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Antigen-Specific Immunotherapeutic Vaccine for Experimental Autoimmune Myasthenia Gravis

Jie Luo and Jon Lindstrom

Myasthenia gravis (MG) and experimental autoimmune myasthenia gravis (EAMG) are caused by Ab-mediated autoimmune responses to muscle nicotinic acetylcholine receptors (AChRs) that impair neuromuscular transmission, thereby causing muscle weakness. Previously, we discovered that i.p. injection of a therapeutic vaccine consisting of bacterially expressed cytoplasmic domains of human AChR subunits reduced the development of chronic EAMG in rats. In this article, we show that immunization with the therapeutic vaccine in adjuvants does not induce EAMG and, thus, is safe. The potency and efficacy of the therapeutic vaccine were greatly increased by s.c. administration of repeated low doses in IFA. Onset of chronic EAMG could be prevented. Established chronic EAMG could be rapidly reversed, modeling therapy of chronic MG. Therapy reduced pathological Abs assayed by immune precipitation of a main immunogenic region chimera. Successfully treated rats exhibited long-term resistance to reinduction of EAMG, suggesting a lasting cure of MG. A long-term effect of therapy was to change the isotype of the pathogenic Ab response from IgG2b, which fixes complement, to IgG1, which does not. Prevention and reversal of chronic EAMG was not caused by the isotype switch, but the isotype switch may contribute to resistance to reinduction of EAMG. Immunization with AChR cytoplasmic domains in adjuvant is promising as a safe, Ag-specific, potent, effective, rapidly acting, and long-lasting therapeutic approach to MG. *The Journal of Immunology*, 2014, 193: 5044–5055.

Myasthenia gravis (MG) and experimental autoimmune MG (EAMG) are caused by T cell-dependent Ab-mediated autoimmune responses to acetylcholine receptors (AChRs) (1–3), in which neuromuscular transmission is impaired by loss of AChRs and disruption of postsynaptic membrane morphology (4, 5). Pathological autoantibodies are directed at extracellular domains of muscle AChRs, especially conformation-dependent epitopes, such as the main immunogenic region (MIR) (6). It is not known what causes the autoimmune response to AChRs in MG. EAMG can be induced by immunization with AChRs from fish electric organs or mammalian muscle or by the MIR sequences in a chimera with acetylcholine binding protein (AChBP) that preserve the native conformation of the MIR (7–9). There is no cure for MG; it is treated with acetylcholinesterase inhibitors (with modest efficacy in improving neurotransmission) and nonspecific immunosuppressants (whose beneficial effects may be delayed for months and can cause severe side effects) (2, 10). Although current treatments for MG can help most patients to achieve clinical remission, a small, but important, proportion of MG patients do not tolerate, or are resistant to, the current treatments (11). There is no specific immunosuppressive therapy (12, 13).

An Ag-specific therapeutic vaccine for MG could avoid side effects of nonspecific immunosuppressive drugs, such as infections and malignancies (13). Therapeutic vaccines using AChR extracellular domain sequences that form epitopes for pathological autoantibodies risk provoking autoimmunity rather than suppressing it (14). Bacterially expressed human $\alpha 1$ subunit extracellular domain 1–210 can induce EAMG (15). Oral administration of human $\alpha 1$ 1–205 extracellular domain peptide suppressed development of EAMG, but administering $\alpha 1$ 1–210 exacerbated EAMG as a result of renaturation of the MIR (16). Removing two major B cell epitopes from the human $\alpha 1$ 1–210 fragment converted the pathogenic fragment into a therapeutic one (17); however, any extracellular Ab epitope is potentially pathogenic (18).

Cytoplasmic domains of the AChR are not accessible to autoantibodies in intact muscle (Fig. 1A). Previously, we reported that a therapeutic vaccine consisting of bacterially expressed cytoplasmic domains of human muscle AChR $\alpha 1$, $\beta 1$, γ , δ , and ϵ subunits was more potent at suppressing EAMG than was a vaccine with both extracellular and cytoplasmic domains, because it excluded pathogenic epitopes (19). However, previous studies were limited to the prevention of chronic EAMG by starting the therapy after acute EAMG and before the onset of chronic EAMG. There are large changes in immune status during the development of chronic EAMG. Autoantibody concentrations are small before the onset of chronic EAMG, but they increase >20-fold during chronic EAMG (20). A therapy that possesses immunosuppressive capacity when started before the development of chronic EAMG may exhibit delayed or no efficacy when the autoimmune cells are fully activated and long-lived plasma cells have matured. Treating pre-existing chronic EAMG is most relevant to treating MG. The ideal therapy would be both Ag-specific and provide long-term or permanent protection against relapse. In this study, we show that vaccination with the therapeutic Ag in adjuvant rapidly suppresses established chronic EAMG and provides long-term, possibly permanent, resistance to the reinduction of EAMG. This approach could provide the long-awaited “cure” for MG.

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Abbreviations used in this article: AChBP, acetylcholine binding protein; AChR, acetylcholine receptor; α Bgt, α bungarotoxin; EAMG, experimental autoimmune MG; MG, myasthenia gravis; MIR, main immunogenic region; Treg, regulatory T cell.

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Materials and Methods

Induction of EAMG

Eight-week-old female Lewis rats (Charles River, Wilmington, MA) were used according to principles outlined in the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*. Studies were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. EAMG was induced by a single s.c. injection of purified *Torpedo californica* AChR emulsified in TiterMax adjuvant (CytRx, Norcross, GA) at the base of the tail (20). Weakness was graded from 0 to 4 as follows: 0, healthy normal rats with no signs of EAMG; 1, mildly decreased activity and weak grip with fatigability; 2, weakness, hunched posture at rest, decreased body weight, and tremor; 3, severe generalized weakness, marked decrease in body weight, moribund; 4, death (21). The rapidly progressing chronic weakness of EAMG that is induced by *Torpedo* AChR can be lethal, because of significant dehydration and body weight loss that is a result of muscle weakness and the resulting problems with eating and drinking, before therapy can inhibit and reverse the pathology. A soft dietary supplement (DietGel 76A; ClearH₂O, Portland, ME) was provided during the chronic phase of EAMG to minimize inhibition of eating and drinking due to muscle weakness, allowing more time for the therapy to work before the weakness became lethal.

Ag preparation

Two contiguous cytoplasmic domain constructs, containing the large cytoplasmic domains of human muscle AChR β 1, α 1, and γ subunits and those of human muscle AChR ϵ , α 1, and δ subunits, were bacterially expressed and chromatographically purified, as described previously (19). Together, these two constructs reflect the order of subunits around the ion channel (Fig. 1A). A human MIR chimera α 1 (1-32, 60-81)/AChBP (MIR/AChBP) was expressed in tissue culture and chromatographically purified, as described previously (6).

mAbs

All subunit-specific mAbs used in this study were characterized and described previously (22). mAb 35 and mAb 210 are directed at the MIR of the AChR α 1 subunit. All mAbs were purified from tissue culture supernatants by protein G affinity chromatography.

Ab assay

Abs to AChRs and to the MIR were measured by immunoprecipitation of 1 nM [¹²⁵I]- α bungarotoxin (α Bgt)-labeled AChRs and [¹²⁵I]-MIR/AChBP chimera, respectively. Serially diluted rat sera were incubated with [¹²⁵I]- α Bgt-labeled AChRs or [¹²⁵I]-MIR/AChBP chimera overnight at 4°C, followed by precipitation using goat anti-rat IgG for 3 h at room temperature. Immediately before centrifugation, samples were diluted to 1 ml with 0.5% Triton X-100 in PBS (pH 7.4). The pellets were washed with 1 ml 0.5% Triton X-100 in PBS prior to a second centrifugation. The washed pellets were assayed by gamma counting. Titers were expressed as nmol toxin-binding sites bound or nmol chimera bound/l serum (nM). All measurements were in triplicate.

ELISA

Abs to the therapeutic AChR cytoplasmic domains were assayed by ELISA, as described previously (19). Microtiter plates (Corning, Lowell, MA) were coated overnight at room temperature with 100 μ l of the constructs (20 μ g/ml in 0.1 M sodium carbonate buffer [pH 9.6]). After blocking with 3% (w/w) BSA and washing, 100 μ l serially diluted rat sera was added for 2 h at 37°C. Bound Abs were detected using biotinylated goat anti-rat IgG and HRP-labeled streptavidin (Kirkegaard & Perry Laboratories, Gaithersburg, MD). HRP activity was measured with a QuantaBlu Fluorogenic Peroxidase Substrate Kit (Pierce, Rockford, IL). Titer is defined as the serum dilution that gives half-maximal binding. All measurements were in triplicate.

IgG isotyping

Anti-MIR IgG isotypes were assayed as previously described, with minor modifications, using microtiter plates (Corning) coated with 100 μ l human MIR/AChBP chimera (20 μ g/ml in 0.1 M sodium carbonate buffer [pH 8.8]) (19). After blocking with 3% BSA and washing, rat sera diluted with normal rat serum to a final anti-MIR titer of 0.3 nM were added and incubated overnight at 4°C. After three washes, HRP-conjugated goat anti-sera to rat IgG isotypes (Bethyl Laboratories, Montgomery, TX), diluted 1/1000 in PBS (pH 7.4), were added for 4 h at 37°C. Bound Abs were detected using the QuantaBlu Fluorogenic Peroxidase Substrate Kit (Pierce). All measurements were in triplicate.

