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Amelioration of Experimental Autoimmune Myasthenia Gravis in Rats by Neonatal FcR Blockade

Liming Liu,1 Ana Maria Garcia,2 Helen Santoro,3 Yixia Zhang, Kevin McDonnell, Jennifer Dumont, and Alan Bitonti

The neonatal FcR (FcRn) plays a critical role in IgG homeostasis by protecting it from a lysosomal degradation pathway. It has been shown that IgG has an abnormally short half-life in FcRn-deficient mice and that FcRn blockade significantly increases the catabolism of serum IgG in mice. Therefore, reduction of serum IgG half-life may have therapeutic benefits in Ab-mediated autoimmune diseases. We have studied the therapeutic effects of an anti-rat FcRn mAb, 1G3, in two rat models of myasthenia gravis, a prototypical Ab-mediated autoimmune disease. Passive experimental autoimmune myasthenia gravis was induced by administration of an anti-acetylcholine receptor (AChR) mAb, and it was shown that treatment with 1G3 resulted in dose-dependent amelioration of the disease symptoms. In addition, the concentration of pathogenic Ab in the serum was reduced significantly. The effect of 1G3 was also studied in an active model of experimental autoimmune myasthenia gravis in which rats were immunized with AChR. Treatment with 1G3 significantly reduced the severity of the disease symptoms as well as the levels of total IgG and anti-AChR IgG relative to untreated animals. These data suggest that FcRn blockade may be an effective way to treat Ab-mediated autoimmune diseases. The Journal of Immunology, 2007, 178: 5390–5398.

Most autoimmune diseases involve both cellular and humoral immune responses to self-Ags in disease pathogenesis. However, there are several autoimmune diseases, such as myasthenia gravis (MG),4 that are predominantly mediated by autoantibodies. MG satisfies the five criteria proposed by Drachman (1) that characterize Ab-mediated autoimmune diseases: 1) autoantibodies are present in patients with the disease; 2) Ab interacts with the target Ag; 3) passive transfer of Ab reproduces features of disease; 4) immunization with Ag produces a model disease; and 5) reduction of Ab levels ameliorates the disease. In fact, MG was the first disease to be identified as being predominantly Ab mediated (2). The disease symptoms include muscle weakness and fatigability which are due to autoantibodies generated against the acetylcholine receptor (AChR) and other neuromuscular Ags. Up to 90% of MG patients have detectable Abs against AChR, whereas ~10–15% of the patients are negative for anti-AChR Abs (seronegative MG; SNMG) (3, 4). However, Igs from sera of SNMG patients bind to muscle cells that do not express AChR (5), and they induce reductions in miniature end-plate potential amplitudes upon passive transfer to mice (6). Moreover, it has been shown that 47–70% of SNMG patients have serum IgG autoantibodies against the muscle-specific receptor tyrosine kinase. Muscle-specific receptor tyrosine kinase mediates the agrin-induced clustering of AChR during synapse formation and is also expressed at the mature neuromuscular junction (7, 8). Therefore, in addition to autoantibodies to AChR, Abs to neuromuscular junction components may also be responsible for pathogenesis of MG. The binding of the IgG Abs to AChR and other muscle Ags recruits complement and triggers inflammatory responses, resulting in damage to the neuromuscular junction which leads to further weakening of muscle contraction (9). It is logical that drugs that lower the autoantibody level may have therapeutic effects on MG. Current therapy involves primarily steroids, immunosuppressant drugs, or cytotoxic drugs which could have severe side effects (10).

More than 40 years ago, it was hypothesized that there existed a receptor that was responsible for the regulation of the catabolism of IgG (11). It was also suggested that these receptors were saturable based on studies that showed increased metabolism of IgG with hyperimmunization (12). The receptor known as neonatal FcR (FcRn) was subsequently cloned and was found to be responsible for the protection of IgG from intracellular metabolism (13, 14). FcRn is composed of two subunits: an H chain (a MHC class I-like molecule); and an L chain, β2-microglobulin (β2m) (13) and is expressed in many tissues including blood vessels, lung, liver, intestine, and kidney (15–17). The current understanding of FcRn function is as follows: IgG is taken up by endothelial cells that line the vasculature by fluid phase pinocytosis, followed by pH-dependent binding to FcRn (18, 19) in acidic endosomes, thereby protecting IgG from releasing into lysosomal endosomes where protein degradation occurs. The IgG-FcRn complex is then shuttled back to plasma membrane where the complex dissociates at neutral pH and IgG is released back to the circulation (20). This protective pathway is responsible for the long circulating half-life observed for IgG. Several groups have provided strong evidence supporting
the role of FcRn in the regulation of IgG catabolism. IgG in β2m-deficient mice have an abnormally short serum half-life compared with normal mice (21–23). FcRn H chain-deficient mice were also compromised in their ability to protect IgG and lacked a robust humoral immune response when immunized with Ag plus adjuvant (14).

Because FcRn has a critical role in maintaining the half-life of IgG, it has been suggested as an attractive target for therapeutic intervention in Ab-mediated autoimmune diseases (24, 25). Akilesh et al. (26) showed that FcRn deficiency conferred protection against arthritis in an FcRn-deficient mice crossed with mice susceptible to Ab-mediated autoimmune arthritis (K/B × N mouse model). High dose i.v. Ig therapy, thought to target FcRn, was also found to ameliorate arthritis disease symptoms, further suggesting that FcRn blockade may have anti-inflammatory effects on arthritis. In a rat model of idiopathic thrombocytopenia purpura, another Ab-mediated autoimmune disease, Getman and Balthasar (27) demonstrated that 4C9, an anti-β2m mAb, induced a transient, dose-dependent increase in the elimination of IgG. However, because β2m is also a subunit of MHC class I, the effects of 4C9 on the IgG catabolism could not be solely attributed to the disruption of FcRn function. A more specific approach is to directly target the H chain subunit of FcRn. Recently, it has been shown that a mutant of human IgG Fc that has greatly enhanced affinity for mouse FcRn significantly increased the clearance of tracer and endogenous mouse IgG (28).

