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Clinical efficacy and cytokine network-modulating effects of tacrolimus in myasthenia gravis

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Abstract

To clarify the long-term efficacy, safety and the cytokine network-modulating effects of tacrolimus in myasthenia gravis, medical records of 86 newly diagnosed consecutive patients and nine steroid-dependent patients were retrospectively reviewed, and peripheral blood mononuclear cells (PBMC) were cultured for the cytokine profile. Steroid reduction effects were observed by using tacrolimus, and no serious adverse effects were observed. The culture study showed reduced IL-12, IL-17, IFN-γ, GM-CSF, TNF-α and MIP-1β, and elevated IL-10 in the PBMC from patients who received tacrolimus, which suggests inhibition of T cells and macrophages, and enhancement of type 1 regulatory T cells.

Keywords: Myasthenia gravis, tacrolimus, cytokine, immunotherapy

1. Introduction

Myasthenia gravis (MG) is an autoimmune disease in which autoantibodies against the nicotinic acetylcholine receptor (AChR) cause a transmission failure at neuromuscular junctions (Conti-Fine et al., 2006). The production of these antibodies in B cells depends upon AChR-specific T cells (Fujii and Lindstrom, 1988). Long-term corticosteroid treatment markedly improves the outcome of MG therapy (Pascuzzi et al., 1984). Additionally, nonsteroidal immunosuppressive agents, such as cyclosporine (Bonifati and Angelini, 1997), azathioprine (Palace et al., 1998), and cyclophosphamide (De Feo et al., 2002), have been used in combination with steroids.

Recently, tacrolimus has also become available for the treatment of MG. It has been reported in non-controlled studies that low-dose administration of tacrolimus is effective and safe in combination with steroids for MG (Evoli et al., 2002; Konishi et al., 2005; Yoshikawa et al., 2002a). A distinguishing feature of tacrolimus is that its effect appears early after its administration. The long-term efficacy of tacrolimus has also been reported (Konishi et al., 2005; Ponseti et al., 2005). Tacrolimus is a macrolide immunosuppressive agent isolated from *Streptomyces tsukubaensis* (Kino et

al., 1987a) and has been used in the field of organ transplantation (Undre et al., 1999). It exhibits immunosuppressive activity by inhibiting production of interleukin (IL) -2 (Flanagan et al., 1991), which is an early T cell activation factor and stimulates cell growth and differentiation (Tocci et al., 1989). However, the pharmacological effects of tacrolimus are not fully understood. It is thought that there is a significant correlation between IgG production and productions of IL-5 and IL-6 by cultured peripheral blood mononuclear cells (PBMCs) from patients with MG (Yoshikawa et al., 2002b). However the effects of tacrolimus on cytokine production by cultured PBMCs have not been evaluated.

In this study, we examined the long-term efficacy and safety of tacrolimus for the treatment of newly diagnosed MG patients in a retrospective case-controlled study. We also examined its efficacy and safety for steroid-dependent patients. In addition, we studied cytokine or chemokine production profiles in PBMCs to clarify the pharmacological effects of tacrolimus in patients with MG.

2. Materials and Methods

2.1 Clinical study 1. -Clinical study of steroid-responsive MG patients in the early stage-

2.1.1 Patients

We retrospectively reviewed the medical records of 86 consecutive MG patients admitted to Kanazawa University Hospital between 1993 and 2004 (Figure 1). Diagnosis of MG was based on their history, neurological examinations, electrophysiological studies (repetitive nerve stimulation test and/or single fiber electromyography), and an edrophonium test. Forty-five sero-positive generalized MG patients were enrolled in this study, and were treated with prednisolone (PSL). Four patients who were resistant to PSL therapy (two of four patients received tacrolimus) were excluded from this study because they received multiple optional therapies such as blood purification therapy (double filtration plasmapheresis, immunoadsorption), high dose intravenous gamma-globulin therapy and/or intravenous methylprednisolone pulse therapy that prevented us from analyzing the efficacy of tacrolimus. We also excluded 12 patients due to their short observation periods (less than 24 months) or insufficient

clinical data.

The remaining 29 patients were analyzed in this study (Figure 1), and divided into two groups: 13 patients treated with PSL plus tacrolimus and 16 patients treated with PSL alone. Tacrolimus (3 mg/day) has been approved in Japan for MG treatment since 2000, therefore, patients who were diagnosed with MG after 2000 were frequently treated with PSL and tacrolimus. There were no significant differences in age, gender, the number of thymectomized patients, thymic histology, and MGFA clinical classification (Jaretzki et al., 2000) between the two groups (Table 1).

2.1.2 Treatment

The patients were admitted to our hospital and received general evaluations. In those who received a thymectomy, no pharmacological intervention was performed before the operation. PSL was orally administered at an initial dose of 5-10 mg on alternate days. This was gradually increased to 40-80 mg (most patients received 60 mg) as the maximum maintaining dose on alternate days. After one-three months of maximum dose and clinical and laboratory improvements, the PSL dose was tapered

gradually. The PSL dose was adjusted to the minimum to maintain a normal quality of life corresponding to the Minimal Manifestation Status (MMS) of the MGFA Postintervention Status (Jaretzki et al., 2000) by the judgment of the neurologist. Tacrolimus was started within six months of the initial administration of PSL and administered orally at a daily dose of 3 mg, which is approved for MG treatment in Japan. Pyridostigmine bromide at up to 180 mg per day was given if required.

2.1.3 Clinical evaluation

Clinical classification was performed according to the MGFA classification (Jaretzki et al., 2000). Clinical severity was evaluated according to the MG-ADL scale (Wolfe et al., 1999). PSL dosage was evaluated by converting to a mean daily dose if the patient received PSL on alternate days. Serum AChR antibodies were measured by an immunoprecipitation assay using ^{125}I-α-bungarotoxin. The pre/post treatment ratio of serum AChR antibodies (pre/post AChRAb ratio) was calculated, referring to a clinical study of azathioprine on MG (Palace et al., 1998). The PSL dose was evaluated at the maximum maintaining phase, 3, 6, 9, 12, 18, 24, 30 and 36 months after

the beginning of PSL administration. The MG-ADL scale was evaluated in the pretreatment state, 6, 12, 24 and 36 months after the beginning of PSL administration. The pre/post AChRAb ratio was evaluated at 3, 6, 9, 12, 18, 24, 30 and 36 months after the administration of PSL.

