticholinesterase agents, thymectomy, immunosuppression with glucocorticoids (GCs) and plasma exchange.

In addition to GCs, tacrolimus (FK506) is currently used for the treatment of MG. The drug acts by inhibiting calcium-calmodulin pathways, which results in a reduction of T cell proliferation. We have previously reported on the individual variations in the suppressive properties of FK506 on in vitro blastogenesis of PBMCs from MG patients.

The MDR-1 gene codes for a drug efflux pump P-gp, which is expressed on the surface of lymphocytes and actively transports these ligands, including FK506, out of target cells, thereby reducing their efficacy. These substrates for P-gp may induce P-gp overexpression in either PBMCs or select drug-resistant cell subpopulations.

MDR-1 gene expression and its corresponding protein membrane density in clinical samples have been studied by polymerase chain reaction (RT-PCR) and by immuno-histochemical techniques. However, the evaluation of MDR-1 or P-gp with these methods was not found to be parallel with the data for functional P-gp, ignoring the possibility that regulation may occur at the transcriptional, translational, and/or post-translational levels. An alternative approach, consisting of the measurement of the extrusion of fluorescent dyes from individual cells, was found to allow for the evaluation of the functional activity of P-gp.

This study was undertaken to examine the influence of FK506 therapy on P-gp function in PBMCs and the relationship between P-gp function and PBMC-sensitivity to FK506 in vitro. In addition, we will also report on the clinical response to FK506 in MG patients.

**MATERIALS AND METHODS**

**Reagents** RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Gibco BRL Co. (Grand Island, NY, U.S.A.). Rhodamine 123 (Rh123) was obtained from Sigma Chem. Co. (St. Louis, MO, U.S.A.). CsA and FK506 were kindly provided by Novartis Pharma Co. (Basel, Switzerland) and Fujisawa Pharmaceutical Co. (Japan), respectively. Ficoll-Hypaque was purchased from Nacalai Tesque K.K. (Kyoto, Japan). [3H]Thymidine (5.55×105 Bq/mmole) was purchased from New England Nuclear Co., U.S.A. The other reagents were of the best available grade.

**Subjects** In this study, 10 MG patients and 18 healthy subjects, as a control, were included. The clinical characteristics of the patients are summarized in Table 1. Six patients received FK506 and four patients were administered prednisolone (PSL) as an immunosuppressant and/or anticholinesterase agents. FK506 was administered at a daily dose of 3 mg. PSL was administered before and during the present study. Most of the patients (8/10) had undergone a thymectomy before immunosuppressive drug administration. Eighteen healthy control subjects (8 male and 10 female; 25.3±6.2 years of age) were concurrently included in the study. Twenty milliliters of venous blood from each subject were obtained and heparinized. The study using human PBMCs was approved by the ethical committee of Tokyo University of Pharmacy and life Science, and consent was obtained from the patients and healthy subjects.

**Isolation and Culture of PBMCs** Twenty milliliters of the heparinized blood was loaded on 6 ml of Ficoll-Hypaque, centrifuged at 2000 rpm for 20 min, and PBMCs were sepa-
The cells were washed and resuspended in RPMI 1640 medium containing 10% fetal bovine serum, 100,000 IU/l penicillin, and 100 mg/l streptomycin to a final density of 1 × 10⁶ cells/ml.

**Lymphocyte Culture and Evaluation of Drug Effect**

These procedures were carried out according to the methods described previously. In brief, 200 µl of the cell suspension, as prepared above, was placed into each of the 96 flat-bottom wells of a microtitre plate. ConA was added as a mitogen to a final concentration of 5.0 µg/ml. Subsequently, 4 µl of an ethanol solution containing FK506 was added to give a final agent concentration of 0.001—1000 ng/ml. Four microliters of ethanol were added to the control wells. The plate was then incubated for 96 h in 5% CO₂/air at 37°C. The cells were pulsed with 9.25 kBq/well of [³H] thymidine for the last 16 h of incubation and then collected on a glass fiber filter paper using a multitriever device and dried. The radioactivity retained on the filter was further processed for liquid scintillation counting. The mean of the counts for a duplicate or triplicate of each sample was determined. The concentration that would give 50% inhibition (IC₅₀) of PBMC-blastogenesis was calculated from the dose–response curve.

