# Normal human immunoglobulin suppresses experimental myasthenia gravis in SCID mice

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Serum IgM has been shown to participate in the control of IgG autoreactivity in healthy subjects. We have recently shown that an immunoglobulin preparation of pooled normal human IgM (IVIgM) contains anti-idiotypic antibodies against disease-associated IgG autoantibodies in autoimmune patients and protects rats from experimental autoimmunity. The aim of the present study was to asses the *in vitro* and *in vivo* immunomodulatory effects of IVIgM in comparison with IgG, in SCID mice reconstituted with thymic cells from a myasthenia gravis patient. Non-leaky SCID mice were injected i.p. with $60 \times 10^6$ thymic cells from a patient with myasthenia gravis and 1 day later boosted with $10^6$ irradiated acetylcholine receptor (AchR)-expressing TE671 cells. On days 14, 21 and 28, mice were treated with IVIgM or with equimolar amounts of human serum albumin. The level of anti-AchR antibodies in the sera of three out of four IgM-treated animals was less than 1 nM. Further, there was a significant decrease in the loss of endplate AchR on the diaphragms of IgM-treated SCID mice. These findings indicate that pooled normal IgM exerts an immunoregulatory role in experimental myasthenia gravis, and suggests that IgM may be considered as an alternative approach in the therapy of autommune diseases.

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## 1 Introduction

Myasthenia gravis (MG) is an autoimmune disorder characterized by muscle weakness and excessive fatigability. In a small number of patients, MG is associated with other autoimmune diseases among which Hashimoto's thyroiditis, rheumatoid arthritis and systemic lupus erythematosus have been the most frequently reported [1]. Antibodies directed to the nicotinic acetylcholine receptor (AChR) are found in the serum of approximately 80 % of patients with MG [2, 3]. Thymic abnormalities are common [2, 3]. Within the myasthenic thymus, thymic myoid cells have been implicated in the immunopathogenesis of MG by virtue of expressing several structures typical of the myogenic lineage including the AchR [4–8]. Some of the immunopathological findings characteristic of MG have been reproduced in severe combined immunodeficiency (SCID) mice populated with either thymic transplants or PBL of myasthenic patients [9–11].

Pooled normal IgG (intravenous immunoglobulin, IVIg) has increasingly been used for the treatment of autoimmune diseases and systemic inflammatory disorders [12–14]. Controlled clinical trials have established the efficacy of IVIg in several neuromuscular diseases, including the Guillain-Barré syndrome, chronic inflammatory demyelinating polyneuropathy, dermatomyositis and the Lambert-Eaton myasthenic syndrome [12, 15–20]. IVIg has also been demonstrated to be effective in the treatment of acute MG [21–24]. Although the mode of action of IVIg in MG is not fully understood, IVIg was shown to inhibit the binding of anti-AchR autoantibodies to AchR *in vitro*, by interacting with idiotypic determinants expressed by pathogenic autoantibodies [25]. We have recently shown that natural anti-idiotypic antibodies present in pooled normal human IgM (IVIgM) also suppress anti-thyroglobulin and anti-DNA autoantibody activity *in vitro* [26].

In the present study, we report on the efficacy of IVIg and IVIgM to suppress anti-AchR autoantibody production *in*

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**Abbreviations: MG:** Myasthenia gravis **AchR:** Acetylcholine receptor **IVIg:** Intravenous IgG **IVIgM:** Intravenous IgM **SCID:** Severe combined immunodeficiency

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*vivo* and to prevent the loss of AchR at the muscle endplates in a reconstituted SCID mouse model of MG.

## 2 Results

### 2.1 Inhibition of the binding of anti-AchR autoantibodies to AchR by IVIg and IVIgM

An ELISA was designed to demonstrate the inhibition of the binding of anti-AchR autoantibodies to AchR by therapeutic preparations of normal Ig. We first demonstrated that IgG purified from the serum of all patients with MG who were tested bound to the lysate of AchR-expressing TE671 cells in a dose-dependent manner, whereas IgG purified from the serum of healthy individuals did not exhibit any binding to the TE671 lysate (data not shown). Co-incubation of biotinylated, purified IgG from patients with increasing concentrations of IVIgM resulted in a dose-dependent inhibition of the binding of patients' IgG to the TE671 lysate (Fig. 1). IVIgM exhibited an equal or higher efficiency than IVIg in inhibiting the binding of purified Ig antibodies from the four patients studied to the AchR-enriched lysate of TE671 cells. HSA used as a negative control did not inhibit the binding of the autoantibodies to the TE671 lysate.