Antigenic modulation

Antigenic modulation of AChRs on the H9c2 cell line (ATCC CRL1446), a rat cell line that expresses muscle AChR, was assayed by incubation with 2 μ l rat sera overnight in 24-well tissue culture plates (Corning) in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Antigenic modulation, measured as loss of [¹²⁵I]- α Bgt binding sites, was determined after 3 h of incubation with 5 nM [¹²⁵I]- α Bgt (23). Background radioactivity was determined using a 100-fold excess of unlabeled α Bgt. Normal rat serum was used as a negative control to determine the total amount of surface AChRs. All measurements were in triplicate. The percentage loss of surface AChR by antigenic modulation was calculated as $(1 - \Delta\text{cpm in the presence of antisera}/\Delta\text{cpm in the presence of normal serum}) \times 100$.

Passive transfer of EAMG

Pooled sera, purified IgGs, or mAbs to the MIR were injected i.p. into 8-wk-old female Lewis rats (Charles River) at time 0. Rats were examined every 12–24 h for weight loss, muscular weakness, and fatigability and scored as described above.

Complement consumption

Serum samples were exposed to 56°C for 6 min to inactivate contaminating complement prior to use. Normal rat serum was used as a homologous complement source to estimate the complement-activating capacity of Ag–Ab complexes. Serial dilutions of serum samples were mixed with a constant concentration of excess MIR/AChBP chimera and incubated at 4°C overnight. Thereafter, equal volumes of diluted normal rat serum were added. The mixture was incubated at 37°C for 60 min, and residual complement activity was determined using the Complement CH50 Assay Kit (HaemoScan, Groningen, The Netherlands), according to the manufacturer's instructions. Complement activity consumed by immune complexes formed between MIR/AChBP chimera and anti-MIR Abs was expressed as the percentage of the available activities in normal rat serum. All measurements were in triplicate.

Statistics

Differences between two groups were assessed with the unpaired Student *t* test using a two-tailed distribution. All results are given as mean $\pm$ SEM. Results were considered statistically significant at $p < 0.05$.

Results

Therapeutic vaccine induced autoantibodies to cytoplasmic domains of rat muscle AChR but did not cause EAMG

The purity of the two cytoplasmic domain components of the therapeutic vaccine was verified by SDS-PAGE (Fig. 1B). The two cytoplasmic domain constructs were combined at a 1:1 ratio (by weight) for use in the following experiments.

Immunization of rats with 0.5 mg of cytoplasmic domains in TiterMax adjuvant, followed by three boosts in IFA at 3-wk intervals, resulted in high titers of Abs to rat muscle AChR (243 nM) but no signs of muscle weakness (mean clinical score 0 throughout the experiment and no loss of weight due to weakness). Lack of cross-reaction with the MIR/AChBP chimera indicates that Abs are directed only against pathologically irrelevant epitopes on the cytoplasmic domains of rat muscle AChR. IgG Abs were purified by protein G Sepharose affinity chromatography from pooled serum of these rats. Three rats/group were injected i.p. with 1 ml of purified IgG (8 mg/ml) containing 186 pmol of autoantibodies to rat muscle AChR at time 0 and examined every 12–24 h for weight loss, muscular weakness, and fatigability (Fig. 1C). A total of 8 mg of IgG purified from normal rat serum was used as a negative control, and 113 pmol of mAb 35, a mAb to the MIR, was injected as a positive control. Autoantibodies from rats immunized with the AChR cytoplasmic domains did not passively transfer EAMG. IgG purified from these rats failed to cause antigenic modulation of AChRs on H9c2 cells, a rat cell line that expresses muscle AChR. These results support the conclusion that immunization with AChR cytoplasmic domains does not induce pathological autoantibodies.

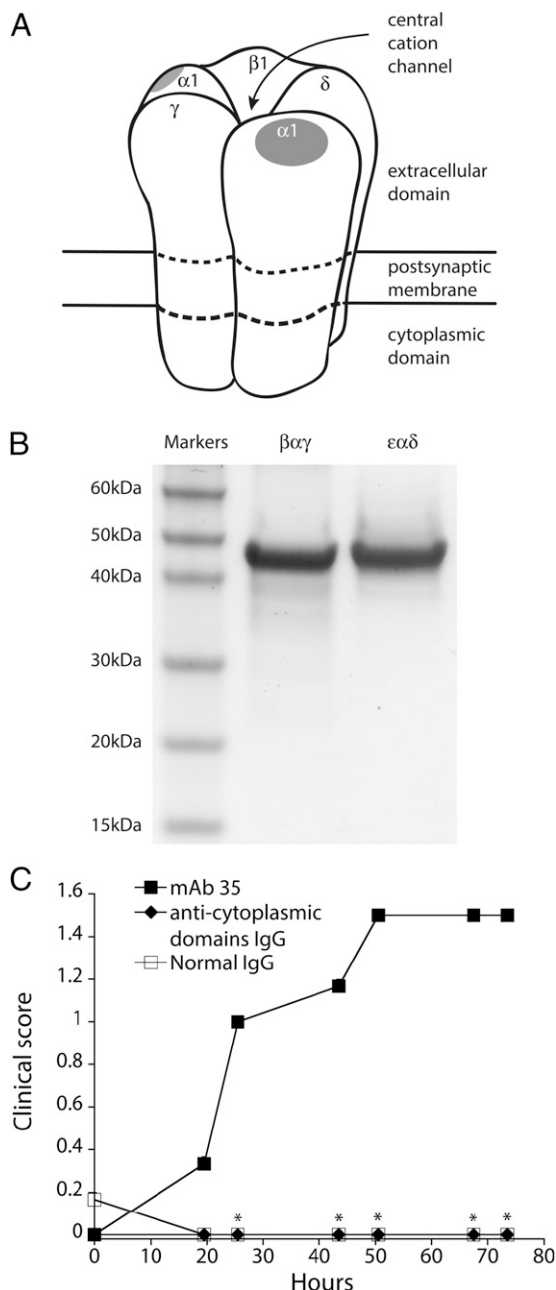


FIGURE 1. Abs to AChR cytoplasmic domains did not passively transfer EAMG. **(A)** Structure of skeletal muscle AChRs (46). Five homologous subunits are organized like barrel staves around a central cation channel whose opening is triggered by binding of acetylcholine to sites located in the extracellular domain at $\alpha 1/\delta$ and $\alpha 1/\gamma$ subunit interfaces. Before innervation and after denervation, γ is replaced by ϵ . The areas in gray highlight the MIR, which is located at the extracellular tip of $\alpha 1$ subunits and is the target of at least half of the autoantibodies to muscle AChRs in human MG and rat EAMG (47). **(B)** The two cytoplasmic domain proteins (5 μ g each) were resolved by 10% SDS-PAGE and stained with SimpleBlue (Invitrogen, Carlsbad, CA). **(C)** IgG Abs purified from rats repeatedly immunized with the cytoplasmic domains of human AChRs did not passively transfer EAMG. Data represent the mean $\pm$ SEM ($n = 3$). The error bars (SEM) are too small to be seen. $*p < 0.02$ versus rats injected with mAb 35.

Adjuvants greatly enhanced therapeutic potency

The efficacy of therapeutic vaccination with cytoplasmic domains at inhibiting the onset of chronic EAMG was greatly increased by adjuvants. All rats, with the exception of adjuvant controls that received an equal volume of PBS emulsified in TiterMax adjuvant,

were immunized with 70 μ g of *Torpedo* AChR emulsified in TiterMax at day 0. After the acute phase of EAMG, these rats were grouped so that both clinical score and body weight distributions were similar between groups, and mean clinical score and average body weight of each group were nearly equal. EAMG control rats received no treatment. Therapy was initiated after the acute phase, 14 d after the induction of EAMG. Therapy consisted of s.c. injection of 1 mg of therapeutic vaccine in adjuvants at four sites at the base of the tail every other week for 6 wk (Fig. 2A). As a comparison, a group of rats received 5 mg/dose i.p. in saline once a week for 5 wk. This was the most effective dose and route of vaccination in our initial report showing that AChR cytoplasmic domains were more effective therapeutically than was a combination of extracellular and cytoplasmic domains (19). Seven weeks after EAMG induction, all six untreated EAMG rats were weak, and five died from EAMG (mean clinical score 3.7) (Fig. 2B). In the group treated with 1 mg of therapeutic vaccine in IFA, two of six rats showed no weakness, and only one died (mean clinical score 1.8). In the group treated weekly with 5 mg in saline i.p., two of six rats did not show clinical signs of EAMG, and two died (mean clinical score 1.7). One milligram of therapeutic vaccine in IFA every other week for 6 wk (3 mg total) was as effective as 5 mg in saline weekly for 5 wk (25 mg total). Thus, IFA increased potency by 8-fold for suppressing development of chronic EAMG.

