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Suppressive potencies of calcineurin inhibitors against the mitogen-induced blastogenesis of peripheral-blood mononuclear cells of myasthenia gravis patients

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Abstract

The calcineurin inhibitors, tacrolimus and ciclosporin, are two useful immunosuppressive drugs for the treatment of myasthenia gravis (MG), for patients who have low responses to glucocorticoids. We have studied the suppressive potencies of tacrolimus and ciclosporin on concanavalin A-induced blastogenesis of peripheral-blood mononuclear cells (PBMCs) obtained from 38 MG patients and 26 healthy volunteers. Differences in the IC50 values of the two calcineurin inhibitors between the patients and the healthy subjects were evaluated. The median (range) IC50 values for tacrolimus and ciclosporin on the blastogenesis of PBMCs of MG patients were 0.06 (0.001–100) and 0.41 (0.09–83.0) ng mL^{-1}, respectively. In contrast, the median (range) IC50 values of tacrolimus and ciclosporin on healthy PBMCs were 0.16 (0.001–0.33) and 5.59 (1.4–31.3), respectively, and thus ciclosporin potencies against PBMCs of MG patients were significantly higher than those against PBMCs of healthy subjects ($P < 0.0001$). The differences in tacrolimus IC50 values between the patients and healthy subjects were not significant. There was a correlation between ciclosporin IC50 values against the blastogenesis of PBMCs of MG patients and the duration of the disease ($r = 0.35$, $P = 0.049$). A significant correlation between the IC50 values of ciclosporin and those of prednisolone against the blastogenesis of PBMCs of MG patients was also observed ($r = 0.56$, $P = 0.003$). Furthermore, the ciclosporin IC50 values significantly correlated with the periods of glucocorticoid administration for MG treatment ($r = 0.42$, $P = 0.038$). Such correlations were not observed with the tacrolimus IC50 values. These results suggested that glucocorticoid administration had an influence on PBMC response to the suppressive efficacy of ciclosporin in MG.

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Introduction

Myasthenia gravis (MG) is an autoimmune disease generally mediated by auto-antibodies to the acetylcholine receptor (AChR-Ab) of skeletal muscles (Vincent et al 2001). In general, four therapeutic strategies are available for MG treatment: enhancement of neuromuscular transmission by anticholinesterase agents, thymectomy, immunosuppression with glucocorticoids, and plasma exchange (García-Crrasco et al 2007). Extended thymectomy is a well-accepted surgical treatment for MG patients. However, AchR-Ab is produced not only in the thymus, but also in external thymic tissues, including peripheral lymph nodes and bone marrow. As such, immunosuppressive therapy, especially high-dose glucocorticoid therapy combined with extended thymectomy, has been proposed and favourable results have been reported (Yamaguchi et al 1987; Sekine et al 2006). Although glucocorticoids are widely used for the treatment of many kinds of autoimmune disorders including MG, individual variations in clinical effects have been observed, and some patients continue treatment with glucocorticoids despite the onset of side effects and poor response (Rivner et al 2002).

Tacrolimus and ciclosporin are currently used for the treatment of MG in patients with low response to glucocorticoid therapy. The drugs act by inhibiting calcium–calcineurin pathways, which results in a reduction of T-cell proliferation (Kino et al 1987). To evaluate the individual therapeutic potential for calcineurin inhibitors, pharmacodynamic approaches using patient peripheral-blood mononuclear cells (PBMCs), in addition to

pharmacokinetics of calcineurin inhibitors, could be an efficient strategy. Indeed, the clinical efficacies of immunosuppressive drugs can be evaluated indirectly in renal transplantation, asthma, and psoriasis using PBMCs of patient origin (Hirano et al 1994, 1997, 1998). This information would be useful for carrying out individually appropriate calcineurin inhibitor therapy.

In this study, we examined the differences and individual variations in anti-proliferative potencies of tacrolimus and ciclosporin on T-cell mitogen-induced blastogenesis of PBMCs obtained from 38 patients with MG and 26 healthy subjects.

Materials and Methods

Reagents

RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Gibco BRL Co. (Grand Island, NY, USA). Ficoll-Hypaque was purchased from Nakarai Co., Japan. Concanavalin A (Con A) was obtained from Seikagaku Kogyo, Japan. [^{3}H]Thymidine (5.55×10^{11} Bq mmol^{-1}) was purchased from New England Nuclear Co., USA. The other reagents were of the best grade available.