2.2 Clinical Study 2. -Clinical study of steroid-dependent MG patients in the chronic stage-

2.2.1 Patients

Medical records of nine outpatients with MG followed up at our clinic were reviewed. They were administered PSL for a long time to maintain MMS and it was difficult to reduce the PSL because of deteriorating myasthenic symptoms. These nine patients were given tacrolimus to enhance the clinical outcome or reduce the PSL dose. The characteristics of the patients are listed in Table 2. All patients had generalized MG and were seropositive for AChR antibodies. They each received a thymectomy before the immunosuppressive pharmacological intervention.

2.2.2 Treatment

All patients were administered tacrolimus orally at a daily dose of 3 mg. The PSL dose was adjusted to maintain the MMS of the patients by neurologists. Pyridostigmine bromide was given at up to 180 mg per day if required. No patient in this study deteriorated over the three years of clinical treatment.

2.2.3 Clinical evaluation

PSL dose, clinical severity, and the AChR antibody titer were evaluated as described in Clinical Study 1. PSL dose, MG-ADL scale, and the pre/post AChRAb ratio were evaluated at the point of tacrolimus initiation, and at 6, 12, 18, 24, 30 and 36 months after the administration of tacrolimus. The same items were also evaluated at three and six months before the administration of tacrolimus.

2.2.4 Adverse effecst of tacrolimus

The patients completed an adverse-effect questionnaire and received physical and laboratory examinations including complete blood count, urea and electrolytes, and

liver function tests at each visit. Unfavorable events occurring after the initiation of tacrolimus were described in this study as an adverse effect of tacrolimus.

2.3 Cytokine production of PBMC

Twenty-one MG patients and eight normal controls were recruited in this study. Seven patients were treated with PSL alone, eight with PSL plus tacrolimus, and six received no therapy. Heparinized peripheral venous blood was obtained from patients and normal controls. PBMCs were separated by density gradient centrifugation using Histopaque 1077 (Sigma-Aldrich Co Ltd., UK) and washed twice with PBS. The cells were suspended in RPMI1640 medium (Gibco BRL, NY USA) supplemented with 5% calf bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco). The cell suspension was adjusted to a concentration of 1×10^6 cells/ml, and cultured in 24-well culture plates in a final volume of 1 ml at 37ºC in 5% CO_2/ 95% air for seven days.

The culture medium was analyzed for 14 different cytokines and chemokines, namely interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-7, IL-10, IL-12 p70, IL-13, IL-17, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, granulocyte colony-stimulating

factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and macrophage inflammatory protein 1β (MIP-1β), using a Bio-Plex multiplex suspension array system (Bio-Rad Laboratories, Hercules, CA USA), which utilizes Luminex fluorescent-bead-based technology, according to the manufacturer's instructions (de Jager et al., 2003; Kellar et al., 2001).

2.4 Statistical analysis

The clinical parameters were assessed by non-parametric analyses. To determine the time point when the effect of tacrolimus appeared, the PSL dosages in the groups of PSL alone and of PSL plus tacrolimus were analyzed by the Mann-Whitney U test (exact test) in Clinical Study 1. An intrapatient comparison was done with the Wilcoxon signed-rank test by comparing the value at each time point with the beginning point of tacrolimus in Clinical Study 2. Differences in cytokine levels between the four groups were evaluated for normal distribution. In cases of normal distribution, the Tukey-Kramer HSD test was applied after oneway ANOVA. In cases of non-normal distribution, the Kruskal-Wallis test was applied.

2.5 Ethics approval

Clinical studies 1 and 2 were carried out following the ethical code of Kanazawa University Hospital. The cytokine study protocol was approved by the Ethics Committee of Kanazawa University, and full informed consent was obtained from all patients.

3. Results

3.1 Clinical Study 1.

The changes in PSL dose, MG-ADL scale, and the pre/post AChRAb ratio are shown in Figure 2. The median PSL dose expressed as the daily amount during the maximum maintenance phase was 30 mg in both groups. The dose of PSL was gradually reduced, and there were no significant differences until 24 months from the beginning of PSL treatment between the two groups. However, the dose was significantly lower in patients treated with PSL plus tacrolimus than with PSL alone at 30 and 36 months (median dose 1.25 vs. 6.0 mg at 30 months, $p<0.05$, 0.0 vs. 5.5 mg at 36 months, $p<0.05$).

Median MG-ADL scales before treatment were 5.0 and 7.0 in the groups treated with PSL plus tacrolimus and PSL alone respectively, and there was no significant difference between them. Six months from the beginning of PSL therapy, all the patients achieved MMS and median MG-ADL scales were 0.0 and 0.5 in those treated with PSL plus tacrolimus and PSL alone respectively. As a result, every patient remained in MMS throughout the course.

The level of AChR antibodies decreased to about a quarter of the pretreatment value within six months in both groups. The pre/post AChRAb ratio remained around 20% in the group treated with PSL alone whereas it gradually dropped in the group treated with PSL plus tacrolimus. There were statistical differences at 30 months (6.32 vs 12.75, $p<0.05$) and 36 months (6.65 vs 15.38, $p<0.05$) from the beginning of treatment (Figure 2).

3.2 Clinical Study 2.

The changes in PSL dose, MG-ADL scale, and pre/post AChRAb ratio are shown in Figure 3. The median dose of PSL at six and three months before tacrolimus was first given was not significantly different from that at the beginning of tacrolimus treatment (12.5 mg at each point). The PSL dose was significantly lower at six months from the administration of tacrolimus (9.0 mg, $p<0.01$) and reduced gradually for three years (8.0 mg at 36 month, $p<0.05$). There were no marked changes in the MG-ADL scale and the pre/post AChRAb ratio over the three years.

