PATHOLOGICAL MECHANISMS IN EXPERIMENTAL AUTOIMMUNE MYASTHENIA GRAVIS

I. Immunogenicity of Syngeneic Muscle Acetylcholine Receptor
and Quantitative Extraction of Receptor and
Antibody-Receptor Complexes From Muscles of Rats With
Experimental Autoimmune Myasthenia Gravis*

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An autoimmune response to acetylcholine receptor (AChR)[1] in the postsynaptic membrane of muscle appears to be responsible for impairment of neuromuscular transmission in humans suffering from myasthenia gravis (MG) and animals immunized with purified AChR. This paper and the accompanying paper (1) investigate how the autoimmune response to AChR is initiated, how the cellular and humoral arms of the immune system act to impair neuromuscular transmission, and how the muscle responds to the immunological assault on its AChR. In this paper we show that experimental autoimmune myasthenia gravis (EAMG) can be induced in rats as well by immunization with purified syngeneic AChR as by immunization with AChR purified from Electrophorus electricus (eel) electric organs, and that the EAMG which ensues in either case involves formation of antibodies to muscle AChR which bind to AChRs at endplates and cause a net reduction in the number of muscle AChRs. In the accompanying paper we show that antibody from rats immunized with AChR can cause EAMG in normal rats by binding to muscle AChR and causing reduction in AChR content, in part by targeting the postsynaptic membrane for destruction by phagocytic cells. In order to put these observations into proper context, general features of both MG and EAMG are reviewed in the introduction of this paper.

Immunization of mammals with AChR purified from electric fish induces EAMG (2-7). Rat antibodies to AChR from eel cross-react only slightly with AChR from rat muscle (8). In this paper we show that rats immunized with purified syngeneic AChR also develop

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[1] Abbreviations used in this paper: AbAChR, antibody-acetylcholine receptor complexes; AChR, acetylcholine receptor EAMG, experimental autoimmune myasthenia gravis; eel, Electrophorus electricus; MG, myasthenia gravis.

EAMG. Specificity of the autoimmune reaction in this case is shown by the observation that antibodies to muscle AChR in these rats cross-react only slightly with AChR from *Electrophorus*. These results show that an individual's AChRs, in suitable adjuvant conditions, can induce an autoimmune response.

Rats develop two phases of EAMG in response to a single injection of *Electrophorus* AChR in adjuvant. 8–10 days after immunization an acute phase of muscular weakness is observed which may be fatal (3). Between 11 and 28 days there is remission from obvious weakness followed by a chronic phase of weakness which usually progresses to death (3). During the acute phase muscle end-plates are invaded by phagocytic cells (9, 10). These cells intrude between nerve and muscle and engulf the postsynaptic membrane resulting in functional denervation of up to 90% of the fibers (9, 10). Phagocytic cells disappear from the end-plate zone after the acute phase. Development of the chronic phase is associated with a dramatic increase in titer of serum antibody to muscle AChR (8). In the chronic phase the area of postsynaptic membrane is decreased and its folded structure simplified (9, 10). End-plates of animals with EAMG bind decreased amounts of ^{125}I-α-bungarotoxin (5), exhibit decreased acetylcholine (ACh) sensitivity (11), increased curare sensitivity (12, 13), and small miniature end-plate potentials (12). Antisera from animals with chronic EAMG inhibit the depolarizing response of muscle (11) and electroplax (8, 14) to cholinergic agonists in vitro. Few (14) or none (15) of the antibodies to AChR, depending on the serum used, are directed at the ACh binding site of the AChR.

Human MG appears to resemble chronic EAMG more closely than acute EAMG. Both MG and chronic EAMG are characterized by weakness and fatigability (3), increased curare sensitivity (12), small miniature end-plate potentials (12), simplified postsynaptic membrane structure, reduced postsynaptic membrane area (9, 10), and reduced ^{125}I-α-bungarotoxin binding at end-plates (16). Both rats with chronic EAMG and humans with MG also have comparable concentrations of serum IgG antibodies directed to determinants on their respective muscle AChRs other than the ACh binding site (8, 17). Decrementing electromyogram responses are a diagnostic feature of MG which is routinely observed in acute EAMG, but only in the most severely effected rats with chronic EAMG (12, 13). This has been attributed to the large safety factor for transmission in rats resulting from the fact that rat nerves release more ACh quanta than do human nerves (12). The massive phagocytic invasion of end-plates characteristic of acute EAMG is not observed in MG (18), though low levels of cellular infiltration might go undetected.

