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Inhibition of α-Bungarotoxin Binding to Acetylcholine Receptors by Antisera From Animals With Experimental Autoimmune Myasthenia Gravis

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Conditions are described for an assay that allows the percent inhibition of α-bungarotoxin binding to acetylcholine receptors by antisera and monovalent antigen-binding fragments of antibody molecules (Fab) to be determined. Anti-Torpedo californica acetylcholine-receptor antisera, prepared in New Zealand White rabbits and Lewis rats, were tested for the ability to inhibit [^{125}I]-α-bungarotoxin binding to membrane-associated and detergent-solubilized T californica acetylcholine receptors. Similar inhibition studies were performed using rabbit antisera and antigen-binding fragments prepared against each of the four acetylcholine receptor subunits. Antisera and antigen-binding fragments prepared against intact receptor could inhibit a maximum of 50% of the α-bungarotoxin binding to solubilized receptor. The results using monovalent antigen-binding fragments indicated that the inhibition was not due to antibody-mediated aggregation of receptor molecules. Rabbits and rats immunized with receptor denatured by sodium dodecyl sulfate all produced antisera that could bind to nondenatured receptor, but none of these animals developed experimental autoimmune myasthenia gravis. These results suggest that the antigenic determinants present on acetylcholine receptors responsible for induction of experimental autoimmune myasthenia gravis are lost with sodium dodecyl sulfate denaturation. A strong correlation was also observed between the presence of experimental autoimmune myasthenia gravis in rats and rabbits and the ability of the antisera from these animals to inhibit 50% of α-bungarotoxin binding to solubilized acetylcholine receptors.

Key words: acetylcholine receptor, experimental autoimmune myasthenia gravis, antigen-binding fragments, subunit antisera

Recent reviews of experimental autoimmune myasthenia gravis (EAMG), discussing possible roles of anti-acetylcholine receptor (AcChR) antibodies in the disease are available [1, 2]. Myasthenic paralysis is believed to be due to loss of AcChR rather than

Abbreviations used: EAMG, experimental autoimmune myasthenia gravis; AcChR, acetylcholine receptor; α-BuTx, α-bungarotoxin; Fab, monovalent antigen-binding fragments of antibody molecules; MF, membrane fragments; MFT, membrane fragments + 1% Triton X-100; MFTS, supernatant from MFT after 100,000g centrifugation for 1 h; MFTS-AcChR, MFTS depleted of AcChR; SACI, Staphylococcus aureus Cowan I strain; SDS, sodium dodecyl sulfate.

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blockade of receptor activation, although partial inhibition of AcChR activity may be very important in situations where the number of AcChR is already reduced.

Differing results have been reported in testing the ability of anti-AcChR antiserum (produced in various experimental animals) to inhibit α-bungarotoxin (α-BuTx) or acetylcholine binding to AcChR. One such study showed that antiserum precipitated [^{3}H]-acetyl receptor more effectively than [^{3}H]-acetyl receptor-α-BuTx complexes, and it was calculated that 25% of the antibodies were unable to bind to toxin-receptor complexes [3]. In another study, no difference in the ability of antisera to precipitate receptor or toxin-receptor complexes was observed [4]. The reported inhibition of [^{125}I]-α-BuTx binding to membrane-associated AcChR has varied from 0% to 100% [4–8], while reports of inhibition of toxin binding to detergent-solubilized AcChR have varied from 50% to 100% [5, 7, 9, 10]. Similar variability (20–100%) has been reported for inhibition of [^{3}H]-acetylcholine binding to membrane-associated and detergent-solubilized AcChR [8, 11]. From 0% to 100% inhibition of [^{125}I]-α-BuTx binding to solubilized and membrane-associated AcChR, using sera from patients suffering from myasthenia gravis, has also been reported [12–14]. In these studies, differences can be found in the sources of antigen, antibody, and substrate, as well as in the type of assay employed. A major objection to all such studies has been that antibodies may aggregate AcChR and impair the accessibility of the toxin-binding site, resulting in an apparent rather than a real inhibition [2].

In this study, antisera prepared in New Zealand White rabbits and Lewis rats against native and sodium dodecyl sulfate (SDS)-denatured Torpedo californica AcChR were tested for the ability to inhibit [^{125}I]-α-BuTx binding to membrane associated AcChR and to Triton X-100-solubilized AcChR. Similar inhibition studies were performed using antisera directed against each of the 4 AcChR subunits. To determine if any of the inhibition seen was due to antibody-mediated aggregation of AcChR molecules, monovalent antigen-binding fragments (Fab) were prepared from the immunoglobulin fractions of antisera directed against AcChR and each of the AcChR subunits and tested for the ability to inhibit α-BuTx binding to AcChR.