3.3 Adverse effects of tacrolimus in Clinical Studies 1 and 2.

Five adverse effects were observed which were considered to be related to tacrolimus (Table 3). A decrease in lymphocyte number was observed in a 56-year old female after three weeks of tacrolimus administration. She had received 60 mg of PSL on alternate days and radiation therapy for thymoma as an aftertreatment for extended thymectomy. After completion of radiation therapy and reducing tacrolimus to daily dose of 2 mg, the lymphocyte number recovered. Other adverse effects were as follows: acute pyelonephritis, hyperkalemia, and decreased creatinine clearance, as well as elevation of glycohemoglobin. These symptoms disappeared after reducing the dose of tacrolimus. No malignancy developed in either clinical study during the observation periods.

3.4 Cytokine production of PBMC

The characteristics of the patients enrolled in this study are listed in Table 4. There were no differences between the four groups in age or gender. The mean PSL dose did not differ statistically between the patients treated with PSL alone and those

treated with PSL plus tacrolimus. There was no difference between the AChR antibody titers in the three subgroups of MG patients.

Levels of cytokines produced by 1 x 10^6 PBMCs, analyzed by Tukey-Kramer HSD test after oneway ANOVA, are shown in Figure 4. IL-4, IL-17 and IFN-γ levels were significantly lower in the group treated with PSL plus tacrolimus compared to the normal control. IL-12 p70, GM-CSF, TNF-α and MIP-1β levels were significantly different between groups (Kruskal-Wallis test). IL-10 levels were significantly higher in the PSL plus tacrolimus group than in the other groups. The levels of the remaining cytokines, IL-1β, IL-2, IL-6, IL-7, IL13 and G-CSF, did not differ between the groups.

4. Discussion

The prognosis for MG has improved in recent years with the introduction of new immunosuppressants (Phillips and Torner, 1996). In addition, modern critical care service and improvements in diagnostic procedures leading to earlier treatment also contributed to a good MG outcome. However, quality of life (QOL) was not adequate because of persistent myasthenic symptoms (Rostedt et al., 2006). Corticosteroids are widely used and are effective in the treatment of MG but have various side effects. Some non-steroidal immunosuppressants can achieve clinical improvements or reduce the side effects of corticosteroids, tacrolimus being one prime example (Evoli et al., 2002; Konishi et al., 2005; Yoshikawa et al., 2002a).

We showed that tacrolimus was an adjunct to PSL in the treatment of antibody-positive generalized MG. Namely, it reduced the maintenance dose of PSL without serious side effects in two clinical studies. In Clinical Study 1, all patients had generalized MG and were seropositive for the AChR antibody. In addition, both groups of patients were matched for age, gender, thymic histology, and severity. Furthermore, all patients were followed up in our affiliated institutions. Therefore, we

consider these two groups to be quite similar in terms of clinical background and comparable for showing the efficacy of tacrolimus.

Recently, a randomized trial showing the efficacy of low-dose tacrolimus for the treatment of untreated de novo MG patients was reported (Nagane et al., 2005). Although our study is retrospective and we did not reduce PSL dose followed by a designed protocol, their results are consistent with our finding. The utilization of low-dose tacrolimus for de novo MG patients from early on in the treatment reduces the maintenance dose of PSL. In Clinical Study 1, three patients stopped receiving PSL at 24 months and six patients at 30 months from the beginning of PSL treatment in the group treated with PSL plus tacrolimus, whereas only one patient discontinued receiving PSL in the group treated with PSL alone. Furthermore, two patients who received PSL plus tacrolimus, discontinued PSL therapy, and after that tacrolimus was withdrawn at 30 months from the beginning of PSL treatment. The administration of tacrolimus from early on in the treatment of MG might be an option to reduce the maintenance dose of PSL long-term. In addition, the PSL dose was automatically increased up to 60 mg on alternate days according to our standard protocol for the

treatment of MG; however, the maximum PSL dose might be reduced significantly by using tacrolimus. Further studies are required to confirm our hypothesis.

We also evaluated the efficacy of tacrolimus for steroid-dependent patients with MG from the standpoint of a long-term treatment strategy (Clinical Study 2). There were no changes in the PSL dose before the administration of tacrolimus for as long as six months and the dose significantly decreased after its administration. These findings indicate that nine patients enrolled in this study were steroid-dependent and in these cases, the dose of PSL was successfully reduced by the administration of tacrolimus. A previous study on tacrolimus for the treatment of MG indicated that clinical improvement was achieved so rapidly that muscle strength improved two weeks after the initiation of tacrolimus (Yoshikawa et al., 2002a). In the present study, patients remained in MMS during the observation periods; however, the PSL dose was reduced within six months from the beginning of tacrolimus administration.

The MG-ADL scale showed no significant differences during the course, and the points were quite low. It is conceivable that the scale at the start point was already low and the patients remained well during the course, so no statistical significance

appeared. Tacrolimus could be useful for steroid-dependent cases to reduce the dose of steroids while maintaining MMS.

Infection and renal toxicity have been reported at the high doses of tacrolimus used for organ transplantation. In this study, five mild adverse effects were reported; lymphocytopnea, acute pyelonephritis, hyperkalemia, decreased creatinine clearance and elevated glycohemoglobin. These effects were transient and resolved by appropriate management and no serious problem occurred. Taken together with a previous report (Konishi et al., 2005), long-term low-dose administration of tacrolimus is an efficacious and safe treatment for MG.

The immunosuppressive activity of tacrolimus is considered to be mediated by the selective inhibition of helper T cell activation (Kino et al., 1987b). Tacrolimus binds to FK506-binding protein 12 (FKBP12) which is the intracellular receptor of tacrolimus (Harding et al., 1989) and the FKBP12-tacrolimus complex inhibits dephosphorylation of nuclear factor (NF)-AT by calcineurin (Liu et al., 1991). As a result, inhibition of calcineurin prevents translocation of NF-AT to the nucleus, which is essential for transcription of IL-2 (Flanagan et al., 1991).

On the other hand, various cytokines, including both T helper 1 (Th1) and T helper 2 (Th2), play important roles in the pathogenesis of MG (Zhang et al., 1997); however, details on each cytokine produced by PBMCs in MG patients and the effects of tacrolimus on cytokine production are still unclear.