Although data are accumulating to suggest that FcRn blockade enhances the clearance of IgG, there is no direct evidence that blockade of FcRn H chain could have therapeutic benefits in Ab-mediated autoimmune diseases. We have investigated the use of a high affinity anti-rat FcRn H chain mAb, 1G3, in the treatment of rat models of MG and demonstrated a significant reduction of disease symptoms for both passive and active models of EAMG.

Materials and Methods

Reagents

Hybridoma cell lines secreting the mAbs 1G3 (CRL 2434), 1745 (CRL 1745) and mAb35 (HB-8857) were purchased from American Type Culture Collection. The 1745 cell line secretes a mAb against porcine parvovirus (29) and was tested with several cell lines and fresh primary cells including human 293 cells, U937 cells, rat and mouse fibroblasts, fresh rat blood leukocytes, and spleen cells; the 1745 mAb did not show staining on these cells in flow cytometry analysis and therefore was used as an IgG1 isotype control (control mIgG1). The 1G3 hybridoma was cloned twice to assure stable expression of functional 1G3 mAb. mAb-secreting hybridomas were grown either in roller bottles in DMEM (Invitrogen Life Technologies), mAb was purified from culture medium using a Hitrap Protein G column (GE Healthcare). Abs were characterized by SDS-PAGE and isoelectric focusing gel electrophoresis. Affinity for soluble rat FcRn was also measured using surface plasmon resonance methods (Biacore) as well as competition with IgG for FcRn binding assay

Rat FcRn-expressing cells (5 × 10⁶/tube/0.1 ml) were incubated on ice with 100 mM Alexa Fluor-488-labeled 1G3 or control mIgG1 at either pH 6.4 for 45 min at 4°C or pH 7.4 for 45 min on ice. After one wash with ice-cold 50 mM sodium phosphate, 100 mM NaCl (pH 6.0 or pH 7.4) with 0.01% surfactant P20 were then injected over the biosensor chip for 4 min at 30 μl/min followed by a 10-min dissociation in sample buffer. Between injections, residual bound FcRn was removed with an 8-s injection of Pierce Ab/Antigen elution buffer followed by a 30-s injection of sample buffer. Kinetic constants were derived from the sensorgram data using the BiaEvaluation software (version 3.1; Biacore). Association and dissociation data were simultaneously fit to models (1:1 Langmuir and heterogeneous ligand-parallel reactions) supplied in the BiaEvaluation package. Equilibrium dissociation constants (Kd) were determined from the ratio of the kinetic constants (k on and k off).

FcRn-binding assay

Rat FcRn-expressing cells (3–5 × 10⁶) were incubated at 4°C with 100 nM Alexa Fluor-488-rat IgG in the presence of various concentrations of 1G3 or mIgG1Ctrl for 45 min in pH 6 binding buffer (calcium and magnesium-free PBS containing 10 mM EDTA, Invitrogen Life Technologies). After one wash with binding buffer, the cells were analyzed in a Beckman Coulter flow cytometer by counting 5000 cells for fluorescence staining. The background fluorescence was established with cells alone. Data are expressed as total mean fluorescence intensity, calculated as the fraction of positive cells multiplied by the mean fluorescence intensity of the positive cells. The concentration of mAb that inhibits binding by 50% (IC50) was calculated using SigmaPlot Software 2000 for curve-fitting analysis (Systat Software).

Flow cytometry analysis of surface marker expression of spleen cells

Spleens were removed from rats and cell suspensions were prepared by teasing apart the spleens in a petri dish containing DMEM. The erythrocytes were lysed with ACK lysis buffer (BioSource International), and the remaining cells were washed three times with PBS containing 1% BSA. Cells were then incubated for 60 min on ice in a 96-well Costar assay plate (1 × 10⁶ cells/well/100 μl) with FITC-labeled mouse Abs against rat leukocyte surface Ags. After one wash, the cells were analyzed for fluorescence staining in a Beckman Coulter flow cytometer.

Measurement of Abs in serum

To measure total rat serum IgG, 96-well ELISA plates (Costar; Corning) were coated with 2.5 μg/ml purified goat anti-rat IgG Fc (Jackson ImmunoResearch Laboratories) in carbonate-bicarbonate coating buffer (Sigma-Aldrich) for 60 min at 37°C. After blocking with 0.1% solution of BSA in PBS (blocking buffer) for 60 min at 37°C, the plates were washed and incubated with 100 μl of PBS containing 0.1% Tween 20 (Sigma-Aldrich) and test sera were diluted in blocking buffer and added to the plates which were incubated at 37°C for 60 min. After three washes, goat anti-rat IgG F(ab′)2-HRP (Jackson ImmunoResearch Laboratories)
was added to the plates that were then incubated at 37°C for 60 min. All plates were developed with SureBlue TMB (Kirkegaard & Perry Laboratories), and the reaction was terminated with stop solution (Kirkegaard & Perry Laboratories). OD450 was determined using a plate reader (Molecular Devices). The concentration of IgG in test sera was calculated from the rat IgG standard curve, and the limit of detection was 12.5 ng/ml.