MATERIALS AND METHODS

Preparation of Antigens

Torpedo californica was obtained locally. AcChR purified by affinity column chromatography was prepared according to the procedure of Vandlen et al [15]. The preparation of AcChR denatured by 1% SDS (AcChR + SDS), AcChR + SDS dialyzed against 0.1% Triton X-100 (AcChR–SDS), and AcChR incubated with saturating amounts of α-BuTx (AcChR + α-BuTx) has been described [16]. The specific details of the preparation of the following antigens has also been described [17]. Membrane fragments were prepared and solubilized in Triton X-100 according to the procedure of Vandlen et al [15] up to the point of application of the material to the quaternary ammonium affinity resin. The supernatant from the 100,000g centrifugation of membrane fragments solubilized in Triton X-100 (MFTS) was depleted of AcChR (MFTS–AcChR) by passage over a resin to which α-BuTx had been conjugated.

Preparation of Antisera

Female New Zealand White rabbits numbers 8, 19, 20, 22–25 (see Table I) were injected and boosted with 0.5–1.0 mg of purified AcChR emulsified in an equal volume of

complete Freund's adjuvant (purchased from Calbiochem). Antigens were administered by subcutaneous injections at multiple sites along the lower back. Animals were exsanguinated when symptoms of EAMG (first described by Patrick and Lindstrom [18]) were observed: paralysis in the hindquarters, drooping ears, and difficulty in breathing. The preparation of rabbit antisera directed against AcChR + SDS (rabbit 7), AcChR–SDS (rabbit 10), AcChR + α-BuTx (rabbit 6), and AcChR (rabbit 5) has been described [16]. The preparation of rat antisera has also been described [17].

Preparation of Fab

Fab were prepared by enzymatic cleavage of ammonium sulfate-fractionated serum with papain, according to the procedure described by Porter [19], with a few modifications described by Putnam et al [20]. To the immunoglobulin fraction of antisera were added 1 M sodium acetate buffer, pH 5.5, to a final concentration of 0.1 M, ethylenediamine tetra-acetic acid to a final concentration of 2 mM, cysteine to a final concentration of 10 mM and mercuripapain (purchased from Sigma Chemical Co.) at an enzyme-to-substrate ratio of 1:100. The mixture was incubated at 37°C for 5 h before the reaction was stopped by the addition of *p*-hydroxy mercuribenzoate at a final concentration of 1 mM. The solution was dialyzed against 10 mM acetate, pH 7.5, and applied to a 215 ml CM-cellulose column (2.6 × 40 cm) equilibrated in the same buffer. Before starting a linear gradient from 10 mM to 500 mM sodium acetate, pH 5.5, in a total volume of 800 ml, 160 ml of 10 mM sodium acetate was applied. Eluted peaks were pooled and dialyzed against 10 mM sodium phosphate, 0.02% sodium azide, pH 7.4. Protein concentration was determined by the method of Lowry et al [21]. Ionic strength was measured with a Radiometer Copenhagen Conductivity meter CDM3.

Inhibition of [^{125}I]-α-BuTx Binding to AcChR

A modification of the procedure described by Greenwood et al [22] was used to prepare [^{125}I]-α-BuTx. The specific activity of the [^{125}I]-α-BuTx used in these assays was 211 Ci/mmole [17]. The preparation of membrane fragments (MF) has been described [15]. Solubilized MF used in the filter disc assay were prepared by solubilizing MF in 1% Triton X-100 (MFT). The DEAE filter disc assay used was essentially the same as the one described by Schmidt and Raftery [23]. Antibody, Fab, and [^{125}I]-α-BuTx incubations with receptor were performed in 1.5 ml polyethylene micro test tubes from Cole Scientific, Inc. Two hundred μl of MF, MFT, or purified AcChR (0.1 pmoles of α-BuTx binding sites) were incubated with 0–100 μl of antiserum or Fab, followed by the addition of 1.1 pmoles of [^{125}I]-α-BuTx. Alternatively, MF, MFT, or AcChR were incubated with [^{125}I]-α-BuTx, followed by the addition of antibody or Fab. One hundred μl of solution were pipetted onto DEAE cellulose filter discs (Whatman DE81, 2.4 cm diameter) and washed in 10 mM sodium phosphate, 0.1% Triton X-100, 50 mM NaCl, pH 7.4, then counted in a Beckman 4000 gamma counter. The percent inhibition of [^{125}I]-α-BuTx binding to AcChR due to antibodies or Fab was calculated as follows:

$$100 - \left[\frac{(R + \text{Ab or Fab} + Tx) - Bg}{(R + Tx + \text{Ab or Fab}) - Bg}\right] 100$$

In the above equation, R denotes receptor, Ab denotes antibody, and Bg denotes background. The background was determined by using receptor saturated with unlabeled α-BuTx. The number of cpm on a filter disc obtained by incubating AcChR with antibody or Fab before the addition of [^{125}I]-α-BuTx was divided by the number obtained

when AcChR was incubated with [^{125}I]-α-BuTx before the addition of antibody or Fab (after background values were subtracted). The number thus obtained was multiplied by 100 then subtracted from 100 to yield percent inhibition.