To elucidate the mechanism by which tacrolimus acts on the immune system, we evaluated the production of various cytokines that constitute the immune network. The secretion of IL-12, IL-17, GM-CSF, IFN-γ, TNF-α, and MIP-1β decreased in patients treated with PSL plus tacrolimus. IL-17, IFN-γ, and GM-CSF are mainly secreted from helper T cells, especially Th1 cells, lead to the proliferation and activation of macrophages. We also showed decreased secretions of IL-12, TNF-α and MIP-1β, which are mainly produced in activated macrophages. These results suggest that tacrolimus specifically inhibits Th1 cells, and as a consequence reduces the activity of macrophages. A recent study showed that treatment with tacrolimus inhibited the expression of Th1 cytokine mRNA in lupus-prone mice (Sugiyama et al., 2004).

A recent study showed that toll-like receptor (TLR) antagonists facilitated production of IL-23 by activated macrophages, which induced T cells to secrete IFN-γ

and IL-17 (Hoeve et al., 2006). The IFN-γ secreted from T cells induces macrophages to secrete IL-12, which inhibit the secretion of IL-17 by activated T cells without affecting IFN-γ production. Considering that TNF-α and MIP-1β are produced from activated macrophages, our results indicate that tacrolimus inhibits the activation of macrophages, and down-regulates T cell activities.

Another interesting result of the current study is the enhanced secretion of IL-10 in the patients treated with PSL plus tacrolimus. IL-10 is mainly produced by Th2 cells (Fiorentino et al., 1989), which also produce IL-4, IL-5, and IL-13. Type 1 regulatory T (Tr1) cells are also representative of IL-10-producing cells, and can secrete transforming growth factor (TGF) –β, leading to peripheral immunotolerance (Groux et al., 1997; Roncarolo and Levings, 2000). A previous report showed tacrolimus enhances the expression of TGF-β1 mRNA and production of TGF-β1 protein (Khanna et al., 1999). Therefore, it is conceivable that IL-10-producing Tr1 cells might be activated by the tacrolimus. In our study, no enhanced production of such Th2 cytokines other than IL-10 was observed in the group treated with PSL plus tacrolimus. Since IL-10 has been described as a strong inhibitor of antigen presentation (de Waal Malefyt et al., 1991), the

inhibition of AChRAb production might be caused by the down-regulation of MHC class II enhanced by IL-10 secretion from activated Tr1 cells.

The value of this clinical study is limited by its retrospective study design and small number of patients, but tacrolimus proved to be a useful for treatment of MG, and was tolerable from our results. Currently, a prospective double-blind randomized controlled multi-center study on tacrolimus for the treatment of MG is ongoing in Japan and the results will give us clearer information regarding the clinical efficacy of tacrolimus. Our study on cytokine production by PBMC aids understanding of the pharmacological effects of tacrolimus.

In conclusion, low-dose tacrolimus is a useful and safe immunosuppressive therapy for the treatment of MG long-term. It is suggested that the immunosuppressive effects of tacrolimus are achieved by the inhibition of activated T cells and macrophages via the cytokine network, and by the enhancement of activities of Tr1 cells. Our results provide useful information for further clinical and experimental studies on the treatment of MG.

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References

Bonifati, D.M., Angelini, C., 1997. Long-term cyclosporine treatment in a group of severe myasthenia gravis patients. J Neurol 244, 542-547.

Conti-Fine, B.M., Milani, M., Kaminski, H.J., 2006. Myasthenia gravis: past, present, and future. J Clin Invest 116, 2843-2854.

De Feo, L.G., Schottlender, J., Martelli, N.A., Molfino, N.A., 2002. Use of intravenous pulsed cyclophosphamide in severe, generalized myasthenia gravis. Muscle Nerve 26, 31-36.

de Jager, W., te Velthuis, H., Prakken, B.J., Kuis, W., Rijkers, G.T., 2003. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. Clin Diagn Lab Immunol 10, 133-139.

de Waal Malefyt, R., Haanen, J., Spits, H., Roncarolo, M.G., te Velde, A., Figdor, C., Johnson, K., Kastelein, R., Yssel, H., de Vries, J.E., 1991. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II major histocompatibility complex expression. J Exp Med 174, 915-924.

Evoli, A., Di Schino, C., Marsili, F., Punzi, C., 2002. Successful treatment of myasthenia gravis with tacrolimus. Muscle Nerve 25, 111-114.

Fiorentino, D.F., Bond, M.W., Mosmann, T.R., 1989. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J Exp Med 170, 2081-2095.

Flanagan, W.M., Corthesy, B., Bram, R.J., Crabtree, G.R., 1991. Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. Nature 352, 803-807.

Fujii, Y., Lindstrom, J., 1988. Regulation of antibody production by helper T cell clones in experimental autoimmune myasthenia gravis. J Immunol 141, 3361-3369.

Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., de Vries, J.E., Roncarolo, M.G., 1997. A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. Nature 389, 737-742.

Harding, M.W., Galat, A., Uehling, D.E., Schreiber, S.L., 1989. A receptor for the immunosuppressant FK506 is a cis-trans peptidyl-prolyl isomerase. Nature 341, 758-760.

Hoeve, M.A., Savage, N.D., de Boer, T., Langenberg, D.M., de Waal Malefyt, R., Ottenhoff, T.H., Verreck, F.A., 2006. Divergent effects of IL-12 and IL-23 on the production of IL-17 by human T cells. Eur J Immunol 36, 661-670.

Jaretzki, A., 3rd, Barohn, R.J., Ernstoff, R.M., Kaminski, H.J., Keesey, J.C., Penn, A.S., Sanders, D.B., 2000. Myasthenia gravis: recommendations for clinical research standards. Task Force of the Medical Scientific Advisory Board of the Myasthenia Gravis Foundation of America. Neurology 55, 16-23.

Kellar, K.L., Kalwar, R.R., Dubois, K.A., Crouse, D., Chafin, W.D., Kane, B.E., 2001. Multiplexed fluorescent bead-based immunoassays for quantitation of human cytokines in serum and culture supernatants. Cytometry 45, 27-36.

Khanna, A., Cairns, V., Hosenpud, J.D., 1999. Tacrolimus induces increased expression of transforming growth factor-beta1 in mammalian lymphoid as well as nonlymphoid cells. Transplantation 67, 614-619.

