A Novel Immunomodulator, FTY720, Prevents Development of Experimental Autoimmune Myasthenia Gravis in C57BL/6 Mice

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Prophylactic oral administration of a novel immunomodulator (immunosuppressant), FTY720 (1 mg/kg, three times a week), completely prevented the development of experimental autoimmune myasthenia gravis (EAMG) in C57BL/6 mice. EAMG has been used as an animal model for human myasthenia gravis, and was established by immunizating the mice with acetylcholine receptor (AChR) from Torpedo californica. FTY720 also suppressed the production of both anti-Torpedo californica AChR antibody and anti-mouse AChR autoantibody by the mice, which were observed in mice in which EAMG had become established. These results strongly suggest that FTY720 is a promising candidate for treatment of human myasthenia gravis.

Key words FTY720; myasthenia gravis; autoantibody; acetylcholine receptor

It has been well established that myasthenia gravis (MG) is caused by autoimmune responses against nicotinic acetylcholine receptor (AChR) on postsynaptic membrane in neuromuscular junctions.1) Experimental autoimmune myasthenia gravis (EAMG) has been used as an animal model for human MG. This model is prepared by immunizing mice or rats with AChR from Torpedo californica; the animals develop MG-like symptoms, including muscle weakness, with production of autoantibody against AChR.2)

The novel immunomodulator (immunosuppressant) FTY720, which was discovered by Fujita et al.3,4) of our group, is a synthetic structural analogue of myriocin (ISP-I), a metabolite of Isaria conclaris.5) The efficacy of FTY720 has been well established in preclinical transplantation models,6) and also recently in renal transplantation in humans.7–10) Although the mechanisms of pharmacological action of FTY720 remain to be fully clarified,11,12) it has been proposed that FTY720 is phosphorylated by sphingosine kinase, and the product, FTY720 monophosphate, suppresses immune response by sequestering lymphocytes from blood and peripheral tissues to the secondary lymphoid tissues.13–16) FTY720 has no inhibitory effect on cytokine production in vitro, in contrast to established immunosuppressants (cyclosporin and tacrolimus hydrate).16) Its mechanism of action is unique and differs from that of the established immunosuppressants. The development of FTY720 was described in our previous report.17)

In this study, we examined the efficacy of FTY720 for preventing the development of EAMG, an animal model of human MG.

MATERIALS AND METHODS

Animals Specific pathogen-free (SPF) C57BL/6 mice (7 weeks of age, females) were purchased from Japan SLC Inc., Shizuoka, Japan. The mice were given γ-ray-irradiated food (CRF-1, Oriental Bio Co., Kyoto, Japan) and distilled water for injection (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan).

FTY720 2-Amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol hydrochloride (FTY720) was kindly provided by Yoshitomi Pharmaceutical Industries, Ltd., Japan.

Acetylcholine Receptor from Torpedo californica Electric organ of Torpedo californica was purchased from Aquatic Research Consultants (San Pedro, CA, U.S.A.), and acetylcholine receptor (tAChR) was purified by the method of Froehner and Rafto using cobrotoxin-coupled Sepharose.18) tAChR concentration was quantified by the Lowry method.

Evaluation of Clinical Symptoms of EAMG Muscle strength was evaluated by the inverted hanging technique as described by Karachunski et al.19,20) Mice with a holding time of 10 min or more were considered normal, and those with a holding time of less than 10 min were considered as having EAMG.

Study Protocol Twelve C57BL/6 mice were immunized intracutaneously on the back with tAChR (5 μg) in the presence of Freund’s complete adjuvant at 8, 10 and 11 weeks of age. Six mice (treated group, Nos. 1—6) out of the twelve were orally given FTY720 in water (1.0 mg/kg) three times a week from the day before the first immunization. The other six mice (untreated group, Nos. 7—12) were given the vehicle (water) alone. Six mice (control group, Nos. 13—18) were immunized with Freund’s complete adjuvant alone in the same way, and given the vehicle. Clinical symptoms of EAMG were evaluated at 11 and 13 weeks of age (3 and 5 weeks after the first immunization). Peripheral blood was collected from the tail veins of mice at 11 and 13 weeks of age, and serum samples were separated by centrifugation. At 13 weeks of age (5 weeks after the first immunization), mice were killed by exsanguination from the vena cava inferior under sodium pentobarbital anesthesia.