**Flow Cytometric Detection of Functional Dye Efflux**

Cells were loaded with Rh123 to yield a final dye-concentration of 5 µg/ml and incubated for 10 min at 37°C in a humidified chamber containing 5% CO₂/air. CsA was used as P-gp specific competitive inhibitor to estimate the amount of Rh123 retention in the absence of P-gp activity. At the end of the loading incubation, cells were washed twice with ice-cold phosphate buffered saline without Ca²⁺ and Mg²⁺. The washed cells were resuspended in 1 ml of culture medium containing 10% FBS. The cells were then allowed to efflux Rh123 for 3 h in the presence of 0.1 µg/ml CsA. Immediately after the efflux period, the intracellular Rh123 mean fluorescence intensity was analyzed using a FACSCalibur analyzer (Becton Dickinson).

**Analysis of P-gp Efflux Data**

The data were analyzed with a FACSCalibur flow cytometer using CellQuest software (Becton Dickinson). Efflux of Rh123 by PBMCs was assessed by analyzing changes in cellular fluorescence after efflux in the presence or absence of CsA. Rh123 is excited at a wavelength of 488 nm. Fluorescence intensity of Rh123 in the PBMCs was detected on fluorescence channel one (FL1) of the FACSCalibur using a 1024 log channel scale. The mean cellular fluorescence of the PBMCs after efflux in the presence or absence of CsA was recorded. Rh123 intensity was assessed by calculating its difference in fluorescence intensity in the PBMCs after efflux in the presence or absence of CsA, measured both by using the Kolmogorov–Smirnov (KS) statistic D. The KS statistic measures the difference between two distribution functions, and generates a D value ranging between 0 and 1.0. A higher D value indicates a greater difference between the distribution functions.

**Evaluation of Clinical Outcome of the Patients**

Clinical evaluation of MG patients was based on the duration of MG, thymus pathology, change in serum anti-ACh-R antibodies titers, and clinical muscle testing (MG score). MG is attributed to circulating antibodies to the ACh-R with additional mediation of the complement system. On estimating antibody titers by immunoprecipitation assay, which detects antibodies directed to all antigenic ACh-R determinants except for its bungarotoxin binding site, a correlation with clinical severity has previously been noted. MG score was assessed by observations of changes in strength (ptosis, facial muscles, swallowing, lung capacity, vital capacity, tongue dyskinesia, outstretching of arms, outstretching of legs, grip, and swallowing). Significant improvement was defined up to 3 points. For calculation, grades of each item are added.

**Statistical Analysis**

For comparison of means, the data were first analyzed by one way ANOVA. Statistics were carried out with a Bonferroni/Dunn multiple comparison test. Relationships between any two indices were analyzed with Pearson’s correlation coefficient test. Calculated p values of less than 0.05 were considered to be significant.

**RESULTS**

**P-Glycoprotein Expression and Function in PBMCs**

P-gp antigen expression in the PBMCs obtained from the three subject groups, i.e., 18 healthy subjects as a control, six MG patients administered FK506 (MG(FK+) group), and four MG patients who had no experience with FK506 (MG(FK−) group) was evaluated. The baseline characteristics for all patients are listed in Table 1. By measuring the amount of Rh123 retained in the PBMCs, the P-gp function on PBMCs was analyzed. After loading Rh123 dye for

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**Table 1. Baseline Characteristics of MG Patients**