Kino, T., Hatanaka, H., Hashimoto, M., Nishiyama, M., Goto, T., Okuhara, M., Kohsaka, M., Aoki, H., Imanaka, H., 1987a. FK-506, a novel immunosuppressant isolated

from a Streptomyces. I. Fermentation, isolation, and physico-chemical and biological characteristics. J Antibiot (Tokyo) 40, 1249-1255.

Kino, T., Hatanaka, H., Miyata, S., Inamura, N., Nishiyama, M., Yajima, T., Goto, T., Okuhara, M., Kohsaka, M., Aoki, H., et al., 1987b. FK-506, a novel immunosuppressant isolated from a Streptomyces. II. Immunosuppressive effect of FK-506 in vitro. J Antibiot (Tokyo) 40, 1256-1265.

Konishi, T., Yoshiyama, Y., Takamori, M., Saida, T., 2005. Long-term treatment of generalised myasthenia gravis with FK506 (tacrolimus). J Neurol Neurosurg Psychiatry 76, 448-450.

Liu, J., Farmer, J.D., Jr., Lane, W.S., Friedman, J., Weissman, I., Schreiber, S.L., 1991. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell 66, 807-815.

Nagane, Y., Utsugisawa, K., Obara, D., Kondoh, R., Terayama, Y., 2005. Efficacy of low-dose FK506 in the treatment of Myasthenia gravis--a randomized pilot study. Eur Neurol 53, 146-150.

Palace, J., Newsom-Davis, J., Lecky, B., 1998. A randomized double-blind trial of prednisolone alone or with azathioprine in myasthenia gravis. Myasthenia Gravis Study Group. Neurology 50, 1778-1783.

Pascuzzi, R.M., Coslett, H.B., Johns, T.R., 1984. Long-term corticosteroid treatment of myasthenia gravis: report of 116 patients. Ann Neurol 15, 291-298.

Phillips, L.H., 2nd, Torner, J.C., 1996. Epidemiologic evidence for a changing natural history of myasthenia gravis. Neurology 47, 1233-1238.

Ponseti, J.M., Azem, J., Fort, J.M., Lopez-Cano, M., Vilallonga, R., Buera, M., Cervera, C., Armengol, M., 2005. Long-term results of tacrolimus in cyclosporine- and prednisone-dependent myasthenia gravis. Neurology 64, 1641-1643.

Roncarolo, M.G., Levings, M.K., 2000. The role of different subsets of T regulatory cells in controlling autoimmunity. Curr Opin Immunol 12, 676-683.

Rostedt, A., Padua, L., Stalberg, E.V., 2006. Correlation between regional myasthenic weakness and mental aspects of quality of life. Eur J Neurol 13, 191-193.

Sugiyama, M., Funauchi, M., Yamagata, T., Nozaki, Y., Yoo, B.S., Ikoma, S., Kinoshita, K., Kanamaru, A., 2004. Predominant inhibition of Th1 cytokines in New

Zealand black/white F1 mice treated with FK506. Scand J Rheumatol 33, 108-114.

Tocci, M.J., Matkovich, D.A., Collier, K.A., Kwok, P., Dumont, F., Lin, S., Degudicibus, S., Siekierka, J.J., Chin, J., Hutchinson, N.I., 1989. The immunosuppressant FK506 selectively inhibits expression of early T cell activation genes. J Immunol 143, 718-726.

Undre, N.A., Stevenson, P., Schafer, A., 1999. Pharmacokinetics of tacrolimus: clinically relevant aspects. Transplant Proc 31, 21S-24S.

Wolfe, G.I., Herbelin, L., Nations, S.P., Foster, B., Bryan, W.W., Barohn, R.J., 1999. Myasthenia gravis activities of daily living profile. Neurology 52, 1487-1489.

Yoshikawa, H., Mabuchi, K., Yasukawa, Y., Takamori, M., Yamada, M., 2002a. Low-dose tacrolimus for intractable myasthenia gravis. J Clin Neurosci 9, 627-628.

Yoshikawa, H., Satoh, K., Yasukawa, Y., Yamada, M., 2002b. Cytokine secretion by peripheral blood mononuclear cells in myasthenia gravis. J Clin Neurosci 9, 133-136.

Zhang, G.X., Navikas, V., Link, H., 1997. Cytokines and the pathogenesis of myasthenia gravis. Muscle Nerve 20, 543-551.

Figure Legends

Figure 1. The 86 patients evaluated at Kanazawa University Hospital from 1993 to 2004. Forty-five MG patients were selected because they had generalized MG, were seropositive for the anti-AChR antibody, and were receiving PSL therapy. Four patients were excluded because they were receiving multiple optional therapies. Twelve of these patients were excluded because of a short observation period or insufficient clinical data. The remaining 29 patients were analyzed in Clinical Study 1.

Figure 2. The clinical course of PSL dose (A), MG-ADL scale (B), and anti-AChR antibody (C) in Clinical Study 1. The course of the anti-AChR antibody is expressed as a ratio (pre/post AChRAb ratio). Each mark indicates the median value of that item. There were no significant differences between the two groups in PSL dose and pre/post AChRAb ratio for 24 months from the beginning of PSL; however, the PSL dose and pre/post AChRAb ratio were significantly lower in the group treated with tacrolimus at 30 and 36 months from the beginning of PSL administration. There were no significant differences during the course between the two groups in terms of MG-ADL

scale.

Figure 3. The clinical course of PSL dose (A), MG-ADL scale (B), and anti-AChR antibody (C) in Clinical Study 2. The course of the anti-AChR antibody is expressed as a ratio (pre/post AChRAb ratio). Each mark indicates the median value of that item. Compared with the PSL dose at the point of tacrolimus initiation (0M), the PSL dose was significantly lower at six months from the beginning of tacrolimus and gradually reduced during the course. Conversely, there were no significant differences in PSL dose before tacrolimus initiation. MG-ADL scale and the pre/post AChRAb ratio showed no significant differences during the observation period.

Figure 4. Cytokine levels (A. IL-4, B. IL-10, C. IL-17, D. IFN-γ) produced by 1 x 10^6 peripheral blood mononuclear cells from MG patients treated with PSL alone, MG patients treated with PSL plus tacrolimus, untreated MG patients, and normal controls assessed by the multiplexed fluorescent bead-based immunoassay. Horizontal bars placed in each group indicate the mean value of the cytokine or chemokine. *

indicates significant difference (Tukey-Kramer HSD test after oneway ANOVA, $p<0.05$).