Impairment of neuromuscular transmission in EAMG and MG could result from decreased AChR content of muscle. Reduced postsynaptic membrane area (9, 10, 18) and decreased ^{125}I-α-bungarotoxin binding to end-plates (5, 16) are consistent with this possibility. However, antibodies bound to AChR in the membrane might also prevent toxin binding and account for decreased ACh sensitivity (11) and increased curare sensitivity (12). Blockage of AChR activity on muscle (11) and electroplax (8, 14) by serum from animals with EAMG is consistent with that possibility. In this paper we show that both antibody-mediated loss of AChR and antibody modification of AChR activity probably occur in EAMG. In chronic EAMG the amount of AChR which can be extracted from muscle is greatly reduced, and much of the AChR which is extracted has antibody attached, but is still capable of binding toxin. Decreased AChR content of muscle in chronic EAMG suggests that antibody to AChR may induce "modulation" (19) of AChR. Antibody-induced removal of surface antigens has been demonstrated in other systems (19). Persistence of AChR unbound with antibody in rats with chronic EAMG having high serum titers of antibody suggests that antibody binding to AChR may also induce increased synthesis of AChR.

Materials and Methods

Preparation of AChR. AChR was solubilized from the electric organ of eel with the nonionic detergent Triton X-100 and purified by affinity chromatography on Sepharose (Pharmacia Fine

Chemicals Inc., Piscataway, N. J.) coupled with the α-neurotoxin of *Naja naja siamensis* as previously described (8, 20). To allow quantitative comparisons between rats, AChR was extracted from the total body musculature. Female Lewis rats were anesthetized with ether, exsanguinated, and decapitated. After removal of skin, tail, and viscera, the remainder of each carcass was homogenized for 1 min at high speed in a Waring blender at 4°C in 300 ml of 0.1 M NaCl, 0.01 M phosphate buffer pH 7.0 (Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.). After centrifugation at 9×10^3 rpm for 20 min in a Sorvall GSA rotor (Ivan Sorvall, Inc., Norwalk, Conn.), the supernate was discarded. Homogenization and centrifugation were repeated on the pellet. The second pellet was extracted in 160 ml 2% Triton X-100, 0.1 M NaCl, 0.01 M phosphate buffer pH 7.0, 0.01 M NaN_3 by homogenizing for 20 s to resuspend, followed by gentle shaking for 1 h. After centrifugation at 10^5 g for 30 min, the resulting supernate was filtered through glass wool to remove floating lipid. Extracts of rats prepared in this way were used for quantitation of total AChR and antibody-AChR complexes, and to provide AChR as antigen for a radioimmunoassay. For use as an immunogen, the AChR was further purified by the same affinity chromatography procedure used to purify AChR from eel. 18 of the group of 22 rats from which AChR was purified had EAMG induced by immunization with eel AChR. The total amount of AChR extracted was 1.2×10^{-9} mol (measured as ^{125}I-α-bungarotoxin-binding sites). This included 1.7×10^{-10} mol of AChR complexed with antibody (from the rats with EAMG). Purified AChR was obtained at a 15% yield (1.7×10^{-10} mol including 1.4×10^{-11} mol complexed with antibody). The amount of protein present was too small to accurately measure without wasting much of the preparation.

Inoculation of Rats. Female Lewis rats aged 10 wk were injected intradermally with AChR emulsified in complete Freund's adjuvant and were given 2×10^{10} *Bordetella pertussis* organisms subcutaneously, as described previously (3). One group of 27 rats (Table I) was immunized with 7.5 $\times 10^{-11}$ mol of eel AChR. Three other groups totaling 73 rats were immunized with 60×10^{-11} mol of eel AChR (Fig. 1 and 2). Sp act of this AChR ranged from $3-5 \times 10^{-6}$ mol ^{125}I-α-bungarotoxin-binding sites/g protein. Three rats were immunized with 2.6×10^{-11} mol of purified rat AChR (Table I). Three rats were given a dose of 2.6×10^{-11} mol of purified rat AChR complexed in vitro with antibody (Table I). The complexes were prepared by mixing the purified rat AChR overnight with crude rat immunoglobulin (prepared as a 35% $(NH_4)_2SO_4$ fraction of 0.53 ml of serum) from a rat with EAMG.