<table>
<thead>
<tr>
<th>Case</th>
<th>FK506 (mg/d)</th>
<th>FK506 duration (month)</th>
<th>Age/sex</th>
<th>Osseumann classification</th>
<th>Duration (year)</th>
<th>Thymectomy</th>
<th>Pathology</th>
<th>Ach-R (nmol/l)</th>
<th>PSL</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>FK506+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 1</td>
<td>3</td>
<td>3.0</td>
<td>50/F</td>
<td>IIb</td>
<td>6.0</td>
<td>+</td>
<td>Hyperplasia</td>
<td>20</td>
<td>5 mg/d</td>
<td>ChE</td>
</tr>
<tr>
<td>Patient 2</td>
<td>3</td>
<td>5.5</td>
<td>52/M</td>
<td>IIb</td>
<td>3.8</td>
<td>+</td>
<td>Hyperplasia</td>
<td>33</td>
<td>ChE</td>
<td></td>
</tr>
<tr>
<td>Patient 3</td>
<td>3</td>
<td>10.0</td>
<td>66/M</td>
<td>IIb</td>
<td>12.6</td>
<td>+</td>
<td>Thymoma</td>
<td>7.6</td>
<td>ChE</td>
<td></td>
</tr>
<tr>
<td>Patient 4</td>
<td>3</td>
<td>2.5</td>
<td>51/M</td>
<td>IIb</td>
<td>3.5</td>
<td>+</td>
<td>Thymoma</td>
<td>29</td>
<td>ChE</td>
<td></td>
</tr>
<tr>
<td>Patient 5</td>
<td>3</td>
<td>7.0</td>
<td>65/M</td>
<td>IIb</td>
<td>12.3</td>
<td>+</td>
<td>Hyperplasia</td>
<td>60</td>
<td>15 mg/d</td>
<td>ChE</td>
</tr>
<tr>
<td>Patient 6</td>
<td>3</td>
<td>7.0</td>
<td>57/F</td>
<td>IIb</td>
<td>33.1</td>
<td>-</td>
<td>Thymoma</td>
<td>7</td>
<td>ChE</td>
<td></td>
</tr>
<tr>
<td>FK506−</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 7</td>
<td>58/M</td>
<td>IIb</td>
<td>0.7</td>
<td>-</td>
<td>0.2</td>
<td>ChE</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 8</td>
<td>65/M</td>
<td>IIb</td>
<td>3.1</td>
<td>+</td>
<td>Hyperplasia</td>
<td>29</td>
<td>80 mg/d</td>
<td>ChE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 9</td>
<td>46/M</td>
<td>I</td>
<td>33.0</td>
<td>+</td>
<td>Hyperplasia</td>
<td>360</td>
<td>80 mg/d</td>
<td>ChE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 10</td>
<td>49/F</td>
<td>IIb</td>
<td>5.8</td>
<td>+</td>
<td>Hyperplasia</td>
<td>20</td>
<td>5 mg/d</td>
<td>ChE</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

M = male, F = female, ACh-R = anti-acetylcholine receptor antibody titer (normal <0.02 nmol/l), PSL = prednisolone, ChE = cholinesterase inhibitor.
Cells were loaded with Rh123 and incubated for 10 min and then allowed to efflux Rh123 for 3 h in the presence or absence of 0.1 μg/ml CsA, as used as P-gp specific competitive inhibitor to estimate the amount of Rh123 retention in the absence of P-gp activity. (A) Rh123 mean fluorescence intensity in PBMCs of six MG patients treated with FK506 (MG(FK+)) was not significantly different compared to that in PBMCs of four MG patients treated without FK506 (MG(FK−)) or the 18 healthy subjects. The distributions of the Rh123 mean fluorescence in PBMCs after 10 min uptake in the MG(FK+) group was higher than those in the MG(FK−) group (p=0.044) and healthy subjects (p=0.012). The differences of the Rh123 mean fluorescence for 3 h efflux in the presence or absence of CsA were found among the MG(FK+) group (p=0.002), the MG (FK−) group (p=0.0057) and the healthy subjects (p<0.0001). (B) Fluorescence intensity of Rh123 in PBMCs after efflux in the presence or absence of CsA (0.1 μg/ml) was analyzed by the Kolmogorov–Smirnov (KS) statistic D. The KS statistic measures the difference between two distribution functions, and generates a D value ranging between 0 and 1.0. A higher D value indicates a greater difference between the distribution functions. P-gp function in PBMCs from MG patients treated with FK506 was significantly lower than that in PBMCs from healthy subjects (p=0.0084).