Treatment in IFA reduced the titer of pathological autoantibodies to the MIR/AChBP chimera 5-fold (Fig. 2C). Using TiterMax adjuvant for the first treatment was nearly as effective as using IFA for reducing weakness, but it was less effective at reducing the Ab titer to the MIR/AChBP chimera. Using TiterMax for every therapeutic injection was much less effective. Treatment with the therapeutic vaccine in either IFA or TiterMax increased Abs to native rat muscle AChR 5-fold (Fig. 2D). Most autoantibodies to rat muscle AChR in treated rats are directed at pathologically irrelevant cytoplasmic epitopes, because the titer to the MIR/AChBP chimera in these rats was reduced by at least half with respect to untreated EAMG (Fig. 2C). The amount of Abs to the MIR was positively correlated with severity of weakness, whereas the amount of Abs to rat muscle AChR, including primarily Abs to cytoplasmic domains, was negatively correlated with weakness.

Optimizing vaccine dose and immunization schedule prevented development of chronic EAMG

The efficacy of specific immunosuppressive therapy at inhibiting the onset of chronic EAMG was improved by optimizing the dose and schedule of therapeutic vaccine in IFA. All rats, with the exception of adjuvant controls, were immunized with 70 μ g of *Torpedo* AChR in TiterMax at day 0. After the acute phase, these rats were grouped so that both clinical score and body weight distributions were similar between groups, and mean clinical score and average body weight of each group were nearly equal. Treatment of EAMG consisted of six s.c. doses of the therapeutic vaccine once weekly (0.25, 0.5, or 1 mg/dose) or three s.c. doses of 1 mg of the therapeutic vaccine, once every 2 wk, starting on day 14 (Fig. 3A). EAMG control rats received equal volumes of an emulsion of PBS and IFA weekly. Each group consisted of six rats.

The therapeutic benefit was dose dependent; 1 mg in IFA at weekly intervals between days 14 and 49 (6 mg total) was the most beneficial and eliminated chronic weakness almost completely (Fig. 3B). Five of six rats showed no clinical signs of EAMG. One rat was transiently affected (peak clinical score 1). Thirteen weeks after disease induction, all six rats were healthy. In contrast, six untreated EAMG rats given IFA only were extremely weak, and four died by 12 wk (mean clinical score 3.6 at day 70). Doses lower than 6×1 mg of vaccine also were beneficial. Overall, only

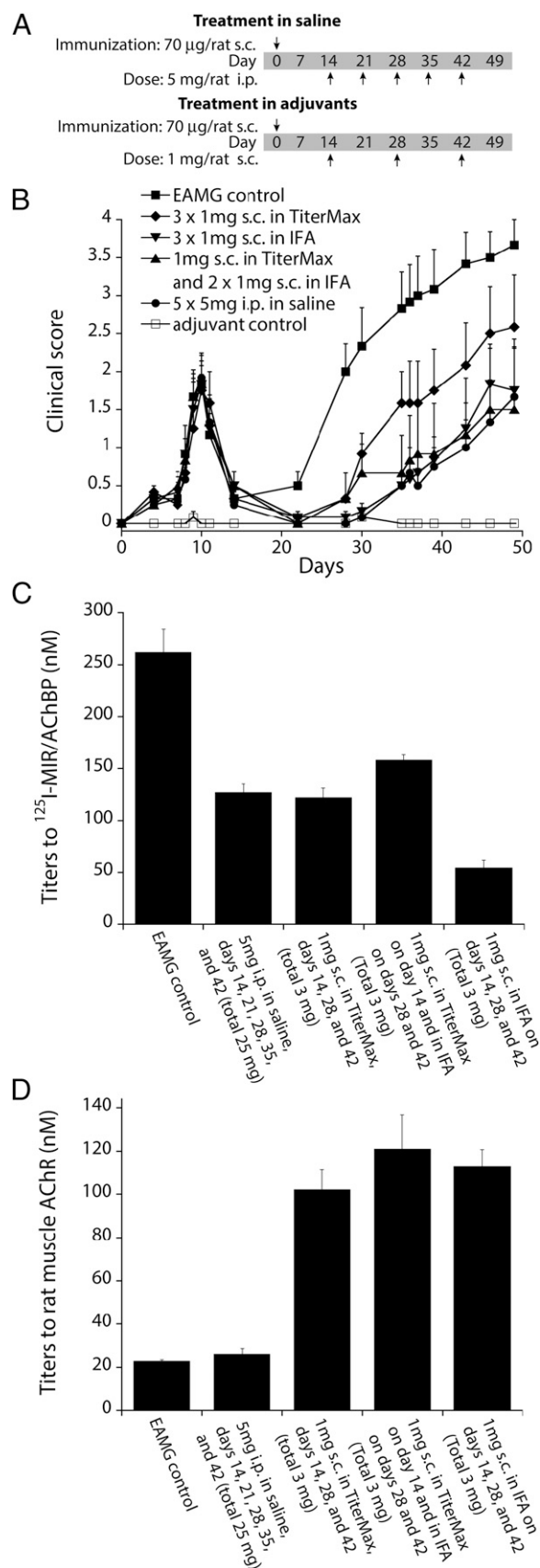


FIGURE 2. Potency of the therapeutic vaccine was greatly increased by emulsion with IFA and changing the route of administration. **(A)** Immunization and treatment scheme. **(B)** Mean clinical scores of groups of rats injected i.p. with 5 mg in saline or injected s.c. with 1 mg in IFA were significantly lower than those of EAMG control rats at all time points after

5 of 24 treated rats from all four treatment groups died from EAMG by 12 wk.

Most of the surviving rats were retained for studies of the long-term effects of therapy. Fourteen weeks after induction of EAMG, two untreated EAMG rats and four rats treated with low doses that still showed signs of weakness were euthanized for ethical reasons. The remaining 15 rats were maintained for another 16 wk. Three rats were euthanized because of infection unrelated to EAMG. All remaining rats continued without signs of weakness for an additional 23 wk after the last therapeutic dose.

Treatment diverted autoantibody specificities away from pathological extracellular epitopes like the MIR

Therapy initiated immediately after the acute phase decreased Abs to the MIR 2 wk after the first dose (Fig. 3C, 3D). One day after the last therapeutic dose (day 50), the most effective treatment (1 mg in IFA weekly between days 14 and 49) reduced the Ab titer to the MIR by a factor of eight relative to untreated EAMG. Even 6 wk later, the level of Abs to the MIR in these rats was only 20% of that in untreated EAMG rats. All therapeutic doses reduced Ab titer to *Torpedo* AChR by half on day 91 (Fig. 3E).

Vaccination increased the titer to therapeutic human AChR cytoplasmic domains by up to 100-fold (Fig. 3F). These non-pathological Abs also increased the titer to rat muscle AChR (Fig. 2D). Six weeks later, the titer to cytoplasmic domains in these rats was still 50-fold that of untreated EAMG rats.

Successfully treated rats were resistant to reinduction of EAMG

To determine whether treatment that prevented the onset of chronic EAMG protected rats from relapse of EAMG, we tried to reinduce EAMG in successfully treated rats by immunizing them again with *Torpedo* AChR in TiterMax. Twenty-three weeks after the last therapeutic dose, 12 treated rats from Fig. 3 were randomly divided into two groups. One group was reimmunized with 35 μ g of *Torpedo* AChR in TiterMax. The other group received an equal volume of PBS emulsified in TiterMax adjuvant. Six rats from the adjuvant control group in Fig. 3 were immunized with 35 μ g of *Torpedo* AChR to form the EAMG control group (Fig. 4A). Treated rats were resistant to attempts to reinitiate EAMG by immunization with *Torpedo* AChR (Fig. 4B). Only two of six treated rats developed muscle weakness after the reimmunization. The others remained healthy throughout the experiment. The two weak rats did not have a typical biphasic course of EAMG. One of the weak rats died at day 21, and the other rat was moderately weak at that time (clinical score 2 at day 21) and recovered 2 wk later. Control rats of the same age were severely affected and

day 28 (both $p < 0.01$). Mean clinical scores of the rats given s.c. injections first in TiterMax and then in IFA were lower than those of the EAMG control rats, but the difference was significant only at the 0.1 level ($0.1 > p > 0.05$ after day 28). Mean clinical scores of the rats given every injection in TiterMax were lower than those of EAMG control rats, but the difference was smaller than for the other treatments ($0.2 > p > 0.05$ after day 28). Data represent the mean $\pm$ SEM ($n = 6$). **(C)** Equal amounts of sera from individual rats from six groups were pooled 7 wk after the induction of EAMG. Ab titer to the MIR was evaluated as described in *Materials and Methods*. Treatments reduced Ab titer to the MIR by approximately half, with the exception of weekly treatment with 1 mg/dose in IFA, which reduced titer to the MIR by a factor of 5. **(D)** Ab titer to rat muscle AChR was evaluated as described in *Materials and Methods*. Treatment with a 5-mg dose in saline had little effect on autoantibody titer to rat muscle AChR, whereas all treatments with the vaccine emulsified in adjuvants significantly increased autoantibody titers by $\sim$ 5-fold relative to untreated EAMG.