Measurement of Anti-AChR IgG in Serum Membrane Extract from Torpedo californica Electric Organ: Electric organ from Torpedo californica was homogenized with buffer A (10 mM Tris–HCl buffer, pH 7.4, containing 1 mM

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EDTA, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride and 10 units/ml Trasylol18) and centrifuged at 10000×g for 1 h. The precipitate was homogenized with buffer A18) containing 1% Triton X-100 in a Potter homogenizer, allowed to stand at 4 °C for 2 h, and centrifuged as above. The supernatant was used as membrane extract from *Torpedo californica* electric organ.

Membrane Extract from BC3H-1 Cells: Cells (4.52×10^7 cells) of a murine smooth muscle-like brain tumor cell line, BC3H-1, which expresses nicotinic AChR,21) were homogenized with buffer A18) in a Potter homogenizer, and centrifuged at 100000×g for 1 h. The precipitate was homogenized with buffer A18) containing 1% Triton X-100 in a Potter homogenizer, allowed to stand at 4 °C for 2 h, and centrifuged as above. The supernatant was used as membrane extract from BC3H1 cells.

Measurement of Anti-AChR IgG in Serum: Polystyrene microtiter wells (EIA flat plate-1, Sanko Junyaku Co., Tokyo, Japan) were coated with α-bungarotoxin (Latoxan, Rosans, France) at a concentration of 5 μg/ml in 0.1 M sodium phosphate buffer, pH 7.5, containing 0.1% NaN₃ at 4 °C overnight. After incubation, α-bungarotoxin solution was removed by aspiration, and the wells were washed 4 times with 0.25 ml of 10 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl. The washed wells were incubated with 0.25 ml of 10 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl, 0.1% bovine serum albumin (BSA, Fraction V, Intergen Co., Purchase, NY, U.S.A.) and 0.1% NaN₃ at 4 °C for 2 h. After incubation, the buffer was removed by aspiration, and the wells were incubated with the membrane extract from *Torpedo californica* electric organ or from BC3H-1 cells, which had previously been diluted 30- or 165-fold, respectively, with the same buffer (0.1 ml) at 37 °C for 3 h. The membrane extract was removed by aspiration, and the wells were washed as above. The wells were incubated with serum samples, which had previously been diluted 10000- or 3000-fold, respectively, with the same buffer (0.1 ml) at 37 °C for 2 h and 4 °C overnight. As regards the dilution extents, to obtain the maximal sensitivity for anti-AChR IgG by the present ELISA, serum samples were diluted to various extents with the same buffer, subjected to the present ELISA, and the ratio22) of the specific binding of anti-AChR IgG to nonspecific binding of nonspecific mouse IgG was examined. The maximal ratio was obtained when serum samples were diluted 10000-fold for anti-tAChR IgG and 3000-fold for anti-BC3H-1 AChR IgG. After incubation, the diluted serum was removed by aspiration, and the wells were washed as above. The wells were incubated with goat (anti-mouse IgG H+L) Fab’-horseradish peroxidase conjugate (Medical and Biological Laboratories Co., Ltd., Nagoya, Japan), which had previously been diluted 95000-fold with 10 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl and 0.1% BSA (0.1 ml) at 37 °C for 3 h. Finally, the conjugate solution was removed by aspiration, the wells were washed as above, and peroxidase activity bound to the wells was measured by colorimetry by using o-phenylenediamine as a hydrogen donor.23) The absorbance at 490 nm was measured with a plate reader (Model 450, Bio-Rad Laboratories, Hercules, CA, U.S.A.). Antibody-positive status was defined as an absorbance due to peroxidase activity higher than the mean value in the control group plus 3SD (n=6).

**Statistical Analysis** The significance of differences in the incidences of EAMG was evaluated by using Fisher’s exact probability test, and that in anti-AChR antibody level was evaluated by using the Mann-Whitney U-test.