The measurement of anti-AChR Ab in serum was the same as that for total IgG measurement except for some assay-specific reagents. Ninety-six-well ELISA plates were coated with 5 μg/ml AChR in carbonate-bicarbonate coating buffer overnight at 4°C. After blocking, either diluted test rat serum or anti-AChR mAb35 (as a standard) was added to the wells. The plates were developed with the same protocol described in the measurement of total rat serum IgG, and the limit of detection was 12.5 ng/ml. Measurement of mAb35 concentration in rat serum in the passive EAMG model followed this same protocol.

To measure total serum IgM, 96-well ELISA plates were coated with 2 μg/ml mouse anti-rat IgM mAb in coating buffer (BD Bioscience) and incubated at 4°C overnight. After blocking, either rat serum or purified rat serum IgM (Sigma-Aldrich) was added to the plates, and the plates were incubated at 37°C for 60 min. Then goat anti-rat IgM-HRP (Jackson ImmunoResearch Laboratories), 1- to 10,000-fold dilutions, was added and incubated at 37°C for 60 min. The plates were developed with the protocol described above. The concentration of IgM in test sera was calculated from the rat IgM standard curve and the limit of detection was 3 ng/ml. The linear range was 6–50 ng/ml.

Measurement of serum 1G3 or control mIgG1 levels was performed in 96-well ELISA plates that were coated with 5 μg/ml mouse anti-mouse IgG (Fab specific). After blocking, 1G3, control mlgG1, or rat serum was added to the plates followed by incubation at 37°C for 60 min. After sample incubation, goat anti-mouse IgG (H + L)-HRP (Jackson ImmunoResearch Laboratories) in 1/10,000 dilutions was added to the plates, which were then incubated at 37°C for 60 min. The plates were developed with the same protocol described above. The concentration was calculated using a 1G3 standard or control mlgG1 standard curve, and the detection limit was 12 ng/ml.

Induction and assessment of passive EAMG

All animal work was approved by the Syntonix Institutional Animal Care and Use Committee. Passive EAMG was induced in female specific pathogen-free (SPF) Lewis rats (Charles River Laboratories) weighing ~100 g, by i.p. administration of mAb35 at 0.75–1 mg/kg. The time of mAb35 administration was defined as time zero. 1G3 or control mlgG1 was administered i.p. at 2 and/or 24 h before disease induction with mAb35. Disease progression was monitored twice daily by measuring disease score, body weight, and grip strength. The visual determination of disease symptoms was graded in a scale of 0–4 (31), where 0 was no symptoms; 1, weak grip, fatigability; 2, general weakness, hunched posture at rest, decreased body weight, tremors; 3, severe weakness, moribund; and 4, death. If needed, 100–150 μl of blood from a tail clip were collected in a Minicollect serum collection tube (Greiner Bio-one). Grip strength was measured with a grip meter (Columbus Instruments). PBS was used as the vehicle in all experiments.
Induction and assessment of active EAMG

SPF female Lewis rats, 6–7 wk old (~150 g at the beginning of experiment), were from Charles River Laboratories. Rats were immunized by s.c. injection at the base of the tail of 50 μg/50 l Torpedo AChR emulsified in CFA containing 10 mg/ml Mycobacterium tuberculosis R37. After immunization, rats were randomized to different groups to receive treatments (8–10 rats/group). Both therapeutic and control Abs were administered i.p. as indicated in the figure legends. The disease progression was monitored twice weekly (Monday and Friday) by recording body weight, grip strength, and disease score.

Statistical analysis

Data were evaluated for statistical significance using Student’s t test in Sigmastat provided by Systat Software.

Results

High affinity binding of 1G3 to Rat FcRn and competition for IgG binding at pH 6.0 and 7.4

1G3 is a mouse anti-rat FcRn mAb developed by Raghavan et al. (32). 1G3 and its isotype control mIgG1 were covalently immobilized onto the surface of biosensor chips, and the binding of soluble rat FcRn to these surfaces was monitored using a Biacore 3000 SPR instrument (Fig. 1). Several methods have been reported for determining the affinity of the FcRn-IgG interaction using Biacore (33–37), and the particular method chosen will yield different values for the association and dissociation constants. Due to the high affinity of the interaction between

FIGURE 3. Competition of 1G3 for binding of IgG to rat FcRn. Rat FcRn-expressing cells were incubated with different concentrations of competitors in the presence of Alexa Fluor-488-labeled rat IgG for 45 min on ice. All competition assays were conducted at pH 6.0. After one wash, cells were analyzed in a Coulter flow cytometer. Results were expressed as total mean fluorescence intensity (TMFI) calculated as the fraction of positive cells multiplied by the mean fluorescence intensity. Data are representative of three separate experiments.

FIGURE 4. Pharmacokinetics of 1G3 in rats. Two groups (four per group) of SPF female Lewis rats (~150 g, 6–7 wk old) were injected i.p. with either 1G3 or control mlgG1 (10 mg/kg). Blood was collected, and serum was prepared at 0.25, 6, 24, 30, 48, 72, 96, 120, and 168 h after the injection. A specific ELISA was used to determine 1G3 and mlgG1 serum concentration as described in Materials and Methods. This experiment was repeated twice with similar results.

FIGURE 5. Effects of 1G3 on serum IgG concentration in rats. Two groups (four per group) of SPF female Lewis rats were injected i.p. with either 30 mg/kg 1G3 or control mlgG1 at 0 and 24 h. Serum was obtained at 0, 24, 48, 96, 120, and 168 h, and total IgG concentration was measured as described in Materials and Methods. Arrows, Time of 1G3 injection. This experiment was repeated once with similar results.