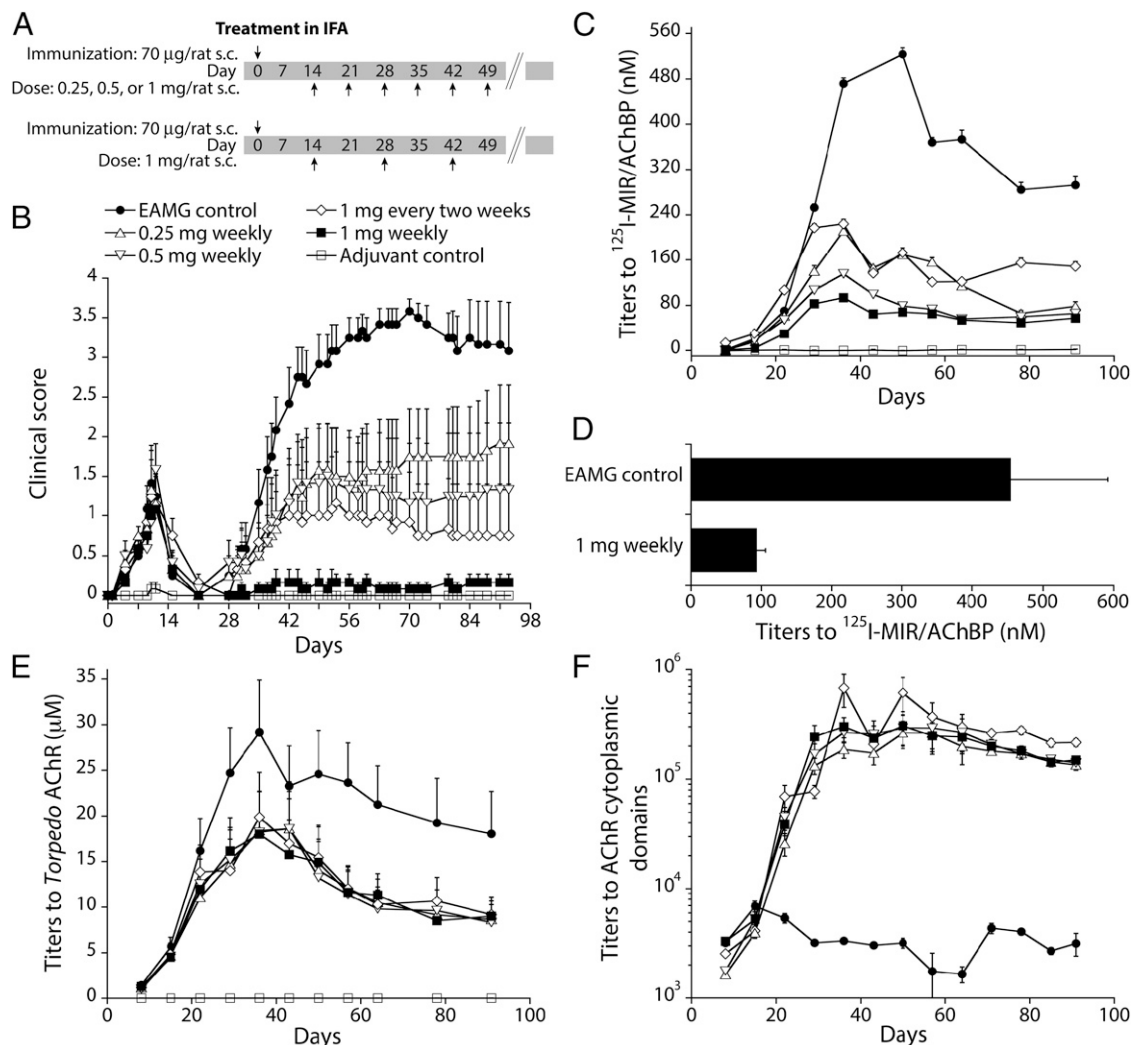


FIGURE 3. Dose and schedule for specific immunosuppression using the therapeutic vaccine in IFA were optimized further. **(A)** Immunization and treatment scheme. **(B)** Mean clinical scores of the rats treated with 0.25 or 0.5 mg weekly were significantly lower than were those of the untreated EAMG rats at all time points after day 56 ($p < 0.05$). Mean clinical scores of the rats treated with 1 mg every other week were even lower ($p < 0.05$ after day 44). Treatment with 1 mg in IFA weekly eliminated chronic weakness almost completely ($p < 0.001$, versus untreated EAMG rats after day 42). Data represent the mean $\pm$ SEM ($n = 6$). **(C)** Equal amounts of sera from individual rats from six groups were pooled weekly after the induction of EAMG. Ab titer of the pools to the MIR/AChBP chimera was evaluated as described in *Materials and Methods*. No cross-reaction was detected with wild-type AChBP. Some error bars are too small to be seen. All treatments substantially suppressed the production of Abs to the MIR 3 wk after their initiation. Treatment with 1 mg weekly in IFA was the most effective. **(D)** Sera of individual rats at day 36 were assayed for Abs to the MIR. Treatment with 1 mg weekly in IFA significantly reduced the production of Abs to the MIR ($p < 0.05$, versus untreated EAMG rats). **(E)** Ab titer of the pools to *Torpedo* AChR was evaluated as described in *Materials and Methods*. All doses of therapeutic vaccine suppressed Ab titer to *Torpedo* AChR similarly. **(F)** All doses of therapeutic vaccine rapidly increased Ab to AChR cytoplasmic domains to similarly high titers. The effect lasted for ≥ 6 wk after the last therapeutic dose.

developed typical biphasic EAMG, and five of six died after immunization with *Torpedo* AChR. Six previously treated rats used as adjuvant controls persisted with little or no detectable weakness.

Successfully treated rats were as susceptible to passive transfer of EAMG by Abs as were normal rats

It was reported that rats that recovered from passive-transfer EAMG by autoantibodies to AChR became refractory to induction of EAMG by a second injection of autoantibodies (8). It was suggested that resistance to repeated passive transfer of EAMG resulted from a low AChR amount or density in the postsynaptic membrane, which was insufficient to initiate further AChR degradation. If our previously treated rats were resistant to reinduction of EAMG for this reason, these rats also should be resistant to passive transfer of EAMG by injection of a mAb to the MIR. Three previously treated and then reimmunized rats, three previously treated rats from the adjuvant control group in Fig. 4B, and

three normal rats at the same age were given 740 pmol/100 g body weight of anti-MIR mAb 210 i.p. Three normal rats at the same age were injected i.p. with normal rat IgG as a negative control. Unlike young rats in which acute weakness usually peaks at day 2 after injection and clears within 1 wk, these older rats showed signs of weakness after day 3 and stayed weak for > 1 wk. All rats given mAb 210 developed acute EAMG of similar severity (Fig. 4C). Because previously treated rats were as susceptible to passive EAMG induced by mAb 210 as were normal rats, it is unlikely that the changes in AChR amount or density in their neuromuscular junctions caused their resistance to reinduction of EAMG.

Treated rats developed no weakness after reimmunization with *Torpedo* AChR but made autoantibodies with pathological specificities

Reimmunization of treated rats with *Torpedo* AChR increased Abs to both *Torpedo* AChR and the MIR but not the therapeutic