Clinical and Electrophysiological Tests. Rats were observed daily for signs of EAMG which were scored, as previously described (3), on a scale ranging from 0 for normal through + for weak grip and cry to +++ for severe generalized weakness. Electromyography was performed before and after intraperitoneal challenge with curare at doses that were without effect in normal rats (8 μg or less), as previously described (13). A decrementing muscle action potential response of 10% or more (fifth response compared with first) to repetitive supramaximal motor nerve stimulation at 5/s was regarded as evidence of EAMG (13).

Antibody Assays. Titers of serum antibodies to AChR from eel and rat were measured by indirect immunoprecipitation using ^{125}I-toxin-labeled AChR as antigen and goat anti-rat immunoglobulin as previously described (2, 8). ^{125}I-α-bungarotoxin was used at sp act of $2-4 \times 10^{17}$ cpm/mol. Antibody concentration was expressed in moles ^{125}I-toxin binding sites precipitated per liter of serum.

Quantitation of AChR in Muscle. The concentration of AChR in Triton-solublized rat musculature was determined by labeling an aliquot of extracted material with ^{125}I-toxin (5×10^{-9} M). The specificity of binding of ^{125}I-toxin to AChR was demonstrated by inhibition with 10^{-3} M benzoquinonium. Labeled AChR was then precipitated by addition of excess rat anti-AChR serum followed by goat anti-rat antibody. This immunoprecipitation method gave the same values as direct measurement of benzoquinonium protectable binding of ^{125}I-toxin using chromatography on Sephadex G-200, showing that ^{125}I-toxin binding to AChR was not prevented by the subsequent addition of excess anti-AChR antibody. Immunoprecipitation assays were performed in triplicate: 0.5-ml aliquots of labeled extract (both with and without benzoquinonium) were mixed with 10 μl of a standard rat anti-AChR serum (titer = 1.7×10^{-7} M). After overnight incubation, goat anti-rat immunoglobulin was added for 5 min (sufficient volume of a 35% $(NH_4)_2SO_4$ fraction to form a maximum precipitate in 5 min). The resulting precipitate was pelleted by 2 min of centrifugation, washed in 1 ml of buffer (0.5% Triton X-100, 0.1 M NaCl, 0.1 M phosphate pH 7.0, 0.01 M NaN_3), and pelleted again. Radioactivity of the pellets was determined by γ-counting, and values with

TABLE I

Comparative Immunogenicity of Eel and Syngeneic Muscle AChR Proteins in Lewis Rats

Immunogen	Dose (× 10⁻¹¹ mol)	Incidence of EAMG		Total Amount per rat (× 10⁻¹¹ mol ± SE)			
				Serum antibody		AChR in muscle	Antibody-AChR complexes in muscle
		Clinical	EMG	Anti-eel AChR	Anti-rat AChR		
None	—	0/6	nt	<0.5	<0.5	7.09 ± 0.20	0.173 ± 0.21
Eel AChR	7.50	12/14	nt	1,510 ± 356	34.0 ± 21	2.07 ± 0.61	1.24 ± 0.55
Rat AChR	2.58	3/3	3/3	11.8 ± 6.1	50.3 ± 28	1.84 ± 0.32	0.906 ± 0.14
Rat AChR-antibody complexes	2.58	1/3	0/3	0.35 ± 0.45	3.09 ± 1.9	3.09 ± 1.1	0.540 ± 0.19

Quantitation of serum antibody, total extractable AChR, and antibody-AChR complexes was performed 35–49 days after immunization. The amount of antibody present was calculated assuming a serum vol of 5 ml.

benzoquinonium were subtracted from the test samples. The concentration of AChR per rat was expressed as moles ^{125}I-toxin-binding sites per liter.