**RESULTS**

**Effect of FTY720 on the Development of Experimental Autoimmune Myasthenia Gravis** Experimental autoimmune myasthenia gravis (EAMG) mice, which were established by immunizing C57BL/6 mice with acetylcholine receptor (AChR) from *Torpedo californica* (tAChR), were used to evaluate the efficacy of FTY720 for preventing the development of myasthenia gravis (MG). Clinical symptoms of EAMG were confirmed by measuring muscle strength using the inverted hanging technique. Mice with a holding time of 10 min or more were considered normal, and those with a hanging time of less than 10 min were considered as having EAMG. Mice (n=18, 8 weeks of age, females) were divided into the following three groups, 1) the treated group (n=6, Nos. 1—6); immunized with tAChR in the presence of Freund’s complete adjuvant at 8, 10 and 11 weeks of age and given FTY720 in water (1 mg/kg orally, three times a week), 2) the untreated group (n=6, Nos. 7—12); immunized in the same way as the treated group and given the vehicle (water) alone, and 3) the control group (n=6, Nos. 13—18); immunized with the adjuvant alone and given the vehicle. In the untreated group, four mice (67%, Nos. 7—10) out of the six developed EAMG at 3 weeks after the first immunization (Fig. 1). In another experiment, the incidence of EAMG was also 67% (sixteen mice out of twenty-four developed EAMG). The incidences in the two experiments corre-
sponded well with the report of Berman and Patrick. In the treated group (Nos. 1—6), EAMG onset was completely suppressed up to 13 weeks of age (5 weeks after the first immunization) (Fig. 1). These results clearly show that FTY720 can prevent the development of EAMG.

Immunological Studies To clarify the efficacy of FTY720 immunologically, IgG antibodies against tAChR and mouse AChR (mAChR) in serum were measured by means of ELISA (Fig. 2). At the period when muscle weakness appeared (3 weeks after the first immunization), anti-tAChR antibody was observed in all mice in the untreated group, and anti-mAChR autoantibody was also detected in four mice (67%, Nos. 8—10, 12) in the untreated group. At 5 weeks after the first immunization, all mice in the untreated group had both anti-tAChR antibody and anti-mAChR autoantibody. In the treated group (Nos. 1—6) and the control group (Nos. 13—18), no antibody against tAChR and mAChR was detected up to 5 weeks after the first immunization (Fig. 2).

DISCUSSION

Patients with myasthenia gravis (MG) have been treated with acetylcholine esterase-inhibitory drugs, glucocorticoids, and by thymectomy. However, acetylcholine esterase-inhibitory drugs are not able to induce a dramatic remission, 20% of MG patients are steroid-resistant, and the efficacy of thymectomy remains controversial. In addition, the adverse effects of glucocorticoids, such as osteoporosis, opportunistic infections, diabetes mellitus, and so on, are serious. Therefore, the development of new means to treat MG and to prevent recurrence is necessary. Although tacrolimus hydrate (FK506) has recently been approved for this purpose, it causes severe adverse effects, such as renal toxicity and opportunistic infection.

The novel agent FTY720 has a number of attractive properties, such as a unique mechanism of immunosuppressive effect, absence of any increase in susceptibility to infectious diseases, and no critical adverse effect at therapeutic doses. The results of the present study show that oral administration of FTY720 can completely prevent the development of experimental autoimmune myasthenia gravis (EAMG) in C57BL/6.

As regards anti-Torpedo californica acetylcholine receptor (tAChR) antibody and anti-mouse AChR (mAChR) autoantibody, even the two mice (Nos. 11, 12) without EAMG symptoms in the untreated group were positive at 5 weeks after the first immunization (Fig. 2). In human MG, anti-human AChR autoantibody is detected in some cases in remission. In our experience (unpublished), 40% of patients with remission (n=16) were sero-positive for anti-human AChR autoantibody. This seems consistent with the sero-positivity in the two symptom-free mice. Thus, it seems that not only the antibodies, but also some other immunological factor(s) (e.g., macrophages, cytotoxic T-lymphocytes, complement, and so on) may be related to EAMG onset. In contrast, anti-mAChR autoantibody could not be detected in one mouse (No. 7) out of four mice (Nos. 7—10) with EAMG symptoms in the untreated group at 3 weeks after the first immunization (Fig. 2, right). This might be due to insufficient sensitivity of the ELISA for anti-mAChR autoantibody. In the treated group (Nos. 1—6), FTY720 suppressed not only anti-tAChR antibody but also anti-mAChR autoantibody (Fig. 2).

In conclusion, the results of this study strongly suggest that FTY720 is a good candidate drug for the prevention of human MG. Although the efficacy of FTY720 to improve established EAMG remains to be defined, FTY720 should at least be useful to prevent the recurrence of MG after thymectomy, or after treatment with glucocorticoid or tacrolimus hydrate.

REFERENCES