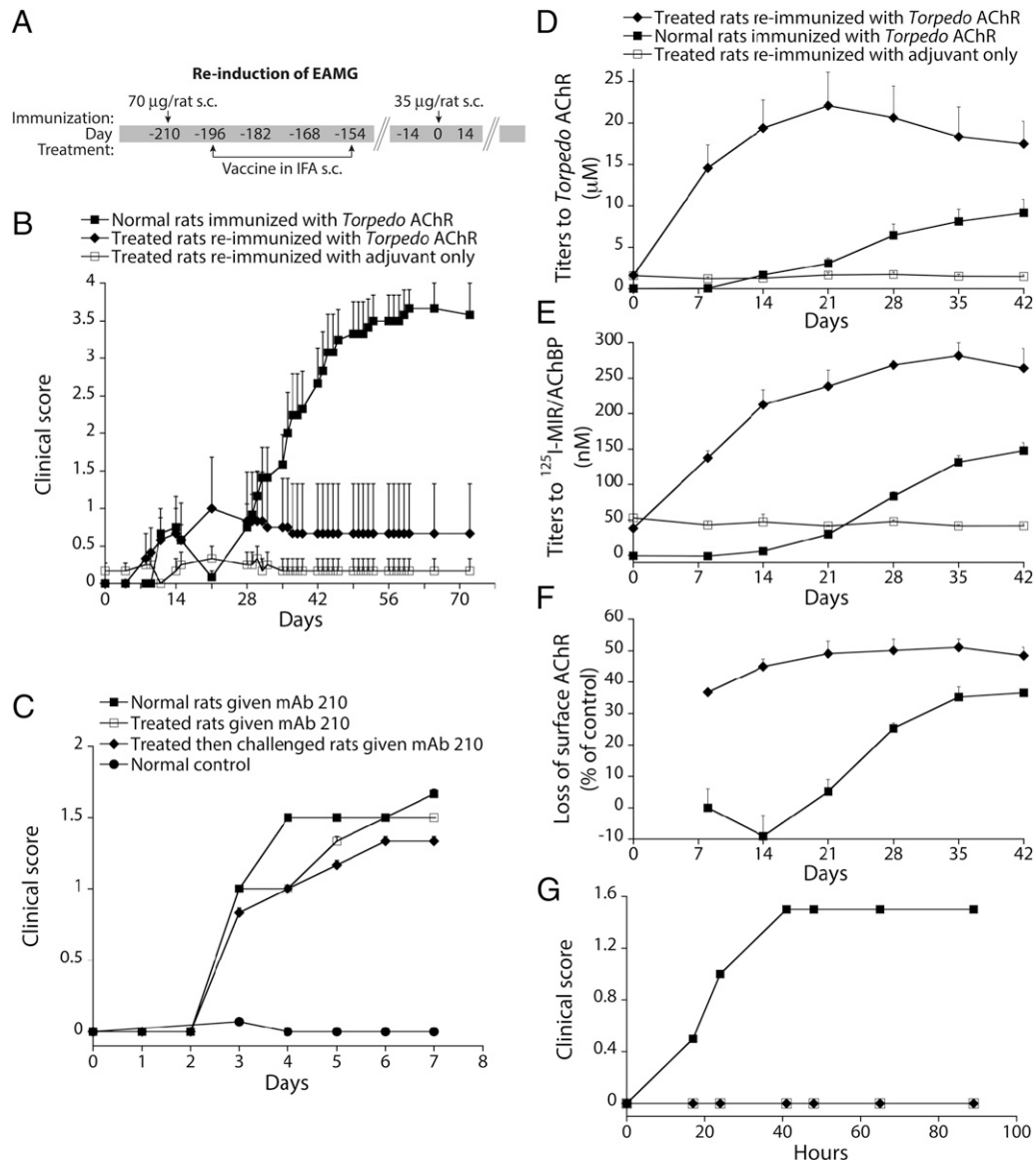


FIGURE 4. Previously treated rats were resistant to reinduction of EAMG. **(A)** Immunization scheme. **(B)** The mean clinical scores of the rats previously treated and then reimmunized with *Torpedo* AChR were significantly lower compared with those of the EAMG control rats at all time points after day 42 ($p < 0.05$ between days 42 and 46, $p < 0.01$ thereafter). Data represent the mean $\pm$ SEM ($n = 6$). **(C)** All three groups of rats receiving mAb 210 were affected similarly ($p > 0.37$). Data represent the mean $\pm$ SEM ($n = 3$). The error bars (SEM) are too small to be seen. **(D)** Equal amounts of sera from individual rats from three groups were pooled weekly after immunization. Ab titer to *Torpedo* AChR was evaluated as described in *Materials and Methods*. Reimmunization with *Torpedo* AChR induced a large increase in Ab titer to the immunogen in the previously treated rats immediately after reimmunization. **(E)** Ab titer to the MIR was evaluated as described in *Materials and Methods*. Time course of Ab titer to the MIR paralleled that of titer to *Torpedo* AChR. **(F)** Two microliters of rat sera were incubated with H9c2 cells and then antigenic modulation, measured as loss of α Bgt binding sites, was determined. Sera from rats treated for EAMG and then immunized again with AChR were more potent at causing antigenic modulation. **(G)** Passive transfer of EAMG was tested using pooled serum aliquots (250 μ l/rat). Data represent the mean $\pm$ SEM ($n = 3$). The error bars (SEM) are too small to be seen. Sera from the rats previously treated and then reimmunized with *Torpedo* AChR caused no weakness, whereas those from EAMG control rats did ($p < 0.05$). Rats injected with sera from the adjuvant control rats showed no weakness, as expected.

vaccine. Twenty-three weeks after the last therapeutic dose, previously treated rats, which had completely recovered, still had Abs to *Torpedo* AChR (~ 1.6 μ M) and to the human MIR (~ 40 nM). Titer to the therapeutic vaccine decreased by a factor of four relative to 23 wk before and remained unchanged after reimmunization with *Torpedo* AChR. Rats developed a strong Ab response to *Torpedo* AChR immediately after reimmunization (Fig. 4D). Ab to *Torpedo* AChR reached a peak of 22 μ M on day 21. By contrast, control rats of the same age developed a modest response 2 wk after immunization, and Ab to *Torpedo* AChR was only 3 μ M by day 21. The time course of Ab titer to the MIR paralleled

that to *Torpedo* AChR (Fig. 4E). Ab to the MIR in previously treated rats was 8-fold higher than in normal rats by 21 d after immunization and was 2-fold higher on day 42. The rapid and strong immune response of the previously treated rats after reimmunization with *Torpedo* AChR suggests that these rats developed memory B and T lymphocytes after the first immunization with *Torpedo* AChR.

Abs in these sera that bind extracellular epitopes of rat muscle AChR were assayed by testing their ability to cause antigenic modulation of AChR on H9c2 cells. Sera from previously treated rats caused more antigenic modulation than did sera from control rats

(Fig. 4F). After reimmunization with *Torpedo* AChR, previously treated rats developed more Abs to the MIR than did control rats. However, despite the presence of autoantibodies with pathological specificities, only control rats developed severe EAMG.

Sera from treated rats could not passively transfer EAMG

The pathogenicity of autoantibodies in the sera of the previously treated rats reimmunized with *Torpedo* AChR was compared with that of the same age control rats initially immunized with *Torpedo* AChR by testing their abilities to passively transfer EAMG. Three rats/group were injected i.p. with pooled serum aliquots (250 μ l/rat) from the rats previously treated and then reimmunized with *Torpedo* AChR, containing 67 pmol anti-MIR Abs. Equal aliquots from the adjuvant control rats (containing 12 pmol anti-MIR Abs) and from the EAMG control rats (containing 24 pmol anti-MIR Abs) were injected as negative and positive controls, respectively. Sera from the previously treated rats were ineffective at passively transferring EAMG, whereas sera from the normal rats were effective (Fig. 4G). Autoantibodies from treated rats were less pathogenic, even though they contained more Abs to the MIR and caused more severe antigenic modulation on H9c2 cells than did those from normal rats. Thus, properties of Abs from treated rats, other than amount or specificity for pathologically significant epitopes, were changed.

Isotype profile of anti-MIR Abs was changed in treated rats after reimmunization with *Torpedo* AChR

AChR loss caused by complement-mediated focal lysis is thought to be more pathologically significant than is accelerated AChR turnover by Ab cross-linking (antigenic modulation) (5). Abs to the MIR from the rats initially immunized with *Torpedo* AChR were effective at fixing complement in response to binding MIR/AChBP chimera, whereas Abs from rats treated for EAMG and later reimmunized with *Torpedo* AChR were not (Fig. 5A). The pathogenicity of Abs depends on their ability to bind to AChRs in muscle and recruit complement. Not all IgG subclasses fix complement equally well. IgG2b has the greatest capacity, IgG2a is less efficient, and IgG1 has little capacity to fix complement (24). This reflects properties of the Fc region of IgG that are characteristic of each isotype. Analysis of isotypes of Abs to the MIR revealed that IgG1 (regulated by Th2 cells in rats) grew from a minor fraction (3.5%) after initial immunization with *Torpedo* AChR to a major part (43.2%) in treated rats that were reimmunized with *Torpedo* AChR. IgG2b (regulated by Th1 cells in rats) went from a predominant isotype (71.2%) in rats initially immunized with *Torpedo* AChR to a minor isotype (19.2%) in treated rats after later reimmunization with *Torpedo* AChR (Fig. 5B). Thus, serum Abs from previously treated rats were less effective at fixing complement because of isotype switching.