Detection and Quantitation of Antibody-AChR Complexes. AChR was identified in Triton-solubilized extracts of rat musculature by its specific binding of ^{125}I-toxin as described above. The size of the toxin-binding material was estimated by sedimentation in a sucrose gradient (20). The association of toxin-binding material with antibody was established by direct immunoprecipitation with goat anti-rat immunoglobulin.

To triplicate 1-ml aliquots (with and without benzoquinonium) were added 5 μl of normal rat serum (as carrier) and goat anti-rat immunoglobulin. The concentration of antibody-AChR complexes was expressed as moles ^{125}I-toxin binding sites precipitated per liter.

Reduction and Alkylation of Antibody In Situ. In an attempt to increase the yield of AChR from muscle of rats with EAMG, homogenized tissues were subjected to reduction and alkylation to cleave bound antibody molecules and thereby circumvent possible insolubility of AChR which might be cross-linked with antibody. This was accomplished by the method used with success to increase to normal values the yield of AChR from eel electroplax treated in vitro with antibody (21). Homogenized muscle was exposed for 10 min to 10^{-3} M dithiothreitol followed by 10 min with 10^{-2} M iodoacetamide. In other preparations more extreme conditions were used consisting of 60 min 4 × 10^{-3} M dithiothreitol in 10% homogenates of tissue followed by 60 min of 4 × 10^{-2} M iodoacetamide.

Results

Immunogenicity of Syngeneic Muscle AChR. Clinical and electrophysiological signs of EAMG were induced in rats by immunization with syngeneic AChR. None of the rats immunized with rat AChR exhibited early signs of weakness and fatigability characteristic of the acute phase of EAMG (3). However, 23–30 days after immunization all three rats inoculated with rat AChR alone developed clinical signs which were typical of chronic phase EAMG (3) and were severe and progressive. One of these showed a spontaneous decrement in muscle response to repetitive nerve stimulation, while the other two exhibited decrementing responses with small amounts of curare (13). One rat of the three immunized with antibody-AChR complexes developed mild clinical signs of EAMG commencing on day 31. On electromyographic testing on days 24 and 32 none of these three rats had a decrementing response, even after curare challenge.

Titers of serum antibody to muscle AChR were considerably higher in animals immunized with muscle AChR alone than in animals immunized with antibody-receptor complexes (Table I). That antibody-AChR complexes were

less effective inducers of an antibody response is consistent with their being less effective in inducing EAMG. Anti-AChR antibodies induced by rat muscle AChR reacted less with eel AChR than with rat AChR (Table I). The absolute amount of anti-muscle AChR antibody in the sera of rats immunized with either eel AChR or rat AChR was more than enough to bind to the total amount of AChR contained in a rat's body.

Decreased Content of AChR in Rats with EAMG. The total amount of AChR extractable from individual normal rats in an age- and treatment-matched group was quite uniform (e.g., $7.09 \pm 0.2 \times 10^{-11}$ mol/rat for six rats). This value was significantly reduced in rats with chronic EAMG. Of 14 rats immunized with 15 μg of eel AChR, 9 had signs of chronic EAMG by day 39 when AChR content was determined. The mean value for total AChR extracted from these 14 rats was 29% of the amount extracted from rats inoculated only with adjuvants (Table I). Further experiments presented below indicated these low values represent an absolute decrease in the amount of AChR present in each rat.

Identification and Quantitation of Antibody-Receptor Complexes in Muscle. Triton-solubilized extracts from rats immunized 90 days earlier with 120 μg of eel AChR were labeled with ^{125}I-toxin and sedimented on sucrose gradients. Instead of sedimenting as a single peak at 9.5S, like AChR from normal or adjuvant-immunized rats (Fig. 1), most toxin-binding material extracted from rats with EAMG sedimented in broad peaks at 18–20S. This indicated that the toxin-binding material had a mol wt of approximately 6–8×10^5. Binding of toxin was inhibited in the presence of benzoquinonium. The high sedimentation value of this toxin-binding material suggested that AChR molecules ($\sim 2.5 \times 10^5$ daltons) from rats with chronic EAMG might be aggregated into small complexes with a few molecules of antibody (1.5×10^5 daltons).