### 2.2 SCID mouse model of MG

Ten SCID mice were injected i.p. with cells isolated from the thymus of a patient with thymoma and myasthenia gravis, along with irradiated TE671 cells, as described in Sect. 4.6. The murine IgG level in SCID mice was less than 3 μg/ml prior to reconstitution, as assessed by ELISA. In post-reconstitution sera on the 14th day, the level of human IgG ranged between 56.8 and over 150 μg/ml.

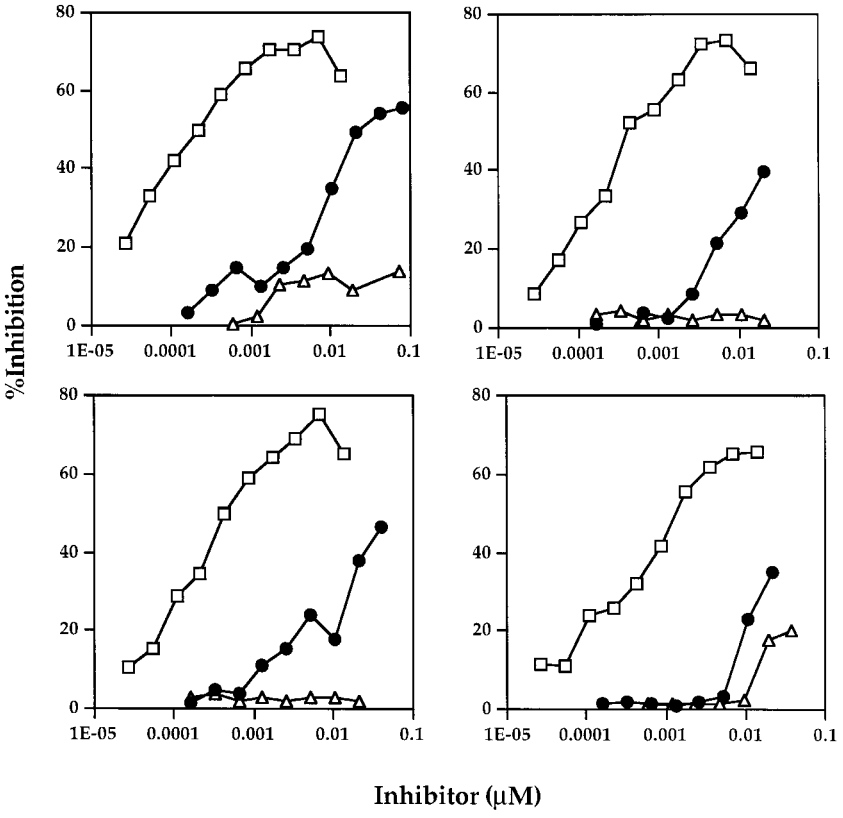


*Figure 1.* Inhibition of the binding of purified IgG antibodies of patients with MG to AchR-expressing cell lysate by therapeutic Ig preparations. A fixed concentration of biotinylated purified IgG from four patients was co-incubated with serial dilutions of IVIg (●), IVIgM (□) or HSA (△) in microtiter plates coated with AchR-expressing lysate of TE671 cells, for 1 h at 37 °C. Bound antibodies were revealed using streptavidin in an ELISA. Each panel represents the results obtained with IgG from one patient.