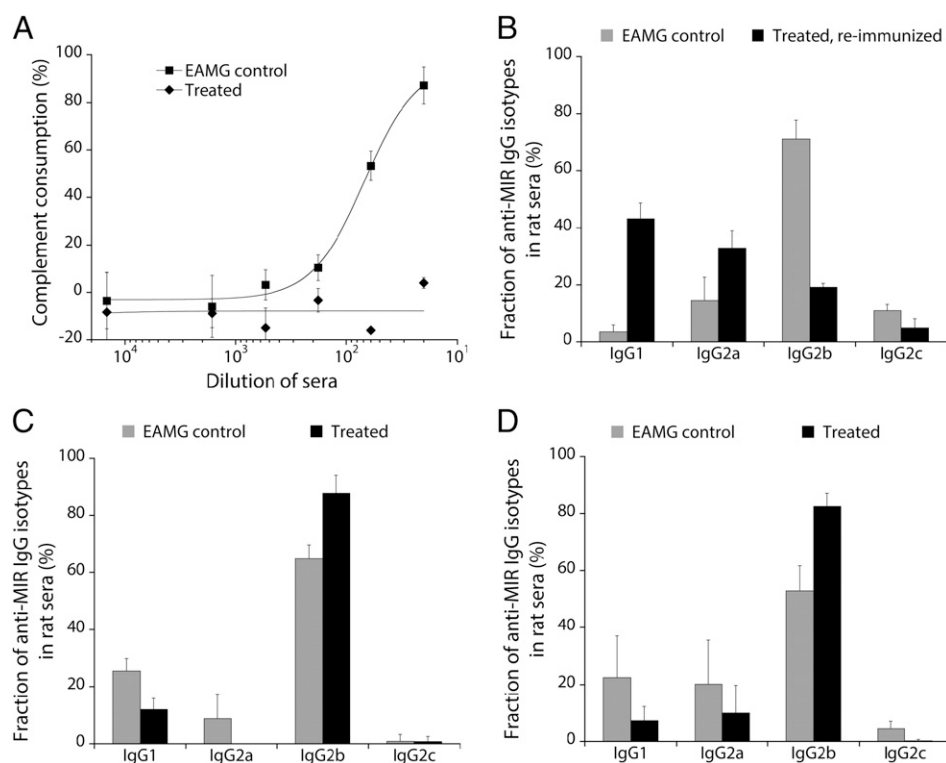


FIGURE 5. Serum Abs from previously treated rats were less effective at fixing complement because of isotype switching. **(A)** Sera (pooled sera used in Fig. 4G) were mixed with excess MIR/AChBP chimera to allow Ab binding and activation of complement, and then complement consumed was assayed. Serum Abs from the normal rats immunized with *Torpedo* AChR were effective at fixing complement. In contrast, serum Abs from the rats previously treated and then reimmunized with *Torpedo* AChR did not fix complement within the tested concentration range, although these sera contained 3-fold more anti-MIR Abs than did sera from the normal rats immunized with *Torpedo* AChR. **(B)** MIR/AChBP chimera was used as Ag to directly measure isotypes of Abs to pathologically significant extracellular epitopes. These are pooled sera used in Fig. 4G, collected 6 mo after initial immunization with *Torpedo* AChR with respect to Fig. 3, and after reimmunization with *Torpedo* AChR. After immunization with *Torpedo* AChR, normal rats primarily produced IgG2b Abs. In contrast, IgG2b Abs significantly decreased in the previously treated rats, whereas IgG1 Abs, which are regulated by Th2 cells and do not fix complement (24, 34), significantly increased after reimmunization with *Torpedo* AChR. **(C)** These are sera collected on day 36 in Fig. 3. Both EAMG control rats and rats treated with 1 mg of the therapeutic vaccine in IFA weekly primarily produced IgG2b Abs, which are regulated by Th1 cells and fix complement (24, 34). **(D)** These are sera collected on day 91 in Fig. 3. Six weeks after the last therapeutic dose, the IgG isotype profile of both groups remained similar.

To determine whether isotype switching occurred during or immediately after the previous therapy, the isotype profile of Abs to the MIR in the sera from both EAMG control rats and treated rats in Fig. 3 was analyzed. Unlike after reimmunization with *Torpedo* AChR, IgG2b was still the predominant isotype in treated rats (87.7% on day 36 and 82.5% on day 91), as well as in untreated EAMG rats (64.8% on day 36 and 52.9% on day 91) (Fig. 5C, 5D). Thus, isotype switching is not an immediate cause of the benefit of therapy but, rather, is a long-term effect of therapy.

Untreated rats that survived EAMG were not resistant to reinduction of EAMG

All untreated EAMG rats in Fig. 3 died from EAMG before reinduction of EAMG. Thus, the experiment of reinduction of EAMG (Fig. 4) did not include a group of untreated EAMG rats. This raises the question of whether the resistance of the previously treated rats to reinduction of EAMG was a result of previous treatment or a result of having survived EAMG. To answer this, 15 rats were immunized with 70 μ g *Torpedo* AChR to induce EAMG, and six rats of the same age received adjuvant only (Fig. 6A). These rats received three injections of an emulsion of PBS and IFA every other week starting at day 35 after immunization. All rats immunized with *Torpedo* AChR developed EAMG. Thirty weeks after the initial immunization, eight rats that survived EAMG were still mildly fatigable, but all had gained weight and muscle strength. Their remaining Ab titer to the MIR was $\sim$ 74 nM. Six of the surviving untreated EAMG rats were immunized again with 35 μ g *Torpedo* AChR. Two others received adjuvant only as a negative control. Six rats that previously received adjuvant were immunized with 35 μ g *Torpedo* AChR as an EAMG control. Unlike EAMG control rats of the same age, which developed typical biphasic EAMG after immunization with *Torpedo* AChR, untreated rats that had survived EAMG developed chronic EAMG only 1 wk after reimmunization with *Torpedo* AChR (Fig. 6B). All six rats were severely affected (mean clinical score 2.3), and one died from EAMG. Five of six rats previously given adjuvant and then immunized with *Torpedo* AChR developed clinical weakness (mean clinical score 2.4); two of them died. Thus, untreated EAMG rats did not show resistance to reinduction of EAMG. These rats developed a strong Ab response to the MIR immediately after reimmunization with *Torpedo* AChR. IgG2b-type Abs were the major part of anti-MIR Abs in these rats after reimmunization, just as after the initial immunization (Fig. 6C). Therefore, therapy, and not having survived EAMG, was responsible for shifting isotypes of Abs to the MIR, causing resistance to reinduction of EAMG in treated rats.

Treatment initiated during the chronic phase rapidly suppressed further development of chronic EAMG and returned most rats to normal

Treatment with the therapeutic vaccine in IFA before onset of the chronic phase of EAMG can prevent it (Fig. 3A). The challenge in treating MG is beginning therapy during the chronic phase. Unlike MG, which is usually of much longer duration and not as rapidly progressive, EAMG initiated with high doses of AChR usually produces rapidly progressing chronic weakness that is frequently lethal. Ab titers to *Torpedo* AChR and to the MIR reached their peaks at day 35 after the initial immunization (Fig. 3C, 3E).

Treatment of ongoing chronic EAMG with three doses of 2 mg of the therapeutic vaccine at 2-wk intervals was effective (Fig. 7A, 7B). All rats, except adjuvant controls, were immunized with 70 μ g of *Torpedo* AChR in TiterMax at day 0. All rats immunized with AChR were treated with 3 mg/kg/d of pyridostigmine in their drinking water; this drug is routinely used for symptomatic ther-

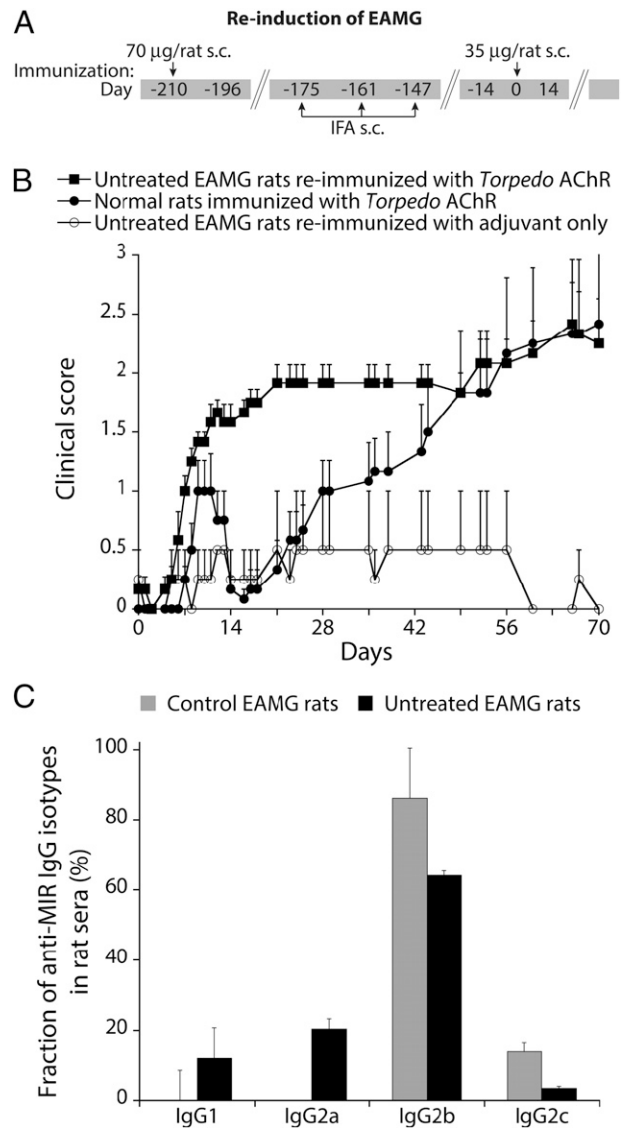


FIGURE 6. Untreated rats that survived EAMG rapidly developed EAMG again when reimmunized with *Torpedo* AChR. **(A)** Immunization scheme. **(B)** The two surviving EAMG rats given adjuvant had little or no weakness. Both the adjuvant control rats and the surviving EAMG rats were very weak after being immunized with *Torpedo* AChR. There was no significant difference in chronic EAMG between these two groups ($p > 0.5$ after day 49). Data represent the mean $\pm$ SEM. **(C)** Sera were collected 42 d after immunization. After immunization with *Torpedo* AChR, both normal rats and untreated EAMG rats primarily produced IgG2b Abs, which are regulated by Th1 cells and fix complement (24, 34).