Table 1.

The characteristics of the patients in clinical study 1.

Patients	Treatment	
	PSL plus tacrolimus	PSL alone
	n=13	n=16
Gender (male/female)	4/9	6/10
Age (y): mean ± SD (range)	44.8 ± 21.6 (15-79)	47.9 ± 23.3 (11-83)
Thymectomized patient: number (%)	11 (84.6 %)	15 (93.8 %)
Thymic histology: (number of patient)		
Thymoma	3	6
Thymic hyperplasia	7	4
Thymic involution	1	5
MGFA classification at disease onset: (number)		
IIa	6	6
IIb	3	3
IIIa	2	3
IIIb	2	4

Table 2.

The characteristics of the patients in clinical study 2.

	Patients, n=9
Gender (male/female)	3/6
Age (y): mean ± SD (range)	48.0 ± 16.1 (26-74)
Disease duration (y): mean ± SD (range)	12.6 ± 11.2 (3-36)
Thymectomized patient: number (%)	9 (100%)
Time from thymectomy (y): mean ± SD (range)	10.2 ± 8.0 (2-24)
Thymic histology: (number)	
Thymoma	3
Thymic hyperplasia	3
Thymic involution	3
MGFA classification at disease onset: (number)	
IIa	3
IIb	2
IIIa	1
IIIb	3
AChR antibody nmol/l: mean ± SD (range)	
at the beginning of tacrolimus	38.5 ± 37.0 (0.3-118)
at 36 months from the beginning of tacrolimus	28.5 ± 24.3 (0.3-66.0)

Table 3

Adverse events due to tacrolimus treatment

patient	age at onset of adverse effect	gender	event	time from tacrolimus initiation	management	Duration to recovery
1	56	F	Lymphocytopnea (300 /μl)	3 W	tacrolimus reduction to 2mg/day	2 M
2	51	M	pyelonephritis	28 M	antibiotics	a few days
3	35	M	hyperkalemia	18 M	tacrolimus reduction to 2mg/day	1 M
4	14	F	decreased creatinine clearance (50ml/min)	5 M	tacrolimus reduction to 2mg/day	2 M
5	43	F	increased glycohemoglobin	2-3 M	tacrolimus reduction to 2mg/day	1 M

Table 4

Classification by patient treatment of in the cytokine study.

	Patients with MG			Normal controls
	PSL alone	PSL plus tacrolimus	No treatment	
Gender (male/female)	3/4	3/5	2/4	3/5
Age (y): mean ± SD	58.7 ± 17.0	49.6 ± 18.4	52.6 ± 24.3	34.6 ± 6.5
PSL dose (mg/day): mean ± SD	6.4 ± 3.5	7.2 ± 2.9	0	0
Tacrolimus dose (mg/day): mean ± SD	0	2.7 ± 0.5	0	0
AChR antibody (nmol/l): mean ± SD	48.0 ± 55.6	44.5 ± 54.7	45.6 ± 71.2	0

Figure 1.

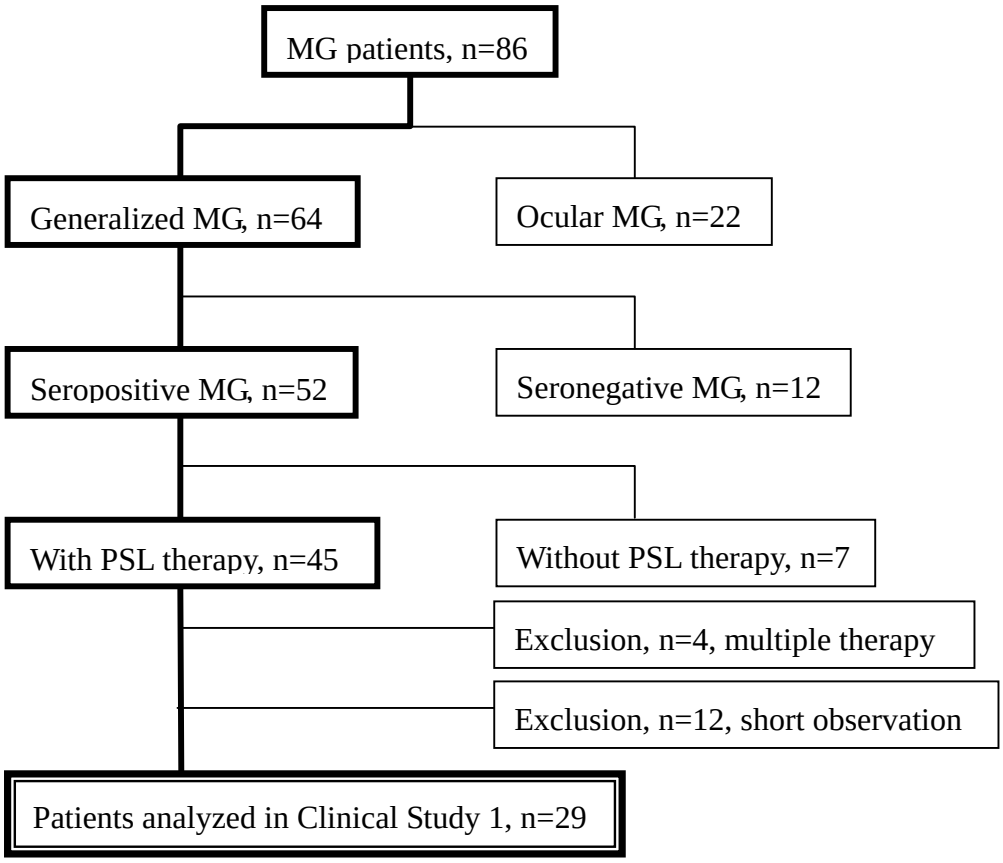

MG patients, n=86
Generalized MG, n=64
Ocular MG, n=22
Seropositive MG, n=52
Seronegative MG, n=12
With PSL therapy, n=45
Without PSL therapy, n=7
Exclusion, n=4, multiple therapy
Exclusion, n=12, short observation
Patients analyzed in Clinical Study 1, n=29

Figure 2.