Precipitation of Antibodies and Fab

Rabbit anti-rat IgG serum was purchased from Cappel Laboratories, Inc. Goat anti-rabbit IgG was purchased from Antibodies Inc., Davis, California. Staphylococcus aureus Cowan I strain (SACI) was prepared according to the procedure described by Kessler [24] and was a generous gift of Deborah Dison Hall. Four hundred μl of 5% SACI were incubated with solutions containing 10 μl of rabbit antiserum for 30 minutes at 25°C. Precipitates were collected by 5-min centrifugations in an Eppendorf 3200 centrifuge (12,000g) and the pellets were washed once by resuspension in 1 ml of 10 mM sodium phosphate, 50 mM NaCl, 0.1% Triton X-100, pH 7.4, followed by a second centrifugation. When the precipitate was obtained using a second antibody, the incubations were carried out for 20–24 h at 4°C or 5–7 at 25°C. Immune precipitates were collected and washed as described for SACI precipitates except that centrifugations were for 30 min.

DEAE-Affinity Chromatography

Three ml DE52 columns, equilibrated in 10 mM sodium phosphate, 0.1% Triton X-100, 50 mM NaCl, pH 7.4, were used to separate AcChR–[^{125}I]-α-BuTx-antibody/Fab complexes from unbound [^{125}I]-α-BuTx. AcChR–[^{125}I]-α-BuTx-antibody/Fab complexes were eluted from the column with 0.3 M NaCl.

RESULTS

Conditions of the Filter Disc Assay

Because much of the reported variability in the percent inhibition of α-BuTx and acetylcholine binding to AcChR by antibodies may be due to differences in the assays employed, we tested various conditions of the filter disc assay [23] that would lead to reproducible results. It was first established by taking time points over a 24-hour period that no further binding of [^{125}I]-α-BuTx or antibodies was seen at 25°C or 4°C with incubation times longer than 1 h. However, for toxin binding to be described by pseudo-first-order kinetics within the 1-hour incubation period, an 8-fold or greater excess of toxin to toxin sites was needed. An 8-, 10- or 20-fold excess of toxin did not alter the effect of antibodies bound to AcChR or the amount of AcChR-toxin complex formed.

Anti-AcChR antibodies were capable of precipitating receptor-toxin complexes without the addition of a second antibody or SACI. For studies of antibody inhibition of α-BuTx binding to AcChR, it was therefore critical that solutions of receptor-toxin-antibody complexes be thoroughly mixed before removing an aliquot for the filter disc assay. Samples were mixed on a Scientific Products Vortex Genie mixer just prior to assay.

Tests for Artifacts in the Disc Assay

Two possible sources of artifacts concerning the use of the filter disc assay were: 1) factors present in serum might prevent receptor-toxin complexes from binding to filter discs and 2) because the filter discs were washed in a pH 7.4 buffer and IgG does not bind to DEAE at this pH, receptor-toxin-antibody complexes might be prevented from binding to filter discs because of the pH. It was determined, using serum from a

nonimmune rat, that receptor-toxin complexes could be prevented from binding to filter discs, but only at high concentrations of serum. This inhibition was presumably due to competition for sites on filter discs. Therefore, in the studies described in this paper, the concentrations of serum used were well below the level at which nonspecific blocking effects were seen. The first test to determine if antibody bound to receptor inhibited receptor binding to filter discs was to add [^{125}I]-α-BuTx to AcChR before adding antibodies. With increasing amounts of antiserum, no inhibition of receptor binding to filter discs occurred within the useful range of the assay. It was possible, however, that a particular fraction of antibodies might be prevented from binding to receptor when α-BuTx was added first and that these antibodies when allowed to bind receptor could prevent receptor from binding to DEAE filter discs. To test the possibility that such a population of antibodies existed, DEAE columns were run on receptor-toxin-antibody complexes and assayed. Details of the columns are described under Materials and Methods. Complexes (with either antibody or toxin bound first) were applied to columns and washed with 3 column volumes of buffer before eluting receptor complexes with sodium chloride. A typical elution profile is shown in Figure 1. No peaks of radioactivity were seen except peak I, which eluted in the void volume, and peak II, which was eluted with high salt. When peak I was pooled and SACI added to the material, SACI did not precipitate any radioactive material from this peak, demonstrating that no IgG bound to receptor-toxin complexes was present in this peak. However, 100% of the radioactive material present in peak II could be precipitated with the addition of SACI. These results indicate that peak I contained the free, unbound [^{125}I]-α-BuTx. If any receptor-toxin complexes were present in this peak, IgG was not attached. In addition, the area under