apy in MG. The intent was to reduce weakness, thereby allowing more time for the treatment to work, and to accurately model how therapy would be used for MG. Immediately before starting treatment, the rats were grouped so that both clinical score and body weight distributions were similar between groups, and mean clinical score and average body weight of each group were nearly equal. Treatment of EAMG consisted of three 2-mg doses of the therapeutic vaccine in IFA every 2 wk starting on day 35 (6 mg total). EAMG control rats received s.c. injection of IFA at the same schedule. Relatively large (2 mg) doses were given with the intention of accelerating the therapeutic effect before EAMG became lethal. Before starting treatment, five of six rats to be treated were sick (mean clinical score 1.3), and four of six EAMG control rats were sick (mean clinical score 1.6). The effect of

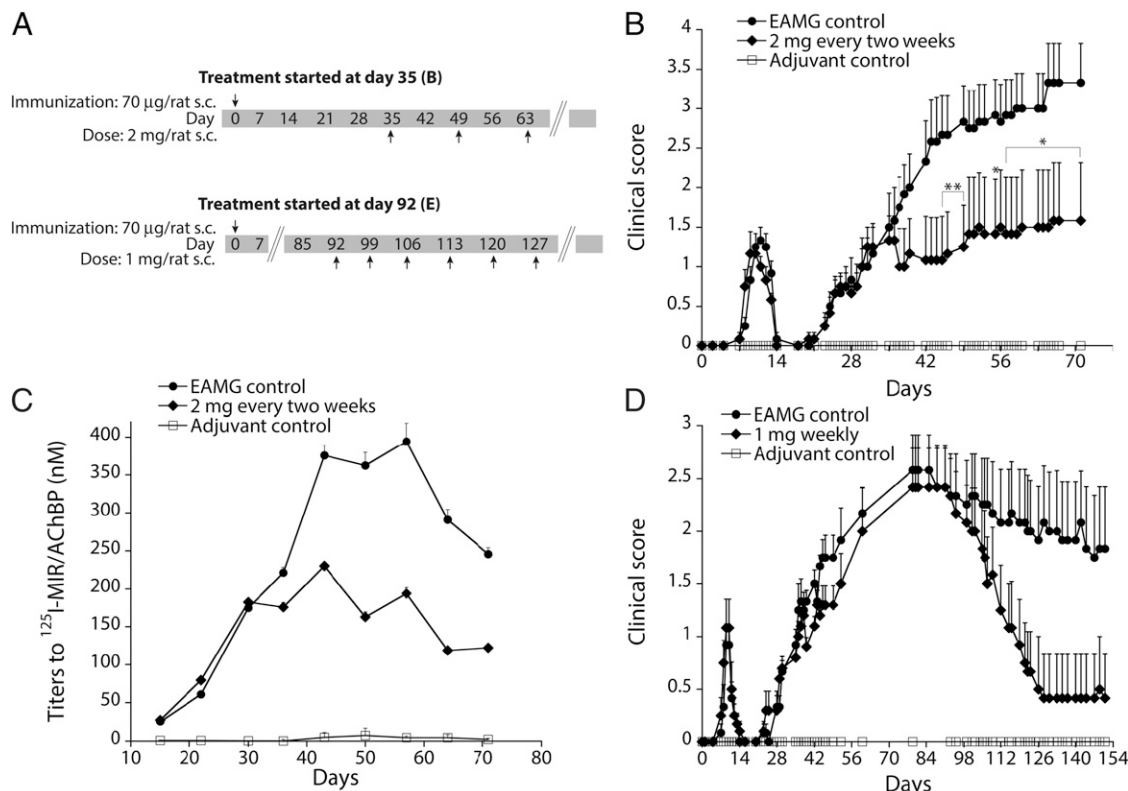


FIGURE 7. Treatments started during the chronic phase rapidly suppressed further development of chronic EAMG and completely reversed weakness in most rats. **(A)** Immunization and treatment scheme. **(B)** When treatments started on day 35, the mean clinical scores of the treated rats were significantly lower compared with those of the untreated EAMG rats at all time points after day 43. Data represent the mean $\pm$ SEM ($n = 6$). **(C)** Equal amounts of sera from individual rats from three groups were pooled weekly after the acute phase of EAMG. Ab titer of the pools to the MIR was evaluated as described in *Materials and Methods*. Treatments substantially reduced the titer to the MIR only 1 wk after the first therapeutic dose. **(D)** When treatments started on day 92, the mean clinical scores of the treated rats were significantly lower compared with those of the untreated EAMG rats at all time points after day 121 ($p < 0.1$ after day 121, $p < 0.05$ between days 128 and 142). Data represent the mean $\pm$ SEM ($n = 6$). * $p < 0.1$, ** $p < 0.05$.

treatment was rapid, but not as extensive as that achieved when it was started before the chronic phase. By the end of the experiment, all six untreated rats with EAMG were weak, and four rats were dead (mean clinical score 3.3), but three of six treated rats had returned to normal, and only one died (mean clinical score 1.6). The untreated rats lost an average of 29.8 ± 11.9 g during the treatment period. By contrast, the treated rats lost an average of 8.5 ± 6.9 g during the same period. Ab titers to the MIR were almost identical between the two groups before starting treatment. Treatment suppressed the production of Abs to the MIR by 1 wk after the first dose. By the end of the experiment, Ab titer to the MIR was reduced by half relative to that in the untreated rats with EAMG (Fig. 7C).

Treatment of established chronic EAMG was highly effective with six 1-mg doses of the therapeutic vaccine at 1-wk intervals (Fig. 7A, 7D). Twenty-one rats were immunized with 70 µg *Torpedo* AChR to induce EAMG and to provide a sufficient number of rats surviving long-term chronic EAMG, and six rats received adjuvant only. All rats immunized with AChR were treated with 3 mg/kg/d of pyridostigmine in their drinking water to provide symptomatic therapy for weakness. Thirteen weeks after the initial immunization, 14 rats (of the initial 21) survived EAMG. Twelve rats surviving EAMG were divided into two groups of six so that the clinical scores and the body weights of each group were similar. Before starting treatment, all rats in both groups were weak (mean clinical scores 2.4 in both groups). Six 1-mg doses of the vaccine in IFA were given s.c. weekly starting on day 92 (6 mg total). EAMG control rats received IFA s.c. at the same schedule. The effect of sustained lower-dose therapy was

rapid. After the last therapeutic dose, five of six treated rats recovered and exhibited no weakness, and only one was weak (mean clinical score 0.5). These treated rats gained an average of 32.2 ± 6.7 g over the 6-wk treatment period. By contrast, five of six untreated EAMG rats were still weak at that time (mean clinical score 1.9). These rats gained an average of 14.8 ± 6.0 g during the same period. Thus, six weekly 1-mg doses in IFA was the most effective dose amount and schedule for both completely preventing the development of chronic EAMG (Fig. 3B) and nearly completely suppressing long-established chronic EAMG (Fig. 7D).

Discussion

We developed a specific immunosuppressive therapy for EAMG with great potential for treating MG. We demonstrated that a therapeutic vaccine using cytoplasmic domains of human AChR $\alpha 1$, $\beta 1$, γ , δ , and ϵ subunits is a safe and powerful tool for suppressing established EAMG and preventing its relapse. Cytoplasmic epitopes should not be pathogenic because of the inaccessibility of the cytoplasmic domains of AChR under physiological conditions. In fact, the vaccine is safe; rats repeatedly immunized with the vaccine in adjuvants did not develop EAMG, although they produced Abs that can bind to solubilized native AChRs, and autoantibodies from these rats did not bind the MIR, passively transfer EAMG, or cause antigenic modulation. IFA greatly increased the potency and efficacy of the therapeutic vaccine. The benefits of therapy are long lasting and may even be permanent. Therapy for 6 wk starting before the onset of chronic EAMG prevented its development, protected the rats from EAMG for ≥ 6 mo afterward, and protected the rats against relapse after

a boost with *Torpedo* AChR 6 mo later. This therapeutic regimen initiated during chronic EAMG prevented its further development and caused rapid improvement in most rats. Such effective therapy of chronic EAMG and prevention of relapse are unprecedented. Its efficacy and safety recommend this therapeutic vaccine for further development in preparation for clinical testing as therapy for MG.