10 min, the cells presented a shift to the right in the histogram of the flow cytometry data, which corresponded to Rh123 accumulation. Subsequently, the cells were incubated for 3 h in the presence or absence of 0.1 μg/ml CsA to evaluate Rh123 efflux mediated by P-gp. The distributions of the Rh123 mean fluorescence in PBMCs after 10 min uptake in the MG(FK+) group was higher than those in the MG(FK−) group (p=0.044) and healthy subjects (p=0.012). Especially, the Rh123 uptake exhibited wide individual variations in the healthy patients (54.6—362.3) beyond the intraindividual differences. Differences in the Rh123 mean fluorescence 3 h post efflux in the presence or absence of CsA were found among the MG(FK+) group (p=0.002), the MG (FK−) group (p=0.0057) and the healthy subjects (p<0.0001) (Fig. 1A). P-gp function of PBMCs as evaluated by KS D value was significantly lower in the MG (FK+) group when compared to the healthy subjects (p=0.0084) (Fig. 1B). However, a significant difference in P-gp function between the MG (FK+) and MG (FK−) groups was not found.

Change of P-gp Function in PBMCs from MG Patients during FK506 Therapy

The P-gp function of the PBMCs in three MG patients was continuously assessed during FK506 therapy. One of the patients (Patient 1) received FK506 (3 mg/d) and PSL (5 mg/d) in combination, and the dye retention ability of PBMCs was evaluated before and 70 d after administration of FK506. Others received FK506 (3 mg/d) without PSL from 78 to 176 d (Patient 2; ■) and from 204 to 302 d (Patient 3; △) after the beginning of FK506 therapy. The change of Rh123 uptake in PBMCs (A), retention for 3 h in the presence (B) or absence (C) of CsA and P-gp function of PBMCs evaluated by KS D value (D) were estimated in these patients. There were statistically significant increases in Rh123 uptakes (p=0.012) and retention in the presence of CsA (p=0.011). The P-gp efflux function of PBMCs (KS D value) did not decrease significantly during the study (p=0.080).
blastogenesis in vitro were compared among the four MG patients who received FK506 (MG(FK+)) group, the four MG patients treated without FK506 (MG(FK−)) group, and the eight healthy subjects. The medians (range) of FK506-IC_{50s} on PBMCs from the MG(FK+) group, the MG(FK−) group, and the healthy subjects were 0.02 (0.001—0.07), 0.12 (0.002—0.27), and 0.16 (0.07—0.51) ng/ml, respectively. The IC_{50} means (S.D.) of these groups were 0.028 (0.031), 0.121 (0.139), and 0.215 (0.160), respectively. When compared with the healthy subjects, the IC_{50s} in the MG(FK+) group were significantly lower (p=0.02) (Fig. 3A). Furthermore, PBMC response to FK506 in vitro (IC_{50}) in MG patients and healthy subjects correlated significantly with P-gp function (p=0.011, n=16).

**The Relationship between Clinical Course and P-gp Function in PBMCs from MG Patients during FK506 Therapy** The total amounts of FK506 administered were 210—906 mg during the course of FK506 therapy (70—302 d) in the MG(FK+) patients. Serum ACh-R Ab levels and MG scores in the four MG patients were monitored to assess the efficacy of FK506 treatment. The changes in their ACh-R Ab levels or MG scores were investigated during the period of FK506 treatment. The mean (range) of anti-ACh-R Ab levels (nmol/l) in these patients was 73.9 (7.6—220) before FK506 treatment. The mean (range) of total MG score in these patients was 13.3 (11—15) before FK506 treatment. MG score improved in these patients during the course of FK506 therapy for four months.