Subjects

In this study, 38 MG patients (5 male and 33 female; aged 47.0 ± 12.0 years (mean ± s.d.)) and 26 healthy volunteers (6 male and 20 female; aged 41.3 ± 5.3 years), as a control, were included. There were no statistically significant differences in age and the male/female ratio between the patient group and the volunteers. Of the 38 MG patients, 31 had undergone a thymectomy before immunosuppressive drug administration. Healthy volunteers had no history of immunosuppressive medication. Venous blood (20 mL) from each subject was obtained and heparinized. The study using human PBMCs was approved by the ethical committee of Tokyo University of Pharmacy and Life Sciences. Consent was obtained from the patients and the healthy volunteers.

Isolation and culture of PBMCs

Heparinized blood (20 mL) was loaded on 6-mL Ficoll-Hypaque, centrifuged at 2000 rev min^{-1} for 20 min, and PBMCs were separated as described previously (Hirano et al 1994). The cells were washed and resuspended in RPMI 1640 medium containing 10% FBS, 100 000 IU L^{-1} penicillin, and 100 mg L^{-1} streptomycin to a final density of 1×10^6 cells mL^{-1}.

PBMC culture and evaluation of drug effect

The procedures were carried out according to the methods described by Rivner et al (2002). In brief, 200 μL cell suspension, as prepared above, was placed into each of the 96 flat-bottom wells of a microtitre plate. Con A was added as a mitogen to a final concentration of 5.0 μg mL^{-1}. Subsequently, 4 μL of an ethanol solution containing tacrolimus, ciclosporin, or prednisolone was added to give a final agent concentration of 0.001–10000 ng mL^{-1}. Ethanol (4 μL) was added to the control wells. The plate was incubated for 96 h in 5% CO_2/air at 37°C. For the final 16-h incubation the cells were pulsed with 9.25 kBq/well [^{3}H]thymidine, and then collected on a glass fibre filter paper using a multiharvester 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.

Statistical analysis

For comparison of means, the data were analysed by two-tailed unpaired *t*-tests. Fisher's exact tests were used to compare the proportion of patients with a given characteristic. Statistics were carried out with a Student's *t*-test and a Mann–Whitney U test to examine differences in IC50 values of calcineurin inhibitors between the two subject groups. Relationships between any two indices were analysed with Pearson's correlation coefficient test. Calculated *P* values of less than 0.05 were considered to be significant.

Results

Comparative study of PBMC responses to calcineurin inhibitors between MG patients and healthy subjects

Typical dose–response curves of tacrolimus and ciclosporin on Con A-induced blastogenesis of PBMCs derived from one MG patient are shown in Figure 1. The extent of PBMC blastogenesis in response to Con A was estimated by the stimulation index (SI), which represents the ratio to the growth of cells in the absence of Con A. The SI was not

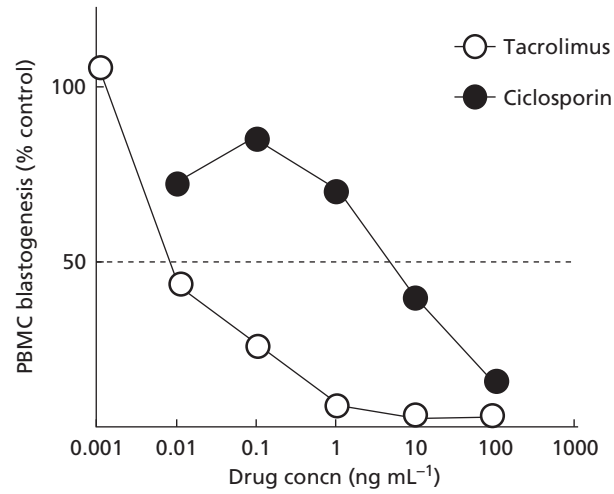

Figure 1 Dose–response curves of tacrolimus and ciclosporin on mitogen-induced blastogenesis of PBMCs from a patient with myasthenia gravis.