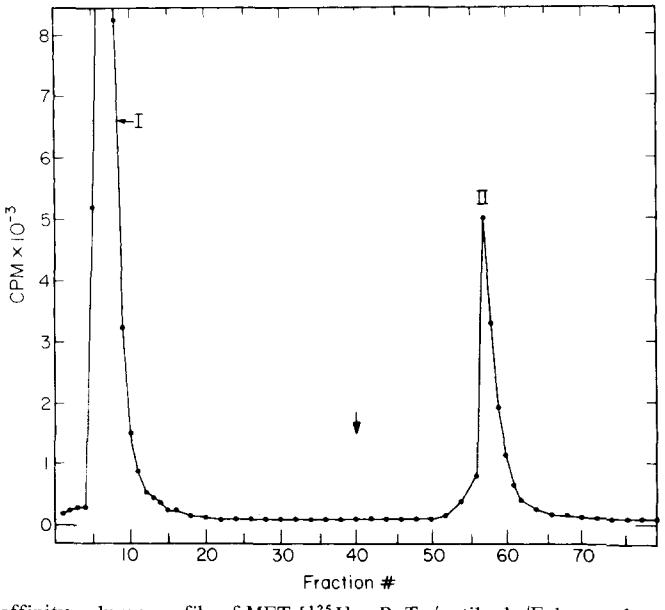

Fig. 1. DEAE-affinity column profile of MFT-[^{125}I]-α-BuTx/antibody/Fab complexes in the presence of an excess of [^{125}I]-α-BuTx. Peak I represents free, unbound [^{125}I]-α-BuTx that does not bind DEAE. The arrow above fraction 40 indicates where 0.3 M NaCl was applied to the column in order to elute MFT-toxin, MFT-toxin-antibody or MFT-toxin-Fab complexes (peak II) from the column.

peak II was greater when toxin was added to receptor first, as compared with the area under the peak when antibody was added first. This result agrees with those obtained by the filter disc assay. A corresponding increase in the free-toxin peak could not be measured because of the large amount of toxin added to the incubation mixture. These results strongly suggest that the DEAE filter disc assay is an appropriate method for determining whether antibodies inhibit [^{125}I]-α-BuTx binding to AcChR.

Preparation of Fab From Immunoglobulins

Profiles of papain digests of the immunoglobulin fractions from the various antisera were essentially identical to those shown by Porter [9] and Putnam et al [20], and the 3 peaks eluted at the same ionic strength as the corresponding peaks described by Putnam et al [20]. Fab (peak I) were tested for the ability to bind antigen but not aggregate it. All of the Fab (except those prepared from the immunoglobulin fraction of a nonimmunized control rabbit) bound AcChR labeled with [^{125}I]-α-BuTx, but this receptor-toxin-Fab complex could not be sedimented by centrifugation at 100,000g for 1 h. The addition of SACI did not precipitate the complex, but the addition of a second antibody (goat anti-rabbit IgG or sheep anti-rabbit IgG) did form a precipitate that could be collected by centrifugation at 12,000g for 30 min. Because SACI binds only to the Fc domain of antibody molecules, these results using SACI and second antibodies demonstrate that the Fab contained no intact antibodies. Fab were tested, in a fashion similar to that just described for antibodies, to determine conditions for their use in the filter disc assay and to test for artifacts in the disc assay. The conditions and results described for antibodies also apply to Fab. Although Fab did not precipitate receptor-toxin complexes without the addition of a second antibody, receptor-toxin-Fab solutions were also thoroughly mixed before removing an aliquot for the disc assay.

Inhibition of [^{125}I]-α-BuTx Binding to AcChR by Antisera and Fab Prepared Against Isolated AcChR Subunits

Antisera directed against each T californica AcChR subunit isolated from SDS polyacrylamide gels and Fab prepared from these subunit antisera had no effect on the binding of α-BuTx to AcChR. No difference in the total amount of [^{125}I]-α-BuTx binding was observed whether toxin, antibody, or Fab was added first, and no difference was observed using MF, MFT, or purified AcChR. In all cases using MFT or purified AcChR as the antigen, subunit antisera and Fab were shown to be bound to receptor-toxin complexes by precipitation with SACI or second antibodies. When subunit antisera were tested in quantities 5 to 20 times the amount needed to precipitate all receptor-toxin complexes, there was still no effect on toxin binding to AcChR.