**DISCUSSION**

This study evaluated the P-gp function of PBMCs in MG patients treated with FK506. When compared with the PBMCs of healthy subjects, P-gp function of PBMCs in MG patients, especially in those treated with FK506, were significantly lower (p=0.0084) (Fig. 1). Furthermore, the P-gp function in PBMCs decreased significantly during FK506 treatment (Fig. 2). From these data, it can be supposed that...
immunosuppressive therapy by FK506 in MG patients impairs P-gp efflux activity and thus augments the amount of immunosuppressive drugs in PBMCs. One of the mechanisms for P-gp attenuation by FK506 is related to the finding that immunophilin-P-gp interaction is required for P-gp processing and function.13) Because immunophilins have been reported to be important for the proper trafficking of rhodopsin,16) ryanodine,17) and inositol 1,4,5-triphosphate,18) it is possible to speculate that immunophilins play a role in the processing of P-gp as well. P-gp function in human T cells is known to be implicated in cytokine secretion, T cell survival and differentiation, and cytotoxic T cell effecter function. However the findings with regard to cytokine secretion are controversial.19) The possibility that another aspect of FK506 action(s) involves interference with interleukin transport catalyzed by the P-gp homologues may be worth considering.

This study also demonstrates that in vitro PBMC sensitivity to FK506 in MG patients treated with FK506 was significantly higher compared with that in healthy subjects. FK506 has also been reported to exert an immunosuppressive action after binding to intracellular FKBP's in T cells.20) Suzuki et al. showed that reduced amounts of FK506 binding protein (FKBP) in T lymphocytes from FK506-treated Bechet's disease patients may be associated with diminished efficacy of the drug after a long period of treatment.21) Dumont et al. (1994) reported that an association of FK506 with FKBP in mouse splenic T cells,22) approximately half of the amount of FK506 taken up by the cells, still continued after 20h of being cultured. The dissociation constant value for the interaction between FK506 and FKBP is reported to be 0.4 nm.23) Though the influence of the intracellular concentration of FK506 on PBMC sensitivity to the drug has not been made clear from our present data, an increase in PBMC response to FK506 in vitro was observed under FK506 treatment. Whereas, there was no statistically significant difference in the PBMC stimulation index (SI) by ConA between MG patients treated with FK506 (114.1±35.3) and healthy subjects (121.3±35.7). Furthermore, PBMC response to FK506 in vitro (IC50) correlated significantly with the P-gp function (p=0.011) (Fig. 3b). These results suggest that long-term administration with FK506 attenuates P-gp function and increases the sensitivity to FK506 in vitro in PBMCs of MG patients. In this study, a significant correlation between the clinical response of MG patients to the therapy assessed by anti-ACh-R Ab levels or total amounts of FK506 administered and Rh123 efflux activity of PBMCs was not found. However, among the MG patients treated by FK506 have undergone successful clinical courses,24,25) and the ACh-R Ab levels or MG scores were improved or preserved in MG patients during the course of FK506 therapy in this study (Fig. 4). The immunoprecipitation assay used to estimate the anti-ACh-R antibody titers detected antibodies directed to all antigenic ACh-R determinants except for the toxin binding site. One previous study reported a poor correlation between anti-ACh-R antibody titers and the clinical severity of MG.26) Thus, the influence of P-gp function in PBMCs on the clinical severity of MG treated with FK506 has yet to be clarified.

P-gp transports drugs generally used for the treatment of autoimmune diseases including GCs and FK506.27) GC is the mainstay for MG treatment.1,28) Richaud et al. described P-gp overfunction in lymphocytes from MG patients,29) and suggested that drug resistance may be induced by long-term or high-dose administration with GCs. The administration of low-dose FK506 combined with GC is gaining in popularity recently.30) In our results, the Rh123 uptake in the MG(FK−) group was lower than in the MG(FK+) group and the influence of CsA as a P-gp inhibitor was found only in the MG(FK+) group (Fig. 1A). Three out of four MG(FK−) patients were treated with PSL continuously, and GCs are suggested to increase P-gp expression or inhibit its function.31) High-dose administration of GCs for the treatment of autoimmune diseases results in increased expression of MDR1 mRNA32) and the related protein33) in PBMCs, which may impair successful therapy in these patients. Thus, FK506 may be helpful for the prevention of P-gp function and subsequent diminution of the efficacy of immunosuppressive drugs in the treatment of MG.

REFERENCES