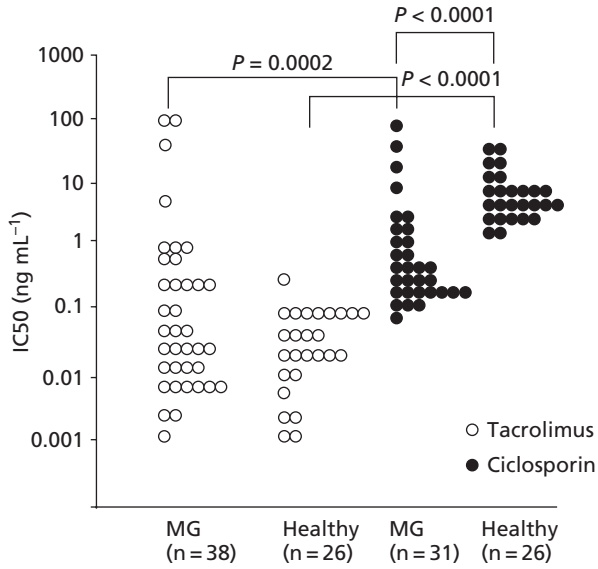

Figure 2 Individual PBMC sensitivity to tacrolimus and ciclosporin in patients with myasthenia gravis (MG) and healthy subjects. PBMC sensitivity to calcineurin inhibitors was expressed as the IC50 value (ng mL^{-1}) for each drug. The potency of ciclosporin on the blastogenesis of PBMCs of the healthy subjects was significantly higher than that of MG patients ($P < 0.0001$). Tacrolimus IC50 values were not significantly different between MG patients and healthy subjects. The IC50 values of ciclosporin were significantly higher than those of tacrolimus in MG patients ($P = 0.0002$) and healthy subjects ($P < 0.0001$), respectively.

significantly different between the MG patients and the healthy subjects (data not shown).

The median (range) IC50 values of tacrolimus and ciclosporin on the blastogenesis of PBMCs from MG patients and healthy volunteers are shown in Figure 2. The median (range) IC50 values of tacrolimus and ciclosporin on the blastogenesis of PBMCs from patients with MG were 0.06 (0.001–100) and 0.41 (0.09–83.0) ng mL^{-1}, respectively. The median IC50 values of tacrolimus and ciclosporin on the blastogenesis of PBMCs from healthy subjects were 0.16 (0.001–0.33) and 5.59 (1.4–31.3) ng mL^{-1}, respectively. When we compared the IC50 values of calcineurin inhibitors between MG patients and healthy subjects, the potency of ciclosporin on the blastogenesis of PBMCs of the healthy subjects was significantly higher than that of MG patients ($P < 0.0001$). However, such a difference was not observed for the tacrolimus IC50 values between MG patients and healthy subjects. When we compared the IC50 values of calcineurin inhibitors, the potencies of ciclosporin on the blastogenesis of PBMCs were significantly higher than those of tacrolimus in MG patients ($P = 0.0002$) and in healthy subjects ($P < 0.0001$).

Relationship between IC50 values of calcineurin inhibitors and clinical characteristics of MG patients

We divided the MG patients into two groups based on their history of thymectomy, and compared their PBMC sensitivity to glucocorticoids between the thymectomized and nonthymectomized group. As shown in Table 1, the median (range) IC50 values of tacrolimus on the blastogenesis of PBMCs from MG patients thymectomized (n = 1) and those nonthymectomized (n = 7) were 0.04 (0.001–100.0) and 0.11 (0.02–43.0) ng mL^{-1}, respectively. The median (range) IC50 values of ciclosporin on the blastogenesis of PBMCs from MG patients thymectomized and those nonthymectomized were 0.40 (0.09–83.0) and 0.55 (0.13–29.2) ng mL^{-1}, respectively. The IC50 value of each calcineurin inhibitor was not significantly different between the two patient subgroups.

As shown in Figure 3, PBMC responses to tacrolimus in-vitro (IC50 values) in MG patients did not correlate with the duration period of the disease (Figure 3A), whereas the IC50 values of ciclosporin on the blastogenesis of PBMCs from MG patients correlated significantly with the duration period of the disease (Figure 3B) (r = 0.35, $P = 0.049$).

We also examined the relationship between the experience of glucocorticoid treatment and the IC50 values of calcineurin inhibitors in these patients. Of the 38 MG patients, 22 had been treated with prednisolone. When we divided these MG patients into two groups based on their history of glucocorticoid treatment, the median (range) IC50 values of tacrolimus on the blastogenesis of PBMCs from MG patients treated with (n = 22) and without prednisolone (n = 13) were 0.04 (0.002–42.3) and 0.11 (0.001–99.9) ng mL^{-1}, respectively. In contrast, the median (range) IC50 values of ciclosporin on the blastogenesis of PBMCs from MG patients treated with and without prednisolone were 0.52 (0.13–29.2) and 0.28 (0.09–17.6) ng mL^{-1}, respectively. Thus, the oral administration of prednisolone did not result in a significant influence on PBMC sensitivity to tacrolimus and ciclosporin. There was no significant correlation between the IC50 values of prednisolone on the blastogenesis of PBMCs and the glucocorticoid treatment period in the MG patients (data not