FIGURE 6. Effects of 1G3 on passive EAMG. SPF female Lewis rats (~100 g, 4–5 wk old) were divided into five groups (six rats per group). Animals were treated i.p. with vehicle, 1G3, or mlgG1Ctrl at various time points before the induction of disease with mAb35. ○, Vehicle treated at −2 h; ▲, 30 mg/kg 1G3 at −2 h; ■, 30 mg/kg 1G3 at −2 h; ●, 30 mg/kg 1G3 at −2 h; ■, control mlgG1 at −2 h. Disease symptoms were scored as described in Materials and Methods. Comparison of disease scores at 24 and 48 h after disease induction: single-dose 1G3-treated groups vs vehicle- or mlgG1Ctrl-treated group, p < 0.05; two doses of 1G3 vs vehicle or mlgG1Ctrl-treated group, p < 0.001.
1G3 and the FcRn, it was necessary to perform the analysis with the IgG immobilized, because the FcRn protein was too sensitive to the conditions needed to regenerate the biosensor chip surface between injections.

At pH 6.0 and 7.4, the 1G3-FcRn binding data fit best to a heterogeneous ligand (two-site) model (total response is due to two independent classes of noninteracting binding sites; the two $K_D$ values each represent some fractional percent, $f_1\%$ and $f_2\%$ of the observed signal). For control mlgG1-FcRn, the binding data were fit to a simple 1:1 Langmuir model (one site; Refs. 33 and 37).

The equilibrium dissociation constants ($K_D$) are calculated from the ratios of the kinetic rate constants ($K_D = k_{off}/k_{on}$). The average values for three experiments are shown in Fig. 1. 1G3 exhibits a high affinity for soluble rat FcRn and binds similarly at pH 6.0 and 7.4. By comparison, the control mlgG1 binds weakly to FcRn at pH 6.0 and there is no apparent binding at pH 7.4. Also, the off-rate ($k_{off}$) for the binding of 1G3 to FcRn at pH 6 is two orders of magnitude slower than for control mlgG1 binding to FcRn.

The binding of 1G3 and control mlgG1 to cells expressing rat FcRn was also examined. Similar to the results obtained using Biacore, 1G3 bound to rat FcRn on the cell surface at pH 6 and 7.4, whereas the control mlgG1 bound only at pH 6 (Fig. 2).

A competition assay using flow cytometry was established to test whether 1G3 could compete for IgG1 binding to rat FcRn-expressing cells. The results showed that 1G3 competed effectively with rat IgG for binding to rat FcRn (Fig. 3).

### 1G3 half-life in vivo

The pharmacokinetics of 1G3 and control mlgG1 were studied in rats after a single i.p. administration. The results showed that 1G3 reached a maximum serum concentration that was similar to the control mlgG1 at 6 h, but then the concentration of 1G3 rapidly declined to unmeasurable levels at 24 h (Fig. 4). In contrast, control mlgG1 had a much longer half-life of $\sim$104 h, similar to the 4- to 5-day half-life found in normal mice (Ref. 38 and Fig. 4).

### 1G3 effect on serum IgG concentration in rats

To determine the effect of 1G3 treatments on endogenous serum IgG concentration, rats were injected with 1G3 at 0 and 24 h, and the serum IgG concentration was measured. As shown in Fig. 5, 1G3 significantly reduced the serum IgG concentration to $\sim$60% of the starting level, whereas the mlgG Ctrl did not affect endogenous serum IgG levels. The effect lasted $\sim$3 days after the last injection.

### Blockade of FcRn inhibits disease symptoms in passive EAMG

Passive EAMG was induced by injecting rats with mAb35, an anti-AChR mAb known to induce the disease (39). We investigated the dose response to mAb35 in this disease model and determined that an i.p. injection of 0.75–1 mg/kg resulted in high clinical scores (score 3 of a possible 4) without significant lethality (data not shown). Disease progression was similar to that described previously, with symptoms typically starting at 12 h, peaking at 48 h and gradually disappearing by 72 h post-mAb35 injection (39). EAMG symptoms were almost completely prevented when 30 mg/kg 1G3 was administered twice at 24 and 2 h before mAb35 injection (Fig. 6). Single doses of 1G3 (30 mg/kg) administered