### 2.3 IVIg and IVIgM suppress serum anti-AchR antibodies in myasthenic SCID mice

Serum levels of human anti-AchR autoantibodies in SCID mice were evaluated by means of a liquid-phase RIA using $^{125}$I-labeled $\alpha$-bungarotoxin. In non-reconstituted mice, the serum level of anti-AchR antibodies remained negligible throughout the period of the study (Fig. 2). The mean level of anti-AchR antibodies in SCID mice implanted with the patient's thymic cells (hu-SCID mice) and treated with HSA increased progressively from day 14 onwards to above the threshold of 1 nM and reached 3.6 nM by day 35 (Fig. 2). In all three myasthenic hu-SCID mice treated with IVIg, the titer of anti-AchR autoantibodies was found to be significantly lower than that in HSA-treated animals throughout the 25 days of the study. In three out of four myasthenic hu-SCID mice treated with IVIgM, the titer of anti-AchR autoantibodies was also significantly lower than that of albumin-treated animals. The titer of antiAchR autoantibodies remainded less than 1 nM throughout the period of the study. These results demonstrate that IVIg and IVIgM reduce significantly the production of anti-AchR autoantibodies in reconstituted myasthenic SCID mice *in vivo*.

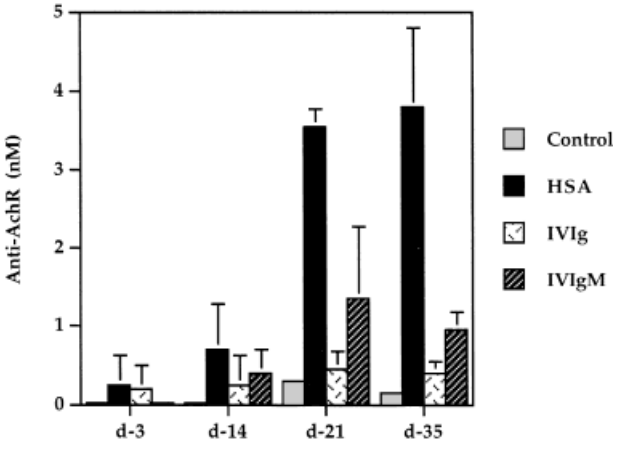


*Figure 2.* Serum titers of human anti-AchR autoantibodies in SCID mice. Myasthenic and control SCID mice were bled at days 3, 14, 21 and 35 after reconstitution. Serum titers of anti-AchR antibodies were determined using a liquid-phase RIA. Grey histograms depict the results obtained in non-reconstituted control mice. Black histograms depict the results obtained in myasthenic mice treated with HSA. Hatched and white histograms depict the results obtained in myasthenic mice treated with IVIg and IVIgM, respectively.

### 2.4 IVIg and IVIgM prevent the loss of endplate AchR in the diaphragms of myasthenic SCID mice

The amount of AchR in the diaphragm of Ig- and HSA-treated myasthenic SCID mice was measured by quantitating the binding of $^{125}$I-labeled $\alpha$-bungarotoxin to diaphragms of animals killed on day 35 following the infusion of Ig or albumin. There was up to 15 % loss of endplate AchR on diaphragms of myasthenic SCID mice that had received human thymus cells and those treated with HSA that were used as controls (Fig. 3). Receptor loss was significantly decreased in the group of animals treated with IVIgM ($p = 0.05$) (Fig. 3). IVIg-treated mice exhibited less pronounced decrease in receptor degradation (Fig. 3), so that the difference in the level of receptor loss between HSA- and IVIg-treated mice was not significant.

## 3 Discussion

Several uncontrolled studies and a recent controlled clinical trial have demonstrated that IVIg is as efficient as plasma exchange in the treatment of acute MG [21–24].