Table 1 Comparison of PBMC sensitivity to calcineurin inhibitors between myasthenia gravis patients treated with and without thymectomy

	Thymectomized	Nonthymectomized	*P* value
Patient number	31	7	
Sex (male/female)	5/26	0/7	NS[1]
Age	46.2 ± 12.9	50.6 ± 6.4	NS[2]
IC50 median (range) (ng mL^{-1})			
Tacrolimus	0.04 (0.001–100.0)	0.11 (0.02–43.0)	NS[3]
Ciclosporin	0.40 (0.09–83.0)	0.55 (0.13–29.2)	NS[3]

[1]Fisher's exact test. [2]Student's *t*-test. [3]Mann-Whitney test. NS, not significant.

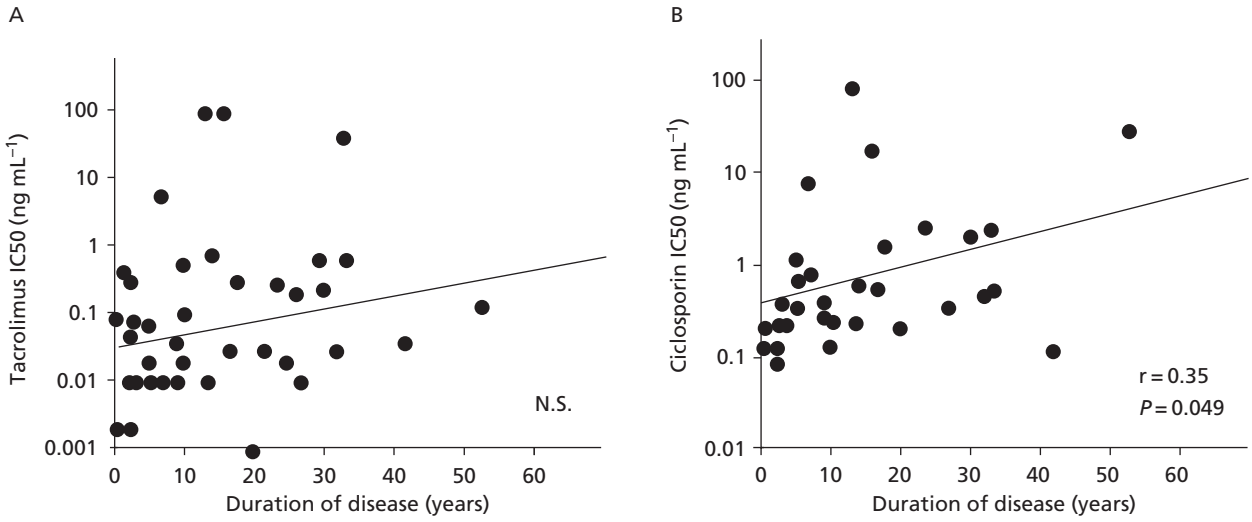

Figure 3 Relationship between PBMC sensitivities to tacrolimus (A) or ciclosporin (B) in-vitro and the duration of disease in myasthenia gravis (MG) patients. PBMC response to ciclosporin in-vitro (IC50) in MG patients correlated significantly with the duration of the disease (years) (r = 0.35, $P = 0.049$).

shown). The IC50 values for ciclosporin and those for prednisolone were significantly correlated (r = 0.56, $P = 0.003$) (Figure 4A). Although the IC50 values of tacrolimus were not significantly correlated with those for prednisolone (Figure 4B), we observed such a tendency in the PBMC sensitivity to tacrolimus.

Furthermore, when we compared the relationship between PBMC sensitivity to calcineurin inhibitors and the periods of glucocorticoid administration, there was a significant correlation between IC50 values of ciclosporin on the blastogenesis of PBMCs and the glucocorticoid treatment period (r = 0.42, $P = 0.038$) (Figure 5B). However, such a correlation was not observed with the tacrolimus IC50 values (Figure 5A).