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1 mg/kg, $p < 0.05$. Grip strength for the 1G3-treated group compared with the vehicle-treated group: 10 mg or 30 mg/kg at 24 and 30 h, $p < 0.001$; 3 mg/kg, $p < 0.05$. 1 mg/kg at 30 h, $p < 0.05$. Body weight for the 1G3-treated group compared with the vehicle-treated group: 30 mg/kg at 24, 30, and 50 h, $p < 0.01$; 10 or 3 mg/kg at 24, 30, and 50 h, $p < 0.05$; 1 mg/kg at 50 h, $p < 0.05$. 1 mg/kg, $p < 0.05$. Grip strength for the 1G3-treated group compared with the vehicle-treated group: 10 mg or 30 mg/kg at 24 and 30 h, $p < 0.001$; 3 mg/kg at 24 and 30 h, $p < 0.05$; 1 mg/kg at 30 h, $p < 0.05$. Body weight for the 1G3-treated group compared with the vehicle-treated group: 30 mg/kg at 24, 30, and 50 h, $p < 0.01$; 10 or 3 mg/kg at 24, 30, and 50 h, $p < 0.05$; 1 mg/kg at 50 h, $p < 0.05$. 1 mg/kg, $p < 0.05$. Grip strength for the 1G3-treated group compared with the vehicle-treated group: 10 mg or 30 mg/kg at 24 and 30 h, $p < 0.001$; 3 mg/kg at 24 and 30 h, $p < 0.05$; 1 mg/kg at 30 h, $p < 0.05$. Body weight for the 1G3-treated group compared with the vehicle-treated group: 30 mg/kg at 24, 30, and 50 h, $p < 0.01$; 10 or 3 mg/kg at 24, 30, and 50 h, $p < 0.05$; 1 mg/kg at 50 h, $p < 0.05$. 1 mg/kg, $p < 0.05$. Grip strength for the 1G3-treated group compared with the vehicle-treated group: 10 mg or 30 mg/kg at 24 and 30 h, $p < 0.001$; 3 mg/kg at 24 and 30 h, $p < 0.05$; 1 mg/kg at 30 h, $p < 0.05$. Body weight for the 1G3-treated group compared with the vehicle-treated group: 30 mg/kg at 24, 30, and 50 h, $p < 0.01$; 10 or 3 mg/kg at 24, 30, and 50 h, $p < 0.05$; 1 mg/kg at 50 h, $p < 0.05$. 1 mg/kg, $p < 0.05$. Grip strength for the 1G3-treated group compared with the vehicle-treated group: 10 mg or 30 mg/kg at 24 and 30 h, $p < 0.001$; 3 mg/kg at 24 and 30 h, $p < 0.05$; 1 mg/kg at 30 h, $p < 0.05$. Body weight for the 1G3-treated group compared with the vehicle-treated group: 30 mg/kg at 24, 30, and 50 h, $p < 0.01$; 10 or 3 mg/kg at 24, 30, and 50 h, $p < 0.05$; 1 mg/kg at 50 h, $p < 0.05$. 1 mg/kg, $p < 0.05$. Grip strength for the 1G3-treated group compared with the vehicle-treated group: 10 mg or 30 mg/kg at 24 and 30 h, $p < 0.001$; 3 mg/kg at 24 and 30 h, $p < 0.05$; 1 mg/kg at 30 h, $p < 0.05$. Body weight for the 1G3-treated group compared with the vehicle-treated group: 30 mg/kg at 24, 30, and 50 h, $p < 0.01$; 10 or 3 mg/kg at 24, 30, and 50 h, $p < 0.05$; 1 mg/kg at 50 h, $p < 0.05$. 1 mg/kg, $p < 0.05$. Grip strength for the 1G3-treated group compared with the vehicle-treated group: 10 mg or 30 mg/kg at 24 and 30 h, $p < 0.001$; 3 mg/kg at 24 and 30 h, $p < 0.05$; 1 mg/kg at 30 h, $p < 0.05$. Body weight for the 1G3-treated group compared with the vehicle-treated group: 30 mg/kg at 24, 30, and 50 h, $p < 0.01$; 10 or 3 mg/kg at 24, 30, and 50 h, $p < 0.05$; 1 mg/kg at 50 h, $p < 0.05$. 1 mg/kg, $p < 0.05$. Grip strength for the 1G3-treated group compared with the vehicle-treated group: 10 mg or 30 mg/kg at 24 and 30 h, $p < 0.001$; 3 mg/kg at 24 and 30 h, $p < 0.05$; 1 mg/kg at 30 h, $p < 0.05$. Body weight for the 1G3-treated group compared with the vehicle-treated group: 30 mg/kg at 24, 30, and 50 h, $p < 0.01$; 10 or 3 mg/kg at 24, 30, and 50 h, $p < 0.05$; 1 mg/kg at 50 h, $p < 0.05$. 1 mg/kg, $p < 0.05$. Grip strength for the 1G3-treated group compared with the vehicle-treated group: 10 mg or 30 mg/kg at 24 and 30 h, $p < 0.001$; 3 mg/kg at 24 and 30 h, $p < 0.05$; 1 mg/kg at 30 h, $p < 0.05$. Body weight for the 1G3-treated group compared with the vehicle-treated group: 30 mg/kg at 24, 30, and 50 h, $p < 0.01$; 10 or 3 mg/kg at 24, 30, and 50 h, $p < 0.05$; 1 mg/kg at 50 h, $p < 0.05$.
pressed active EAMG symptoms (Fig. 8).

Treatment with 30 mg/kg 1G3, three times per week, significantly suppressed disease symptoms whereas 1 and 3 mg/kg 1G3 suppressed the disease symptoms but to a lesser extent (Fig. 7A). Animals treated with 1G3 gained weight and did not lose grip strength, unlike the control group that exhibited significant declines in both of these parameters (Fig. 7, B and C).

We then tested doses of 1, 3, 10, and 30 mg/kg and found that 1G3 reduced disease symptoms in a dose-dependent manner. Treatments with 1G3 at either 10 or 30 mg/kg given 24 and 2 h before disease induction almost completely prevented EAMG disease symptoms whereas 1 and 3 mg/kg 1G3 suppressed the disease symptoms but to a lesser extent (Fig. 7A). Animals treated with 1G3 gained weight and did not lose grip strength, unlike the control group that exhibited significant declines in both of these parameters (Fig. 7, B and C).