Inhibition of [^{125}I]-α-BuTx Binding to AcChR by Antisera and Fab Prepared Against AcChR

When MFT or purified AcChR were used an antigens, anti-AcChR antisera and anti-AcChR Fab could inhibit up to 50% of [^{125}I]-α-BuTx binding to AcChR. Figure 2 is a plot of the effects of adding increasing amounts of anti-AcChR Fab on toxin binding to AcChR. It was shown that 100% of the receptor was bound with a 150 μl addition of Fab. Approximately 50% inhibition of toxin binding was seen with 200 μl of Fab, and no further inhibition was obtained with the addition of twice as much Fab, demonstrating that the inhibition seen was at a maximum. With anti-AcChR antiserum, a 50-fold excess of antiserum over the minimum amount needed to inhibit 50% of the toxin binding again did not result in any further increase in the amount of inhibition obtained (Fig. 3).

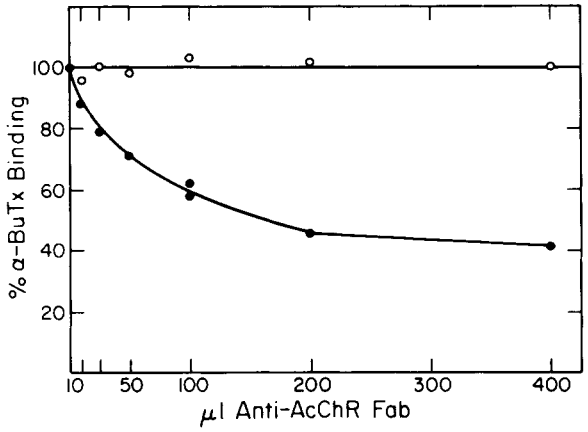

Fig. 2. Inhibition of [^{125}I]-α-BuTx binding to MFT by rabbit anti-AcChR Fab. The Fab used were at a protein concentration of 0.9 mg/ml and the MFT contained 2.2×10^{-13} moles of α-BuTx binding sites. When increasing amounts of anti-AcChR Fab were added to MFT labeled with [^{125}I] α-BuTx, the line drawn through open circles (○) was obtained. When increasing amounts of anti-AcChR Fab were added to MFT before the addition of [^{125}I]-α-BuTx, the line drawn through closed circles (●) was obtained.

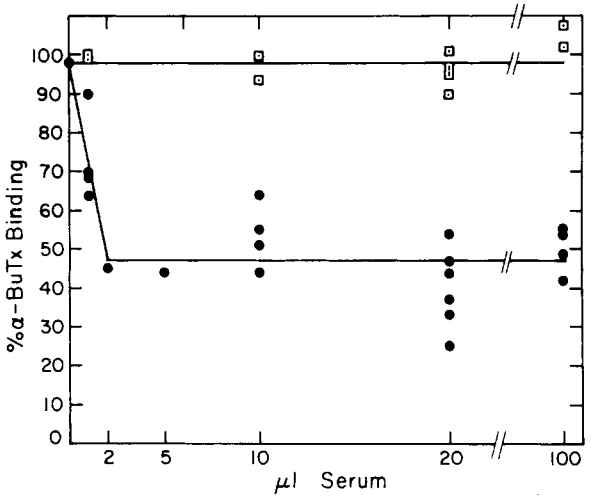

Fig. 3. Inhibition of [^{125}I]-α-BuTx binding to MFT by rat antisera directed against AcChR, AcChR + SDS, MFTS, and MFTS + SDS. The MFT contained 1.1×10^{-13} moles of α-BuTx binding sites. Incubations of MFT with each of 5 anti-AcChR + SDS antisera and each of 5 anti-MFTS + SDS antisera before the addition of [^{125}I]-α-BuTx are represented by open squares (□). Incubations of MFT with each of 5 anti-AcChR antisera and each of 5 anti-MFTS antisera before the addition of [^{125}I]-α-BuTx are represented by closed circles (●).