Discussion

In this study, we evaluated the PBMC response to the suppressive efficacy of calcineurin inhibitors in MG patients. Our results suggested that MG patients were likely to show a high response to ciclosporin therapy as compared with healthy subjects, although the potency of tacrolimus was higher than that of ciclosporin in MG patients and in healthy subjects. The duration period of the disease correlated significantly with the IC50 values of ciclosporin on the blastogenesis of PBMCs in the MG patients. Our results suggested the influence of long-term glucocorticoid treatment

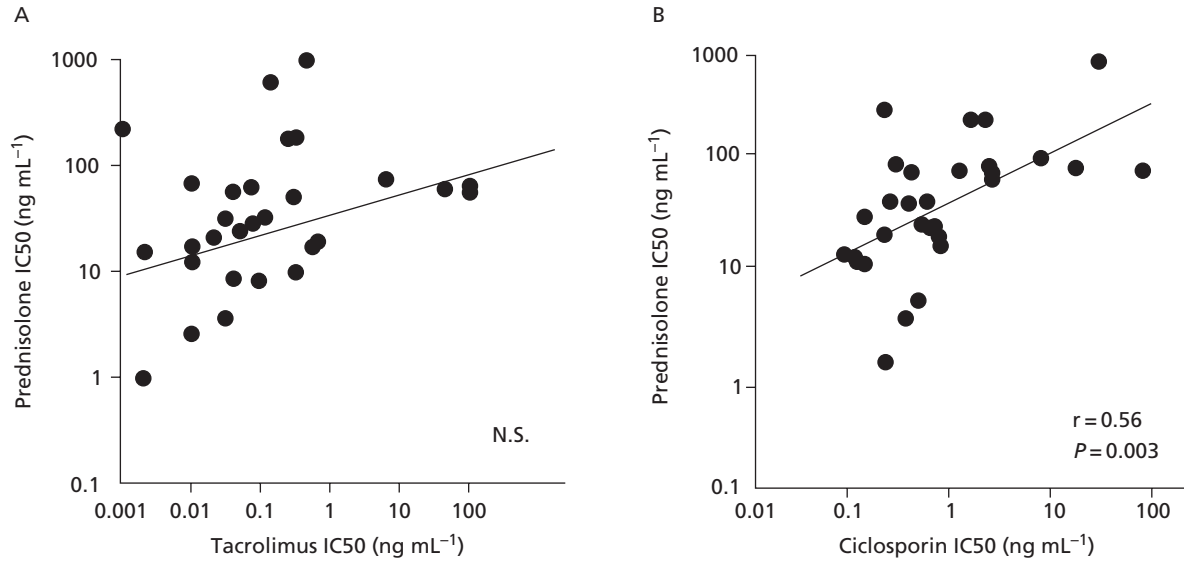

Figure 4 Relationship between PBMC sensitivity to tacrolimus (A) or ciclosporin (B) and those to prednisolone in-vitro in myasthenia gravis (MG) patients. PBMC sensitivity to ciclosporin in-vitro (IC50) in MG patients correlated significantly with that to prednisolone (r = 0.56, $P = 0.003$).