To investigate whether 1G3 has therapeutic benefit in a chronic model of EAMG, we immunized rats with AChR in CFA. Treatments with 1G3 at either 10 or 30 mg/kg given 24 and 2 h before disease induction almost completely prevented EAMG disease symptoms whereas 1 and 3 mg/kg 1G3 suppressed the disease symptoms but to a lesser extent (Fig. 7A). Animals treated with 1G3 gained weight and did not lose grip strength, unlike the control group that exhibited significant declines in both of these parameters (Fig. 7, B and C).

**Table I. Serum mAb35 concentration after 1G3 treatment**

<table>
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<tr>
<th>Time (h)</th>
<th>Vehicle</th>
<th>1 mg/kg 1G3</th>
<th>3 mg/kg 1G3</th>
<th>10 mg/kg 1G3</th>
<th>30 mg/kg 1G3</th>
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<td>3</td>
<td>10.9 ± 1.1</td>
<td>10.6 ± 2.3</td>
<td>10.4 ± 2</td>
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<tr>
<td>7</td>
<td>8.9 ± 0.8</td>
<td>8.6 ± 1.3</td>
<td>8.6 ± 1.8</td>
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<td>24</td>
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<td>5.7 ± 1.6</td>
<td>5.2 ± 0.6*</td>
<td>5.5 ± 0.4**</td>
<td>4.4 ± 0.5***</td>
</tr>
<tr>
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<td>5.8 ± 0.9</td>
<td>5.3 ± 0.4*</td>
<td>3.9 ± 0.4***</td>
<td>3.3 ± 0.6***</td>
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<td>83</td>
<td>68</td>
<td>41</td>
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</table>

* Sera were obtained from rats used in the experiment described in Fig. 7. Serum mAb35 concentrations were determined as described in Materials and Methods. The time indicates hours after mAb35 injection. 1G3-treated groups compared with the vehicle-treated group: *, p < 0.01; **, p < 0.03; *** p < 0.001.

To investigate whether the protective effect of 1G3 on disease symptoms was reflected in the serum concentration of the disease-inducing Ab, serum levels of mAb35 were measured. The concentration of mAb35 in serum was significantly reduced in animals treated with 3, 10, and 30 mg/kg 1G3, and the reduction was dose dependent (Table I). A dose of 1 mg/kg also reduced serum levels of mAb35 but did not reach statistical significance.

**1G3 reduces the disease severity of active EAMG**

Autoimmune diseases such as MG are generally chronic. To investigate whether 1G3 has therapeutic benefit in a chronic model of EAMG, we immunized rats with AChR in CFA. Treatments were initiated 21 days after immunization with AChR, when disease symptoms started to appear. A dexamethasone treatment group was included because steroids such as prednisone or dexamethasone are commonly used in the treatment of MG (10). Treatment with 30 mg/kg 1G3, three times per week, significantly suppressed active EAMG symptoms (Fig. 8A). The 1G3- and dexamethasone-treated groups retained grip strength, and although vehicle-treated animals were noticeably weaker in these tests, statistical significance was not reached (Fig. 8B). All three treatment groups increased in body weight during the first 37 days (Fig. 8C). After that time, dexamethasone-treated rats experienced a progressive decline in body weight that persisted until the experiment was terminated 62 days after immunization. In a separate experiment, mIgG1 Ctrl was found to have no significant impact on the disease progress (data not shown).

To investigate whether the FcRn blockade affected the serum immunoglobulins in immunized animals, serum levels of total IgG, IgM and specific anti-AChR IgG were measured (Table II). The baseline level of total serum IgG in SPF rats was ~1.2 ± 0.1 mg/ml, and this increased markedly over the course of the experiment, reaching a high of 17.1 ± 4.2 mg/ml by day 62. 1G3 suppressed the

**FIGURE 8.** Effects of 1G3 on active EAMG. Female Lewis rats (~150 g, 6–7 wk old) were immunized with AChR and CFA as described in Materials and Methods. Twenty-one days after immunization, rats were randomized into three groups (10 per group) and treated three times per week with 30 mg/kg 1G3, 1 mg/kg dexamethasone, or vehicle. Disease scores, grip strength, and weight changes were recorded as described in Materials and Methods. (A) Disease scores; (B) grip strength. –– ––, Average starting grip strength; C, body weight. –– ––, Average starting body weight. Comparison of disease scores between the 1G3-treated group and the vehicle-treated group on day 37, p < 0.05. These data are representative of three separate experiments.
serum IgG and anti-AChR IgG were measured with ELISA as described in Methods and Materials. CR3-expressing cells approximately significantly reduced in the 1G3-treated group when compared with those of the control group. CR3-expressing cells (primarily T lymphocytes), whereas the His24-expressing populations in the spleen by flow cytometry analysis. Treatment had no effect on serum IgM levels (data not shown).

It is well known that steroid treatment has broad immunosuppressive effects; therefore, we compared the effects of 1G3 to dexamethasone treatment on various indicators of the immune system. The weights of spleens and draining lymph nodes of animals treated with 1G3 remained at normal levels, whereas treatment with dexamethasone reduced the spleen and lymph node weight significantly, as expected (Table III). It was also of interest to know whether FcRn blockade affects specific cells of the immune system. Therefore, we measured a number of major immune cell populations in the spleen by flow cytometry analysis. Treatment with 1G3 did not affect the percentages of CD4* and CD8* cells (primarily T lymphocytes), whereas the His24-expressing population (presumably mostly B cells) was significantly increased. The number of CR3 (stained with OX42mAb)-expressing cells was significantly reduced in the 1G3-treated group when compared with those of the control group. CR3-expressing cells approximately doubled in the dexamethasone-treated group.