Thus, anti-AcChR antiserum and Fab could inhibit a maximum of 50% of the toxin binding to receptor. In contrast, anti-AcChR antisera from three rabbits and one rat all failed to inhibit toxin binding to nonsolubilized MF as did anti-AcChR Fab prepared from a fourth rabbit using concentrations of serum within the useful range of the disc assay. Up to 20% inhibition of toxin binding to MF by anti-AcChR antiserum could be seen, however, in the concentration range where normal serum nonspecifically blocked receptor

binding to sites on the filter disc. The filter disc assay is limited by the amount of protein that can be applied per disc. In this study, the usefulness of the assay was limited by the amount of serum that could be applied per disc. To investigate the ability of anti-AcChR antibodies to inhibit α-BuTx binding to nonsolubilized MF, a modification of the current assay, a different assay, or [^{125}I]-α-BuTx with a higher specific activity than was used in this study would be necessary. Filter discs with a larger surface area would allow more material to be applied, or [^{125}I]-α-BuTx with a higher specific activity would require less receptor, and therefore less serum could be used.

Inhibition of [^{125}I]-α-BuTx Binding to AcChR By Antiserum Prepared Against Modified AcChR

The antisera from 11 different rabbits immunized with various forms of AcChR (AcChR + SDS, AcChR–SDS, and AcChR + α-BuTx) were tested for the ability to inhibit α-BuTx binding to solubilized T californica membrane fragments. Including multiple bleedings from some rabbits, a total of 19 sera were tested. The results are summarized in Table I. The average percent toxin inhibition from the antisera of the eight rabbits injected with purified AcChR was 49% ± 13.3%. The average percent in-

TABLE I. Percentage Inhibition [^{125}I]-α-BuTx Binding to MFT by Various Rabbit Antisera

Rabbit no.	Antigen	% Inhibition[a] of α-BuTx binding	No. of immunizations	Comments
5	AcChR	42 (57 ± 8.6)[b]	1	paralysis in 22 days
22	"	50	2	paralysis in 19 days
23	"	62	3	paralysis in 32 days
24	"	60	2	paralysis in 23 days
25	"	54	2	paralysis in 23 days
8	"	62	6	died, unobserved 21 days after a 6th injection
19	"	70	2	paralysis in 26 days
19	"	59		"
20	"	34 (35 ± 5.7)	6	no paralysis during 2 years
20	"	39		"
20	"	26		"
20	"	38		"
20	"	40		"
10	AcChR–SDS[c]	10 (6 ± 5)	5	no paralysis during 9 months
10	"	3		"
7	AcChR + SDS[d]	11 (8 ± 4.2)	4	no paralysis during 3 months
7	"	5		"
6	AcChR + α-BuTx[e]	17	1	paralysis in 22 days

[a] 20 μl of serum were incubated with 1.1×10^{-13} moles of α-BuTx binding sites.
[b] Values used throughout the text indicate the mean percent ± SD.
[c] AcChR denatured by 1% SDS then extensively dialyzed against 0.1% Triton X-100.
[d] AcChR denatured by 1% SDS.
[e] AcChR incubated with saturating amounts of α-BuTx.

hibition from the two rabbits injected with AcChR denatured in SDS was only 7% ± 3.9%.

An interesting observation was made with antisera from rabbit 20. This animal had been injected 6 times over a period of 15 months with different purified AcChR preparations emulsified in complete Freund's adjuvant, incomplete Freund's adjuvant, or no adjuvant. Although the animal became irritable approximately 5 days after an injection, it never developed paralysis. The antisera from 5 bleedings were tested for the ability to inhibit toxin binding to AcChR. All antisera did inhibit toxin binding, but the percent inhibition (35% ± 5.7%) was consistently lower than the average of the anti-AcChR antisera from animals displaying EAMG (57% ± 8.6%). These results suggest that a population of antibodies that is capable of preventing toxin binding to AcChR may be important in the induction of EAMG. To further investigate this possibility, a rabbit was immunized with receptor-toxin complex to see if EAMG would be induced.

Antiserum from the one rabbit injected with AcChR + α-BuTx (#6) could inhibit only 17% of the toxin binding to AcChR. The receptor used for injection into this rabbit was the same preparation used (free of toxin) for injection into rabbit 5. As shown in Table I, antiserum from rabbit 5 inhibited toxin binding to AcChR by 42%. Apparently α-BuTx bound to AcChR can prevent the induction of a population of antibodies directed against the toxin binding site. This antiserum did not prevent all of the toxin from binding to AcChR however, and the animal did become paralyzed. Because α-BuTx was not covalently bound to AcChR when used as the immunogen, several possible explanations for the 17% inhibition exist. Most likely, some of the toxin sites on AcChR were unoccupied by toxin when exposed to the animal's immune system. For future studies on the effect of blocking toxin binding sites on receptor and the induction of EAMG, α-BuTx should probably be covalently bound to receptor.