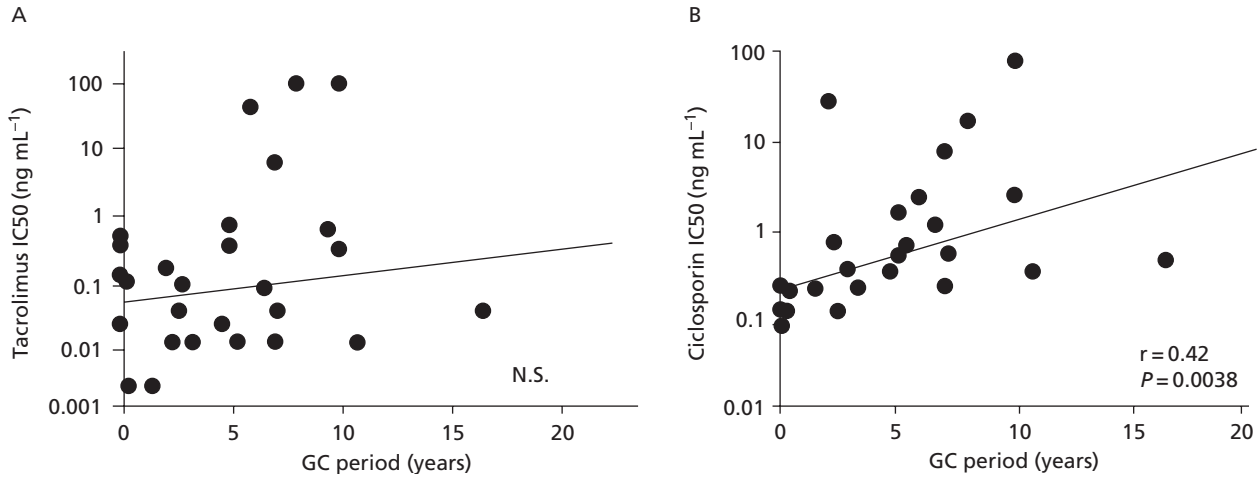

Figure 5 Relationship between PBMC sensitivity to tacrolimus (A) and ciclosporin (B) in-vitro and the periods of glucocorticoid (GC) administration in myasthenia gravis (MG) patients. PBMC responses to ciclosporin in-vitro (IC50 values) correlated significantly with the period (years) of glucocorticoid treatment in MG patients (r = 0.42, P = 0.038).

on the inhibitory effect of ciclosporin for PBMC proliferation. From these results, it was suggested that the potency of ciclosporin against the blastogenesis of PBMCs of MG patients in the early stages might be high, as compared with those against the blastogenesis of PBMCs of MG patients in the later stages. Previous studies have shown a correlation between the in-vitro response of PBMCs to the suppressive efficacy of glucocorticoids or calcineurin inhibitors and the clinical efficacy of the drugs in organ transplantations and several autoimmune disorders (Hirano 2007). Thus, it could be suggested that long-term administration of glucocorticoids may have decreased the response of MG patients to ciclosporin therapy.

As shown in Figure 1, differences in PBMC-suppressive potencies between the two calcineurin inhibitors were significant. In general, tacrolimus is known to be 10–100-times more potent than ciclosporin (Spencer et al 1997). Although these drugs suppress signal transduction at the calcineurin level, this is achieved via complex formation with different binding proteins (Plosker & Foster 2000). In addition, tacrolimus acts by a variety of different mechanisms, which include inhibition of calcineurin (Plosker & Foster 2000). Thus, these differences of actions of the two drugs may have been implicated in the different potencies of these drugs observed.

MG is an autoimmune neuromuscular disorder with a chronic clinical course that requires long-term multiple modality therapy, besides glucocorticoid therapy (García-Crrasco et al 2007). Thymectomy is also one of the most effective MG therapies. Accordingly, the extended thymectomy combined with glucocorticoid would be of benefit in MG treatment. When we compared PBMC sensitivity with calcineurin inhibitors between the thymectomized and nonthymectomized groups, there were no differences in the IC50 values of tacrolimus and ciclosporin (Table 1). Thus, our data suggested that thymectomy in MG patients did not alter the efficacy of calcineurin inhibitors to suppress PBMC proliferation.

A drug efflux pump P-glycoprotein (P-gp) is expressed on the surface of lymphocytes (Chaudhary et al 1992) and actively transports glucocorticoids out of target cells, thereby reducing the drug efficacy (Bourgeois et al 1993). Richaud-Patin et al (2004a) described P-gp overfunction in lymphocytes from MG patients, and suggested that drug resistance may have been induced by long-term or high-dose administration with glucocorticoids. High-dose administration of glucocorticoids for the treatment of autoimmune diseases results in increased expression of MDR1 mRNA and P-gp in PBMCs, which may impair successful therapy in these patients (Richaud-Patin et al 2004b). It was reported that alternate-day administration of high-dose prednisolone may reduce the risk of post-thymectomy myasthenic crisis (Sekine et al 2006). We showed a correlation between the potencies of ciclosporin and prednisolone in-vitro to suppress the blastogenesis of PBMCs of MG patients. Calcineurin inhibitors are also transported by P-gp (Saeki et al 1993). We reported that tacrolimus treatment attenuated P-gp function in the PBMCs of MG patients (Tanaka et al 2007). In contrast, Koziolek et al (2001) reported that the P-gp expression increased in the brush border, arterial endothelia, and Bowman's capsule after ciclosporin treatment. The difference of the influence on P-gp between these calcineurin inhibitors might have been the cause of the difference in the potencies of these drugs against the blastogenesis of PBMCs of MG patients treated with glucocorticoids. Future studies should focus on the efficacy of calcineurin inhibitors on steroid resistance in MG patients and elucidate the cause of the different actions between glucocorticoid and calcineurin inhibitors.

Conclusion

The suppressive potency of ciclosporin against the blastogenesis of PBMCs of MG patients was higher compared with that of ciclosporin against the blastogenesis of PBMCs of healthy subjects. The data suggested that the ciclosporin response in

PBMCs of MG patients was decreased by the administration of glucocorticoids. Such a tendency was not observed with tacrolimus.

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