### Table II. Total serum IgG and AChR-specific Abs in active EAMG

<table>
<thead>
<tr>
<th>Time of Measurement</th>
<th>Vehicle (mg/ml)</th>
<th>1G3 (mg/ml)</th>
<th>Dexamethasone (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preimmune</td>
<td>1.21 ± 0.70</td>
<td>1.30 ± 0.20</td>
<td>1.10 ± 0.10</td>
</tr>
<tr>
<td>Total IgG</td>
<td>4.90 ± 0.70</td>
<td>4.70 ± 1.90</td>
<td>6.70 ± 2.40</td>
</tr>
<tr>
<td>Anti-AChR</td>
<td>0.56 ± 0.17</td>
<td>0.45 ± 0.07</td>
<td>0.71 ± 0.15</td>
</tr>
<tr>
<td>Day 34</td>
<td>6.40 ± 2.40</td>
<td>4.60 ± 1.50</td>
<td>7.50 ± 1.30</td>
</tr>
<tr>
<td>Total IgG</td>
<td>12.90 ± 3.20</td>
<td>7.00 ± 2.50</td>
<td>11.90 ± 5.50</td>
</tr>
<tr>
<td>Anti-AChR</td>
<td>0.29 ± 0.02</td>
<td>0.20 ± 0.02</td>
<td>0.12 ± 0.03</td>
</tr>
<tr>
<td>Day 62</td>
<td>17.10 ± 4.20</td>
<td>9.20 ± 2.70</td>
<td>11.30 ± 3.90</td>
</tr>
<tr>
<td>Anti-AChR</td>
<td>0.15 ± 0.07</td>
<td>0.15 ± 0.08</td>
<td>0.12 ± 0.10</td>
</tr>
</tbody>
</table>

* Sera were obtained from rats used in the experiment described in Fig. 8. Total serum IgG and anti-AChR IgG were measured with ELISA as described in Materials and Methods.

** Discussion

Existing evidence shows that FcRn plays a critical role in the homoestasis of serum IgG and that FcRn blockade enhances the clearance of IgG (14, 28). However, there is no direct evidence showing that a pH-independent FcRn H chain blocker could ameliorate Ab-mediated autoimmune diseases. In this article, we show that 1G3, a high affinity and pH-independent blocker of rat FcRn H chain, enhanced the clearance of pathogenic Abs and had significant therapeutic effects on both passively and actively induced EAMG.

Depending on the disease severity, MG patients can be categorized into two groups: patients who have developed myasthenic crisis; and patients who have generalized MG but not in crisis (9). Passive EAMG induced by mAb35 resembles the disease Characteristics of MG crisis, which is severe and has a fast onset. When given either 24 or 2 h before mAb35 injection, 30 mg/kg 1G3 almost completely prevented the EAMG symptoms. When given twice, at 3 or 1 mg/kg, 1G3 treatment significantly suppressed the disease symptoms. The therapeutic effects on disease symptoms were reflected in the grip strength and body weight changes, which are a more objective measurement of the disease severity. Importantly, there was a dose-dependent reduction of mAb35 at 48 h in the serum after 1G3 treatment, which strongly suggests that the mechanism of 1G3 action was due to the enhanced clearance of mAb35 by FcRn blockade. The reason why the serum mAb35 reduction lagged behind the disease symptom suppression is unclear. It is known that 50% of IgGs are distributed extravascularly (38). Therefore, it is possible that 1G3 effects occurred first in the extravascular space (for which there is no easy and reliable measurement) and manifested in the intravascular space later.

The pathogenic effect of mAb35 may be dependent on its serum concentration and residence time. Loutrari et al. (40) reported that mAb35 IgG induced EAMG readily, whereas Fab were not capable of inducing disease symptoms and F(ab')2 induced minimal disease. This disease induction pattern was attributed to the serum residue time of these molecules in that Fab, and to a lesser extent F(ab')2, were rapidly removed from the circulation whereas the full mAb IgG persisted for a long time (40). Subsequently, Poulas et al. (41) reported that papain, which cleaves the IgG into free Fab and Fc fragments, was able to reduce the severity of EAMG when injected in vivo and that the papain-treated animals had a significant reduction of serum mAb35 concentration. Our data in the passive EAMG model also show the correlation between therapeutic effects and the reduction of serum mAb35. In this regard, our data are consistent with prior observations.
One of the intriguing observations from this study is that 1G3 itself has a very short serum residence time and is completely gone 24 h after injection, whereas the mouse IgG1 control has a long half-life (104 h). Biacore and flow cytometry experiments showed that 1G3 bound to rat FcRn with high affinity and had a slow off-rate at both pH 6 and pH 7.4. This high affinity, pH-independent binding and the rapid disappearance of 1G3 from circulation suggest that once 1G3 binds to FcRn in vivo, it does not release rapidly. A similar observation was reported previously in a study involving human FeC mutants (42). Mutant FeC fragments that were pH-independent, high affinity binders of mouse FcRn had abnormally short serum residence time when injected into mice (42).

Passive EAMG is a transient disease; therefore, therapeutic effects in this model may suggest a potential treatment option for myasthenic crisis. To investigate the effects of FcRn blockade on chronic MG, we used an active EAMG model that involved immunization of rats with AchR and CFA. Administration of 1G3 when disease symptoms appeared significantly suppressed disease symptoms. The active EAMG model involves both humoral and cellular arms of the immune response, whereas the passive EAMG model is a pure Ab-mediated disease. This may explain why the therapeutic effects on active EAMG are not as striking as in passive EAMG. Dexamethasone, a steroid used in many autoimmune diseases including MG, also suppressed the disease symptoms but with significant side effects: a body wasting syndrome; and leukopenia manifested by the significant reduction in weight of the spleen and lymph nodes.