The antisera from 29 Lewis rats immunized with AcChR, AcChR + SDS, MFTS, MFTS + SDS, MFTS – AcChR and buffer were tested for the ability to inhibit α-BuTx binding to MFT. The results are summarized in Table II. After a 3-month immunization period, skeletal muscle AcChR was extracted and quantitated in each animal [17]. An animal was judged to have EAMG if it had ~ 30% of the number of extracted muscle AcChR as compared with the number extracted from a control rat [25]. Antisera from the 15 rats that did not have EAMG could inhibit [^{125}I]-α-BuTx binding to AcChR by only 2.7% ± 2.8%. Antisera from the 10 rats that did have EAMG inhibited [^{125}I]-α-BuTx binding to AcChR by 55% ± 9%. The antisera from the 4 rats injected with MFTS–AcChR could not inhibit toxin binding to AcChR but they contained only 70% of the number of muscle AcChR as compared with control rat muscle AcChR. Saturating amounts of antisera (enough to precipitate all receptor-toxin complexes) were not present in all tests. Saturating amounts were, however, present in antisera from the AcChR and AcChR + SDS groups. Although saturating amounts were present in the AcChR + SDS group, none of these antisera could inhibit toxin binding to AcChR. Because this difference in the ability of antisera to inhibit toxin binding persists in the presence of saturating amounts of antisera, it reflects a difference in the specificities of the antisera and not the quantities of antibodies.

Inhibition Due to IgG

The experiments described above suggest, but do not prove, that IgG is responsible for the inhibition seen. A different class of immunoglobulin, or possibly a specific protease, present in the serum of animals with EAMG and able to destroy the toxin binding could

TABLE II. Percentage Inhibition of [^{125}I]-α-BuTx Binding to MFT by Various Rat Antisera

Animal no.	Antigen	% Inhibition[a] of α-BuTx binding	Mean % inhibition	Comments[b]
41	Buffer control	0–1	1 ± 0.9	No EAMG
42		0		"
43		2		"
44		0		"
45		2		"
11	AcChR + SDS	5	3 ± 2.6	No EAMG
12		0		"
13		4		"
14		0–6		"
15		4		"
31	MFTS + SDS	5	4 ± 3.7	No EAMG
32		0		"
33		10		"
34		4		"
35		3		"
36	MFTS–AcChR	0	1 ± 1.8	± EAMG
38		0–2		"
39		4		"
40		0		"
46	AchR	56–58	54 ± 6.0	EAMG
47		67		"
48		46		"
49		45–51		"
50		56		"
26	MFTS	46–49	55 ± 11.7	EAMG
27		75		"
28		63		"
29		45		"
30		53		"

[a]20 μl of antiserum were incubated with 1.1×10^{-13} moles of α-BuTx binding sites.
[b]EAMG as determined by quantitation of the number of muscle AcChR extractable from each animal. No EAMG indicates all AcChR present; ± EAMG indicates 70% present; EAMG indicates 30% present.

be responsible. The following experiments eliminate some of the possibilities. When α-BuTx was added to AcChR before the addition of Fab, there was no change in the total amount of toxin bound over a 53-h period. When toxin was added after preincubation with Fab, the amount of toxin binding slowly increased from 50% to 77% over the 53-h time period. When receptor-Fab-toxin complexes were allowed to incubate for long periods of time, toxin apparently bound slowly to additional sites initially unavailable for toxin binding. This experiment demonstrates that the inhibition of toxin binding is reversible, making it very unlikely that the inhibition seen was due to loss of the site by proteolytic digestion. If the inhibition were due entirely to the IgG class of immunoglobulins, then it should be possible to pre-clear serum of IgG by the addition of SACI. By adding increasing amounts of pre-cleared anti-AcChR antiserum to MFT before the addition of [^{125}I]-α-BuTx, the percent inhibition was seen to decrease from 54% to 17%,

where the inhibition plateaued. This experiment demonstrates that IgG is clearly involved in the inhibition seen. It is possible that other subclasses of immunoglobulins that are not bound by SACI contribute to the remnant inhibition.

DISCUSSION

Anti-AcChR antisera and anti-AcChR Fab could inhibit toxin binding to MFT and purified AcChR to a maximum of ~ 50%. Some inhibition of toxin binding to non-solubilized MF was also observed but only with concentrations of antiserum beyond the useful range of the assay described in this study. A 50-fold excess of antibody over the minimum amount needed to inhibit 50% of toxin binding to MFT did not increase the percent inhibition. The result that anti-AcChR Fab could inhibit the same amount of toxin binding as anti-AcChR antibodies demonstrated that the inhibition was not due to antibody-mediated aggregation of receptor. Antibodies directed against Triton X-100-solubilized AcChR could inhibit toxin binding to receptor. Although antibodies directed against subunits isolated from SDS polyacrylamide gels or AcChR denatured in SDS all bound Triton X-100-solubilized AcChR, none could inhibit toxin binding. Antibodies bound to particular antigenic sites present on nondenatured AcChR are capable of inhibiting 50% of the toxin binding capacity of the AcChR. It is not known whether these antibodies are preventing toxin binding by steric or allosteric interactions at or near the toxin binding site. Similar findings using a modified AcChR preparation were reported by Bartfeld and Fuchs [26]. In that study, antiserum prepared against reduced and carboxymethylated AcChR bound to solubilized AcChR but could not inhibit toxin binding to AcChR.