Most serum autoantibodies to AChR are produced by long-lived plasma cells residing in spleen and bone marrow. These terminally differentiated cells are resistant to most immunosuppressive drugs (10, 25). Thus, in MG there is typically a delay of up to 18 mo between initiation of immunosuppressive therapy and patient improvement. Ab titer is low 2 wk after induction of EAMG, when populations of plasma cells are minimal. Suppression of B cell differentiation would prevent further production of plasma cells. Treatment with 1-mg doses in IFA weekly starting at that time prevents muscle weakness. Even without treatment, Ab titer decreases gradually after it peaks 35 d after induction, and spontaneous remissions occur ~90 d after induction, if rats have not died from EAMG. However, unlike treated rats, untreated rats that survive EAMG do not achieve a complete remission. This suggests that the B cell proliferation rate could be significantly decreased 90 d after induction, and remaining long-lived plasma cells could play a crucial role in maintaining the Ab level at that time. Treatment starting at day 92 rapidly reversed existing weakness. This suggests that our therapy also could inhibit or apoptose plasma cells. Apoptosis of plasma cells can be induced by Foxp3⁺ regulatory T cells (Tregs) or by cross-linking FcγRIIb on the plasma cells by immune complexes (26, 27). These mechanisms could explain the rapid effects of our therapy. This property of therapy provides another clinically meaningful advantage over existing immunosuppressive therapy. Unlike MG, which is usually much longer in duration, EAMG induced with high doses of AChR usually produces rapidly progressing chronic weakness that is often fatal. Most deaths occurred between 5 and 11 wk after induction. The observed resistance to therapy in some rats might be a result of irreversible damage caused by rapidly progressing extensive weakness. This adverse characteristic may not extend to human therapy.

Drachman et al. (28) tried to eliminate the autoimmune response in both EAMG and MG and provide long-term benefit by largely ablating the existing immune system with high-dose cyclophosphamide. They found that some memory lymphocytes survived the treatment and were capable of responding to Ag challenge. Thus, follow-up conventional immunotherapy would be required to prevent relapse in treated patients. Our Ag-specific immunotherapy resulted in rapid and durable improvement without dramatically altering the entire immune system. When reimmunized with *Torpedo* AChR, treated rats developed an anamnestic Ab response that was similar to that in untreated EAMG rats. However, unlike untreated EAMG rats, which developed a Th1-regulated Ab response, treated rats primarily produced Th2-regulated Abs that do not fix complement and, thus, are not pathogenic. Rather than “rebooting” the immune system, our Ag-specific immunotherapy actually “reprograms” it so that it responds to a subsequent challenge with a boost of *Torpedo* AChR in a different manner.

We discovered evidence for the mechanisms by which the therapy may work. Induction of immune responses can be blocked by passive transfer of Ab (29). For example, Ab to the D Ag is used clinically to prevent hemolytic disease of the fetus and newborn (30). Large amounts of Ab to the Ag bound to B lymphocytes can cross-link FcγRIIb on B cells, thereby triggering apoptosis (29, 31, 32). This process is Ag specific, but not epitope specific, because the therapeutic Abs are not directed at the same epitopes of

the autoimmunogen involved in the pathological autoimmune response. This explains why Abs to epitopes on the extracellular surface can be suppressed by cytoplasmic domain fragments. Induction of EAMG with human MIR/AChBP chimeras (which have no cytoplasmic or transmembrane domains) induced formation of Abs to the cytoplasmic domains of muscle AChRs (9). Abs to the extracellular domain of AChRs cause focal lysis of the postsynaptic membrane and shedding of AChRs into the synaptic cleft (5). These shed muscle AChRs can both provoke the immune response to cytoplasmic domains and boost the response to extracellular domains (9). This feed-forward cycle of autoimmune stimulation to muscle AChRs could sustain EAMG and MG. This vicious cycle also could be the target of our therapy. Therapeutic vaccine protein, present in much greater amounts than shed AChRs, produces high concentrations of Abs to cytoplasmic domains, reversing the vicious cycle by driving apoptosis rather than stimulation of pathogenic B lymphocytes. We observed that weekly administration of a mix of mAbs to the cytoplasmic domains of AChR subunits, but not normal rat IgG, partially suppressed development of the chronic phase of EAMG (J. Luo, unpublished observations). This suggests that Ab-mediated feedback suppression contributes to the benefits of therapy.

Therapy rapidly reduced Abs to the MIR and, after reimmunization with *Torpedo* AChR, shifted predominant Ab isotypes from IgG2b (which fixes complement) to IgG1 (which does not). During therapy, suppression of Ab response to the MIR was not isotype-specific. After reimmunization with *Torpedo* AChR, resistance of treated rats to reinduction of EAMG was accompanied by a rapid increase in Abs to the MIR. These data suggest that a distinct mechanism may be responsible for the long-term effect of therapy. Administering the therapeutic vaccine in IFA is more effective than in TiterMax. IFA induces a predominantly Th2-biased response, whereas TiterMax induces a strong Th1 response (33). The shift in IgG subtypes toward the Th2-regulated IgG1 isotype by therapy, together with the preference for a Th2-promoting adjuvant, suggests that therapy may involve downregulation of Th1 responses specific to extracellular epitopes. In EAMG rats, only a tiny fraction of total Abs induced by immunization with *Torpedo* AChR cross-react with rat muscle AChR (19, 20), and only those autoantibodies specific to extracellular epitopes and capable of fixing complement are pathogenic. Therapy that suppressed pathogenic autoantibodies to the MIR increased therapeutic autoantibodies to rat muscle AChR cytoplasmic domains. The IgG isotype profile of responses to the therapeutic proteins does not parallel that of autoimmune responses to rat muscle AChR (19). All of these data suggest that the pathogenic autoimmune response to extracellular epitopes and the pathologically irrelevant autoimmune response to cytoplasmic domains are regulated individually, probably by different subpopulations of T cells. The T cell subpopulation that specifically modulates the pathologically relevant autoimmune response remains to be determined.

Th1 cells induce synthesis of complement-fixing Abs, which damage the postsynaptic membrane (34). Antigenic modulation is caused by cross-linking of AChRs by Abs independent of complement. In MG patients, the predominant isotypes of anti-AChR Abs are IgG1 and IgG3 (which fix complement) (35). Complement-mediated destruction of AChRs may be more pathogenically significant than antigenic modulation (36, 37). Autoantibodies from previously treated rats reimmunized with *Torpedo* AChR were ineffective at fixing complement and, thus, failed to cause or passively transfer EAMG although they caused antigenic modulation. A similar observation was made when EAMG was induced by human MIR/AChBP chimera. Unlike *Torpedo* AChR,

which primarily produced IgG2b Abs to the MIR, the chimera generated very little IgG2b Abs, but high levels of IgG1 Abs, to the MIR (J. Luo, unpublished observations). Thus, although the chimera produced much higher Ab titers to the MIR chimera than did *Torpedo* AChR, EAMG induced by the chimera developed more slowly and was less severe than that induced by equivalent amounts of *Torpedo* AChR (9). Autoantibodies to AChR are detected in 90% of MG patients and are used as a diagnostic test for MG (38). There is not a close correlation between anti-AChR concentration and severity of MG (39). In addition to variability in specificities of the Abs to AChR, differences in isotypes of the Abs to AChR in various patients may contribute to this lack of correlation.

Suppression of ongoing EAMG by oral administration of bacterially expressed human or rat AChR $\alpha 1$ extracellular domains was associated with downregulation of the Th1 response (40). An increase in Foxp3 expression in EAMG rats following the oral treatment suggested involvement of Foxp3⁺ Tregs in the Ag-specific immune suppression (17). It was reported that Ag-specific Tregs suppress protective Th1 responses in infectious diseases (41). Administration of Ag to compartments rich in Tregs results in specific suppression of the autoimmune response (42). Tregs also could be activated by Treg epitopes in Abs in response to binding Ag (43). The contributions of Tregs to Ag-specific immunosuppression by treatment with cytoplasmic domains of AChRs remain to be determined.

Specific immunosuppressive therapy with human AChR cytoplasmic domains may be even more effective in MG patients than it is in rats with EAMG. Rats strongly avoid making autoantibodies. For example, rats immunized with human MIR/AChBP chimera develop EAMG; although only 4 of the 42 (10%) human muscle AChR $\alpha 1$ subunit amino acids in the chimera differ from rat $\alpha 1$, 82% of the Abs to AChR are selective for human versus rat muscle AChR (9). Cytoplasmic domain sequences differ between humans and rats by 13%. Thus, specific immunosuppressive therapy of MG with human cytoplasmic domains might be >5-fold more potent than therapy of EAMG.

Translation of the therapy to human MG requires a substitute adjuvant for IFA. Aluminum salts (alum) are the most widely used adjuvants for human vaccines and will be evaluated in rats with EAMG. Alum induces a strong Th2 response with little or no Th1 response (44). It is likely that alum will be as effective as IFA, considering the beneficial effects of the Th2 response in the therapy.

The basic approach used in this study to suppress a pathological autoimmune response to the extracellular domain of the autoimmunogen with cytoplasmic domains of the autoimmunogen should be applicable to other Ab-mediated autoimmune responses to transmembrane proteins (e.g., autoimmune responses to glutamate receptors) (45).

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Disclosures

A patent has been filed on this approach to specific immunosuppressive therapy for MG.

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