Immunization with AchR and CFA induced not only an anti-AchR response but also a high magnitude immune response to mycobacterial Ags that are the major components of CFA (43). Naïve rats had a low amount of total IgG in the serum (~1 mg/ml); whereas serum IgG of immunized rats increased substantially (up to 17 mg/ml). Treatment with 1G3 suppressed the increase in total serum IgG by ~50% when compared with the vehicle-treated group 48 and 62 days after immunization. It took several weeks of 1G3 treatment to establish IgG reduction, presumably due to the faster rate of synthesis of IgG compared with its clearance rate in this hyperimmune situation. Dexamethasone also appeared to suppress the increase in IgG in the serum, although the magnitude of reduction was less than that induced by 1G3 treatment.

The kinetics of anti-AchR IgG is apparently different from that of total IgG in that the anti-AchR IgG response appeared to have reached the peak on day 20 and then declined gradually. Saoudi et al. (44) reported a similar kinetics of anti-AchR IgG Ab response after immunization with AchR and CFA. Two weeks after treatment began, serum anti-AchR IgG in the 1G3-treated group was reduced significantly when compared with vehicle treated group and this probably contributed to the therapeutic effects observed. It is predicted that FcRn blockade should affect only the catabolism of IgG and not that of IgM, given that other Ig classes do not bind to FcRn. Our results show that serum total IgM level was comparable throughout the study period between the 1G3-treated group and the vehicle-treated group.

Roopenian et al. (14) showed that FcRn H chain-deficient mice are not capable of mounting a robust humoral immune response but that there is no impairment in the cell-mediated immune response. Our results also suggest that FcRn blockade affects primarily the IgG Ab response and not the overall immune system. Spleen weights from 1G3-treated and vehicle-treated groups were comparable, whereas the weights for the dexamethasone-treated group were significantly reduced. The weights of draining lymph nodes increased initially after immunization for all animals. After the treatment began, the lymph node weights were comparable between the 1G3-treated and the vehicle-treated groups; however, treatment with dexamethasone resulted in a reduction in lymph node weight, due to the known immunosuppressive effects of the steroid.

CR3 expression is increased in inflammatory situations, and the expression is reduced when the inflammation wanes (45, 46). It has also been shown that anti-CR3 mAb treatments (administration of OX42) significantly reduced the inflammation of acute colitis in rats (47). In our study, CR3-expressing cells were significantly reduced in the 1G3-treated group when compared with the vehicle-treated group. Thus, the reduction of CR3-expressing cells may have contributed to the therapeutic effects of 1G3. Several mechanisms might be responsible for the reduction of CR3-expressing cells: 1) 1G3 treatment might have inhibited the function of the major inflammatory mediator, polymorphonuclear cells. A recent report showed that FcRn may play a major role in IgG-mediated phagocytosis by polymorphonuclear cells (48); 2) 1G3 treatment could have suppressed the Ag processing and presentation by FcRn-expressing monocytes and macrophages, resulting in reduced inflammation. It has been shown that monocytes and macrophages express functional FcRn that may positively influence Ag processing and presentation (49); 3) 1G3 treatments suppressed the up-regulation of Ag-specific IgG, resulting in reduced Ag presentation. Ag-specific IgG complexed with its Ag can potentiate Ag presentation through FcγR binding (50, 51).

Although MG is primarily mediated by autoantibodies, the underlying cellular immune responses also play an important role in autoantibody responses because the anti-AchR Ab response is T cell dependent (44, 52). T cell response to AchR was elevated in MG patients (52–54). Thus, ideal therapeutics for Ab mediated autoimmune diseases such as MG may be the combination of therapies that can reduce the autoantibody titer rapidly and, at the same time, control the underlying cellular immune response. In this regard, FcRn blockade could be used in combination with lower doses of steroids (steroid sparing). FcRn blockade could potentially lower the autoantibody level below the threshold that triggers disease symptoms and a lower dose of steroids or other immunosuppressant could control the underlying cell-mediated response. This combination therapy might achieve maximum disease control with significantly reduced side effects. There is evidence that i.v. Ig therapy has a steroid sparing effect in pemphigus patients, reducing the dose of steroid or, in some cases, eliminating steroids completely (55).

Rituximab, an anti-CD20 mAb, has been tested in some Ab-mediated autoimmune diseases. Although rituximab substantially depleted CD20-expressing B cells in patients with systemic lupus erythematosus, elevated autoantibody titers persisted (56). This may be due to the presence of low levels of residual autoreactive memory B cells and/or long-lived autoreactive plasma cells. In agreement with such observations, it has been shown that rituximab depletes short-lived plasmablasts but not long-lived plasma cells, which continue to produce autoantibodies (57). In this situation, FcRn blockade may have an advantage of rapidly controlling autoantibody levels.

In summary, we have shown that 1G3, a high affinity and pH-independent blocker of IgG binding to rat FcRn, substantially ameliorated passive EAMG and significantly reduced the symptoms of active EAMG in rats without nonspecifically suppressing the immune system. These results suggest that FcRn blockade may be an effective and a novel way to treat autoantibody-mediated autoimmune diseases.

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**Disclosures**

L. Liu, Y. Zhang, K. McDonell, J. Dumont, and A. Bitonti are current employees, and A. M. Garcia and H. Santoro are former employees of Syntonix Pharmaceuticals.
EFFECTS OF FcRn BLOCKADE ON RAT EAMG