To date, EAMG has been induced in 23 of 24 New Zealand White rabbits and Lewis rats in our laboratory using crude, Triton X-100-solubilized, or highly purified AcChR preparations. We have been unable to induce EAMG in 20 of 20 rabbits and rats using crude, Triton X-100-solubilized, highly purified, or isolated subunits of AcChR denatured in SDS. It has previously been reported that AcChR denatured by SDS or isolated AcChR subunits do not induce EAMG in rats or rabbits [16, 17, 27, 28]. In contrast, Lindstrom et al [29] have reported induction of EAMG in rats with isolated AcChR subunits in SDS. Other studies with modified AcChR have shown that reduced and carboxymethylated AcChR does not induce EAMG [26], but the tryptic digestion product of AcChR does [30].

An average value of 49% toxin inhibition was seen with antisera from 8 rabbits (6 of them paralyzed) injected with nondenatured T californica AcChR, while the average from 2 rabbits (not paralyzed) injected with AcChR + SDS was only 7%. Similarly, the average percent toxin inhibition with antisera from 10 Lewis rats injected with AcChR or MFTS (and suffering from EAMG) was 55% and again an average of only 3% inhibition was seen with antisera from 10 rats injected with AcChR + SDS or MFTS + SDS (and not suffering from EAMG). A striking correlation between the presence of EAMG in Lewis rats and the ability of their antisera to inhibit toxin binding to AcChR was therefore observed. A similar correlation probably also applies to rabbits. Zurn and Fulpius [5] have shown that the antiserum from one rabbit, which was bled during the course of multiple injections of AcChR, demonstrated an increase in the ability to inhibit [^{125}I]-α-BuTx binding of AcChR concomitant with the onset of paralysis in the rabbit. A similar correlation was not observed for anti-AcChR antibodies directed at sites other than the toxin binding site. This latter result agrees with those reported by

Lennon et al [31] for rats; however, the patterns obtained from the anti-AcChR antibody titers vs time profiles differed markedly in the 2 studies. Bartfeld and Fuchs [26], based on studies with reduced and carboxymethylated AcChR, have also suggested the possibility that anti-toxin site antibodies may be relevant in causing neuromuscular damage in rabbits with EAMG.

The results showing 1) that antiserum directed against SDS denatured AcChR can bind to and precipitate solubilized AcChR but cannot inhibit toxin binding, 2) that anti-AcChR antiserum can inhibit toxin binding, and 3) the correlation of these data with the presence of EAMG, together with 4) the observation that anti-Electrophorus electricus AcChR antiserum can inhibit carbamylcholine-induced depolarization of isolated eel electroplaques [3, 32], provide evidence that the population of antibodies causing this inhibition has a physiological significance. Objections to attaching a physiological significance to any impairment of [^{125}I]-α-BuTx binding to solubilized AcChR by anti-AcChR antiserum have been twofold [2]: 1) Receptors may become aggregated by antibodies, resulting in impaired access by toxin and 2) any effect due to antibodies directed at intracellular determinants would be without effect in vivo. The first objection has been overcome by the results of the Fab experiments described in this paper. The second objection may still be valid; however, the observation described in point 4 above tends to overcome this objection also.

This study demonstrated that anti-AcChR antibodies could inhibit maximally ~50% of α-BuTx binding to solubilized AcChR. The inhibition was due primarily, if not totally, to IgG; the use of Fab demonstrated that the inhibition was not due to aggregation by immunoglobulins. A correlation was observed between the presence of EAMG in rats (and probably also rabbits) and the ability of the antisera from these animals to inhibit 50% of α-BuTx binding to AcChR. This study also indicated that particular antigenic determinants on AcChR could induce EAMG, and that these antigenic determinants were lost with SDS denaturation. It will be important in future research to ascertain which antigenic determinants these are and where these determinants are located in the AcChR molecule. This problem can be addressed from both genetic and biochemical approaches. Studies of the specificity of antisera produced in inbred animal strains in which EAMG cannot be induced [33, 34] can be expected to lead to critical insight into the nature of autoimmune myasthenia gravis. Further studies of AcChR-α-BuTx complexes used as the immunogen may also contribute key information about the disease.

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