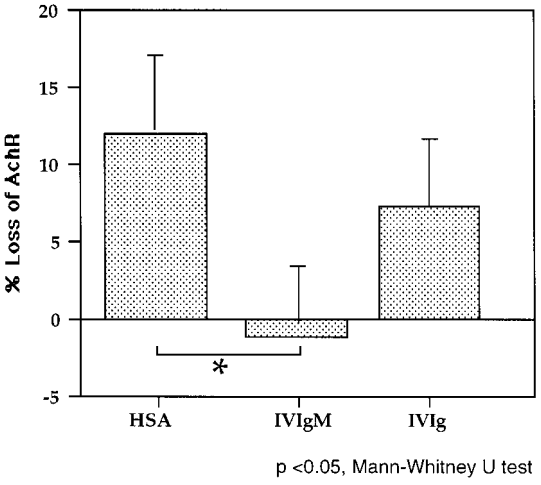


*Figure 3.* Loss of AchR in diaphragms of myasthenic SCID mice treated with IVIg and IVIgM. SCID mice were killed at day 35. The binding of $^{125}$I-labeled $\alpha$-bungarotoxin to diaphragms was assessed as a measure of receptor expression. Three animals were treated with HSA, three animals were treated with IVIg and four animals received IVIgM. The results are shown as the mean ratio between the binding of $^{125}$I-labeled $\alpha$-bungarotoxin to diaphragms of the treated mice and the binding of $^{125}$I-labeled $\alpha$-bungarotoxin to diaphragms of the control non-reconstituted mice ± SD. Statistical significance was calculated by using the Mann-Whitney U test.

In the present study, we have assessed the immunomodulatory effect of IVIg and of IVIgM in an *in vivo* model of MG, using SCID mice reconstituted with cells isolated from a thymoma of a patient with MG.

Experimental MG where fragments of human MG thymus with lymphofollicular hyperplasia were transplanted into SCID mice has been described [10, 27]. Human anti-AchR IgG autoantibodies are produced in this model for up to 6 months. SCID mice transplanted with thymoma cells fail to produce autoantibodies directed against AchR, whereas SCID mice transplanted with fragments of hyperplastic thymi from myasthenic patients produce anti-AchR autoantibodies [28]. Thymic abnormalities are common in MG and thymic myoid cells were shown to provide a source of AchR autoantien [29]. The experimental approach that we used included boosting the animals with irradiated AchR-expressing TE671 cells at the time of injection of the MG thymoma cells, so as to stimulate the engrafted human cells towards anti-AchR antibody production. Our data suggest that both AchR-specific T- and B cells are present in the thymoma of the donor MG patient since circulating AchR-specific antibodies were produced in the reconstituted SCID mice. In addition, loss of AchR in the diaphragm of SCID mice repopulated with MG thymoma cells was observed, further validating the SCID mouse model to study the immunopathology and therapy of MG.

A controlled trial has recently confirmed IVIg to be safe and effective for the treatment of acute MG [22]. IVIg exhibits a broad range of immunomodulatory properties, including anti-inflammatory activity, neutralization of autoantibodies through idiotypic interactions, down-regulation of antibody-producing B cell clones and modulation of T cell functions, through interaction with B cell- and T cell antigen receptors and several other membrane molecules on lymphocytes and APC that are critical for immunoregulation [12, 14]. Here we confirm that IVIg inhibits the binding of AchR-expressing TE671 cells of antibodies of MG patients *in vitro*. Since the half-life of IVIg in the mouse approximates 18 days, our results indicate that IVIg acts by suppressing the production of anti-AchR antibodies in addition to neutralizing circulating autoantibodies as a result of the passive transfer of anti-idiotypic antibodies present in IVIg preparations [25, 30, 31]. Although IVIg was effective in reducing the levels of circulating anti-AchR antibodies in myasthenic SCID mice, it had no effect on the loss of AchR in the diaphragm as compared to the IgM preparations, suggesting different mechanisms that underlie the beneficial effect of the two preparations.

Serum IgM plays a critical role in regulating IgG autoreactivity under physiological conditions [32–34]. We have recently shown that a preparation of IVIgM, processed from the plasma of over 2500 healthy donors, suppresses the activity of several IgG autoantibodies purified from the serum of patients with autoimmune diseases *in vitro* through idiotypic interactions [26]. The inhibitory effect of IVIgM was greater or equivalent to that of IVIg on a molar basis. The present study indicates for the first time, that IVIgM down-regulates anti-AchR autoantibody activity *in vitro* and *in vivo* in a SCID mouse model of MG at a dose equimolar to that of IVIg. Availability of an experimental model described in this report would allow for evaluating different concentrations of Ig preparations in *in vivo* studies. Serum IgM mostly consists of natural germ-line-encoded antibodies, in contrast with serum IgG that also contains significant amounts of immune antibodies directed to non-self antigens that may not be of direct relevance to regulation of autoreactivity. Taken together, our data thus substantiate the concept that IgM may be considered as an alternative/additional approach for immunotherapy of MG and other autoimmune diseases.