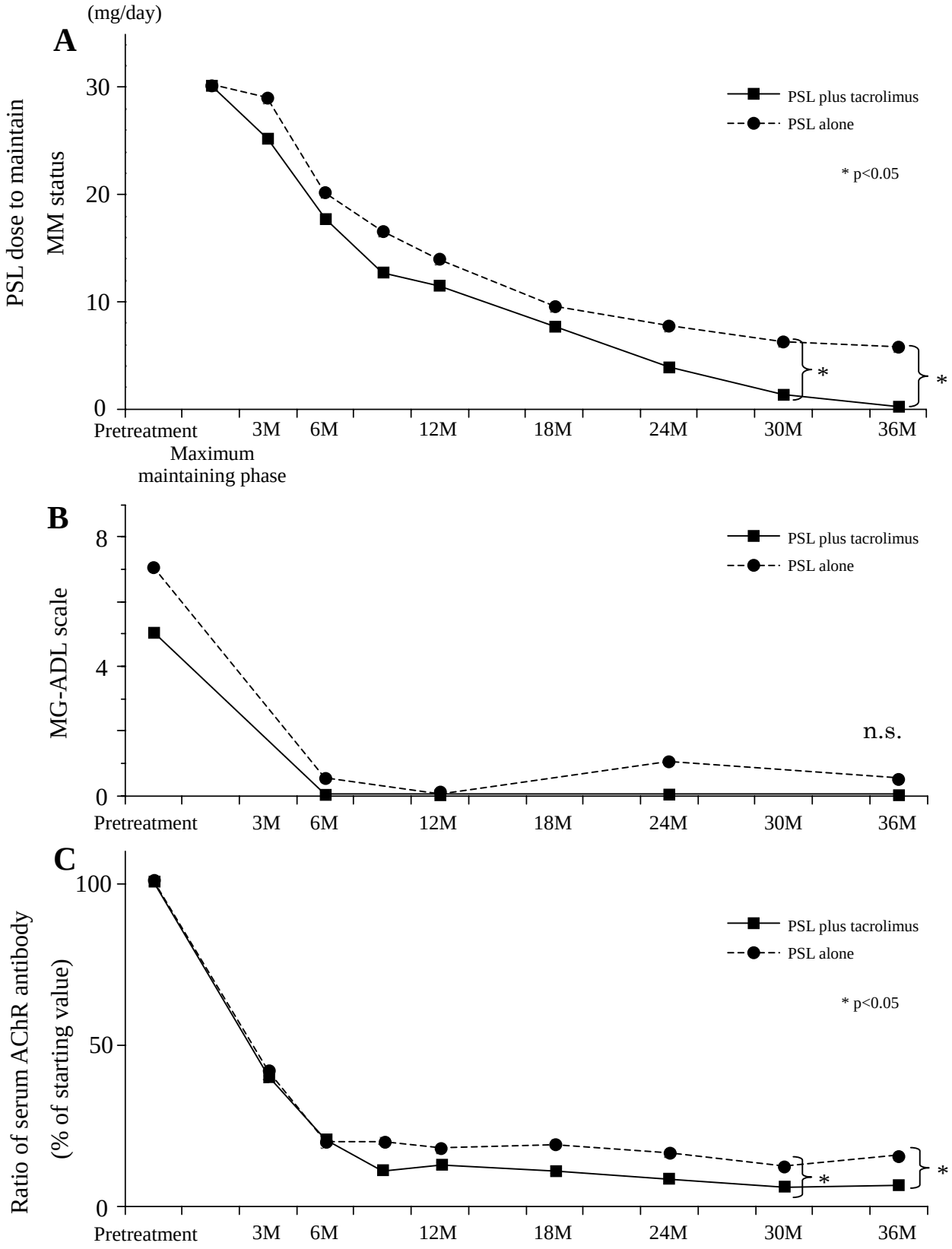

A
(mg/day)
PSL dose to maintain MM status
30
20
10
0
PSL plus tacrolimus
PSL alone
* p<0.05
*
*
Pretreatment
3M
6M
12M
18M
24M
30M
36M
Maximum maintaining phase
B
MG-ADL scale
8
4
0
n.s.
C
Ratio of serum AChR antibody (% of starting value)
100
50
0

Figure 3.

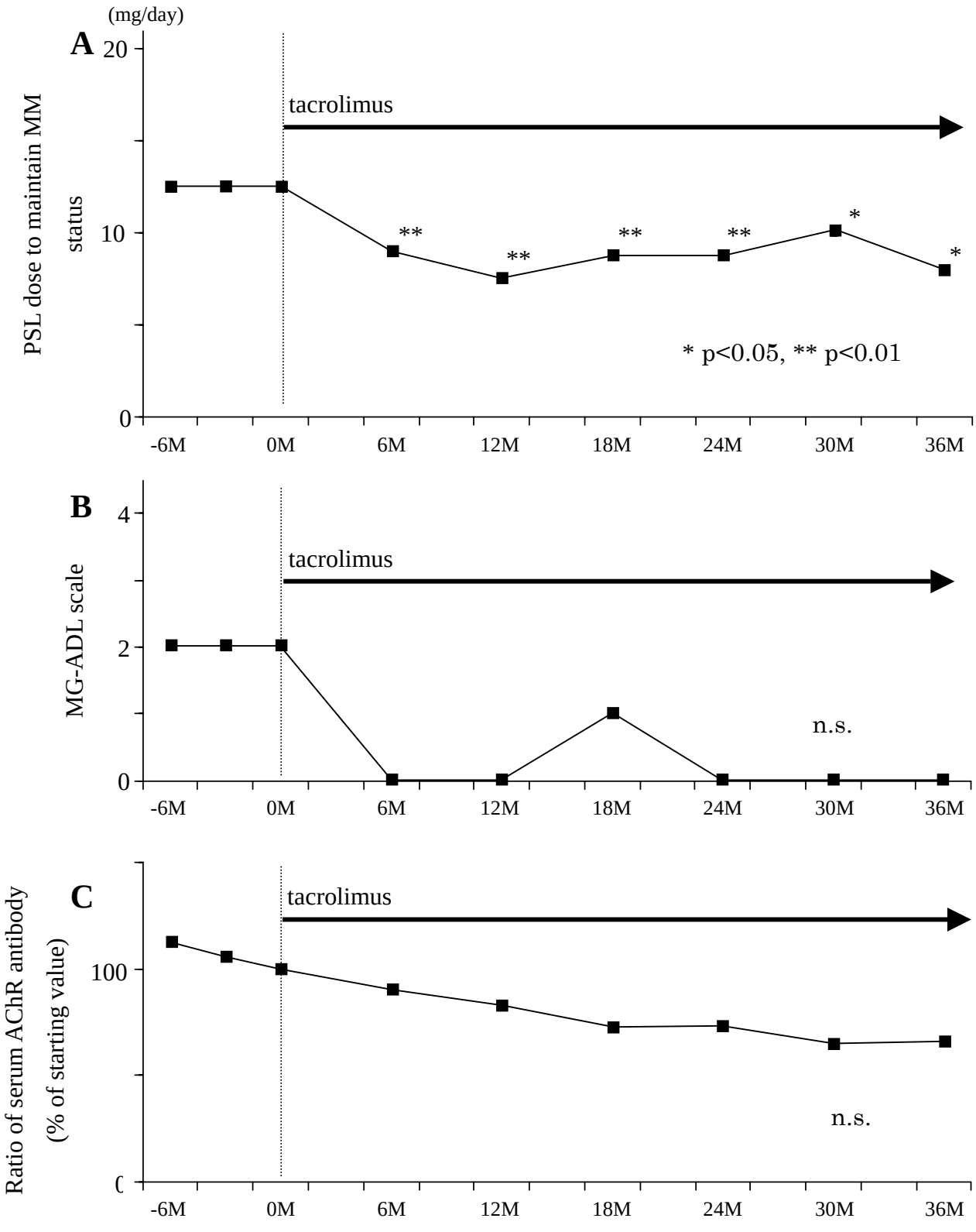
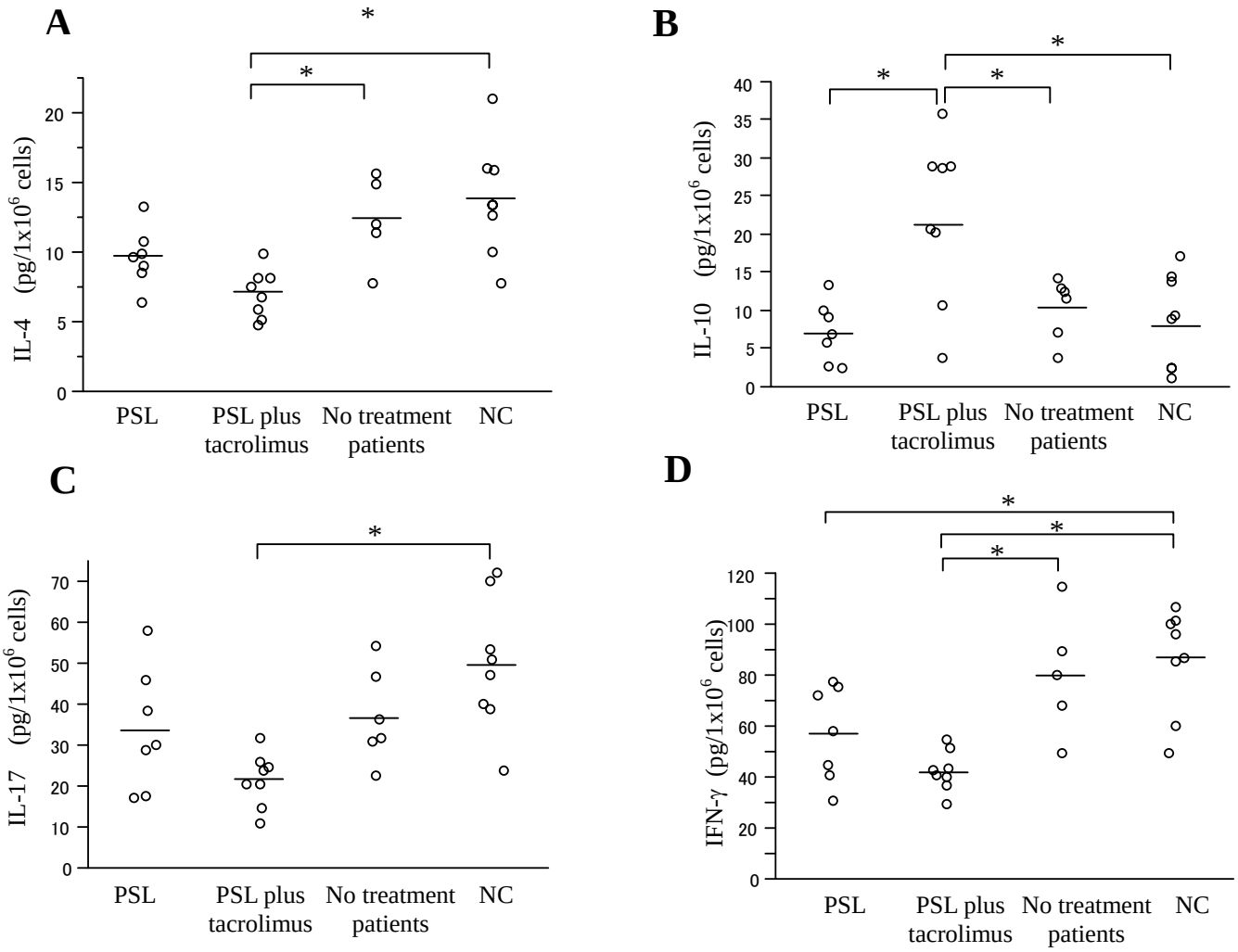

(mg/day)
A
PSL dose to maintain MM status
20
10
0
tacrolimus
**
**
**
**
*
*
* p<0.05, ** p<0.01
-6M
0M
6M
12M
18M
24M
30M
36M
B
MG-ADL scale
4
2
0
tacrolimus
n.s.
C
Ratio of serum AChR antibody (% of starting value)
100
tacrolimus
n.s.

Figure 4.



* p<0.05