## 4 Materials and methods

### 4.1 Ig preparations

IVIg for therapeutic use was Sandoglobulin$^{R}$ (a gift of the Central Laboratory of the Swiss Red Cross, Bern, Switzerland). IVIgM was processed by the Laboratoire Français du Fractionnement et des Biotechnologies (Les Ulis, France) from pooled plasma of over 2500 healthy donors by using a modified Deutsch-Kistler-Nitschmann's ethanol fractionation procedure followed by octanoic acid precipitation and two successive steps of ion-exchange chromatography. The final preparation contained over 90 % pure IgM, as assessed by ELISA using isotype-specific antibodies, gel filtration, immunoelectrophoresis and SDS-PAGE. The protein content of the preparations was measured spectrophotometrically at 280 nm.

### 4.2 Cell lines

The human rhabdomyosarcoma cell line TE671 (ATCC), which expresses AchR, was maintained in RPMI 1640 supplemented with 1 % L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 10 % FCS (Dutscher, Strasbourg, France).

### 4.3 Anti-AchR antibody titers

The anti-AchR antibody titers were determined using a liquid-phase RIA using $^{125}$I-labeled α-bungarotoxin, as described earlier [35, 36]. Briefly, 100 fmol $^{125}$I-AchR were incubated with 10 μl mouse sera or different dilutions of

standards for 1 h at room temperature and 4 h at 4 °C. The complex formed by $^{125}$I-AchR and anti-AchR autoantibodies was then precipitated using anti-human IgG antibodies (Southern Biotechnology, Birmingham, AL). The amount of radioactivity in the precipitate was proportional to the level of autoantibodies against AchR. The titers of anti-AchR antibodies were expressed as nmol/l.

### 4.4 Inhibition of the binding of anti-AchR autoantibodies to AchR by therapeutic Ig preparations

An ELISA was developed to demonstrate the inhibition of the binding of anti-AchR autoantibodies present in the sera of patients to AchR by therapeutic Ig preparations, as previously described with minor modifications [37]. For preparation of lysates of cells which express AchR, the TE671 were grown to preconfluent stage. The washed cell pellets were lysed in cold lysis buffer containing 1 % NP40 (Biorad) in 20 mM Tris pH 7.5, 1 mM EDTA, 140 mM NaCl and a cocktail of protease inhibitors (Boehringer) by incubating the pellet for 45 min at 4 °C with regular vortexing. Pellets were centrifuged at 10 000× g for 10 min at 4 °C. The microtiter plates were coated with 20 μg/ml cell lysate overnight at 4 °C. After saturation of the reactive binding sites with 1 % PBS-BSA, a fixed concentration of biotinylated purified IgG from patients that yields 50 % binding was coincubated with serial dilutions of IVIg (Sandoglobulin) or IVIgM for 1 h at 37 °C. Plates were then incubated with streptavidin (1:3000) (Amersham, Les Ulis, France) and revealed using orthophenylene diamine dihydrochloride.

### 4.5 SCID mice

Eight-week-old female C.B.-17 scid/scid (SCID) mice were obtained from IFFA CREDO, L'Arbresle, France and maintained under sterile conditions. The animals were tested for leakiness by the supplier. The levels of human IgA, IgG and IgM in the sera of SCID mice were determined by means of a sandwich ELISA against standard curves obtained from reference preparations.

### 4.6 Experimental myasthenia gravis in SCID mice

Non-leaky female SCID mice were injected i.p. with $6 \times 10^7$ thymoma cells isolated from a surgically removed thymoma of a patient with a clinically and electrophysiologically proven MG. One day later, the mice were injected i.p. with $10^6$ irradiated (6000 rad) TE671 cells. The animals were bled from the retro-orbital plexus and treated on days 14, 21 and 28 with equimolar concentrations of HSA (8 mg/mouse), IVIg (8 mg) or IVIgM (44 mg). On day 35, the animals were killed and the loss of endplate AchR on their diaphragms evaluated by measuring the binding of $^{125}$I-labeled α-bungarotoxin (0.2 mg with a specific activity of 1 mCi/mg per half of diaphragm, obtained from Dupont De Nemours-New England Nuclear, Les Ulis, France) as described [38].

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