That AChR in extracts of muscle from rats with EAMG was indeed complexed in situ with antibody was demonstrated by the ability of goat anti-rat immunoglobulin to directly precipitate the ^{125}I-toxin-labeled material (Table II). Thus, the specificity of the material binding ^{125}I-toxin, as judged by its inhibition with benzoquinonium, its large sedimentation coefficient, and its precipitability by anti-rat immunoglobulin antibodies provide strong evidence that AChR is complexed with antibody in situ in EAMG. The possibility that the antibody-AChR complexes were formed during the extraction process rather than in vivo was ruled out, since addition of an excess of exogenous rat anti-AChR antibody during extraction of AChR from normal rat muscle did not yield antibody-AChR complexes detectable in the final extract.

The fact that extracted antibody-AChR complexes were still capable of binding ^{125}I-toxin is evidence that anti-AChR antibody does not bind directly to the ACh-binding site. This fact also makes it unlikely that reduction in the amount of AChR extractable from rats with EAMG was apparent (due to inhibition of toxin binding) rather than real. Another possible explanation for the reduced absolute yield of AChR from rats with EAMG could be cross-linking of AChR by antibodies into a matrix too large to be solubilized. In that case reduction of inter-heavy chain disulfide bonds of some of the antibodies cross-linking AChR should cleave them into half molecules and partially break up the matrix. Alkylation of the reduced disulfides should prevent recross-linking. Reduction and alkylation was performed on muscle homogenates from rats with chronic
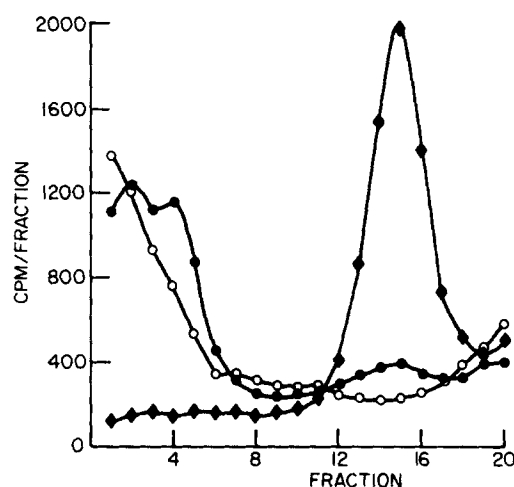
FIG. 1. Sucrose density gradient centrifugation of AChR extracted from normal and EAMG muscle. Aliquots (0.1 ml) of muscle extracts were incubated with ^{125}I-toxin (3 × 10^{-9} M), layered on sucrose gradients, and centrifuged. Fractions 21–34 at the top of the gradients containing unbound ^{125}I-toxin are not shown. (◆) AChR extracted from adjuvant control rat muscle peaked in tube 15, indicating a sedimentation coefficient ~9.5S. (O, ●) AChR extracted from two rats with chronic EAMG (90 days after immunization) sedimented more rapidly, peaking at tube 4 or lower in the gradient. These 5-ml, 5–20% sucrose gradients were centrifuged 8.3 h at 5 × 10^4 rpm in a Beckman SW50.1 rotor (Beckman Instruments, Inc., Fullerton, Calif.). Centrifugation for 4 h failed to completely resolve control AChR from unbound ^{125}I-toxin, but then the antibody-AChR complexes sedimented in the middle of the gradient. (●) appeared as two peaks ~18S and ~20S, while (O) appeared as a single peak at ~20S.

TABLE II

Typical Assay Results for Concentration of AChR and Antibody-AChR Complexes

	Binding of ^{125}I-α-bungarotoxin	
	AChR assay	Antibody-AChR complex assay
Test aliquots	2.07 × 10^4 cpm	1.77 × 10^4 cpm
Aliquots plus benzoquinonium	0.507 × 10^4 cpm	0.195 × 10^4 cpm
Difference ± benzoquinonium	1.56 × 10^4 cpm	1.58 × 10^4 cpm
AChR concentration	1.51 × 10^{-10} M	7.55 × 10^{-11} M

Assay results are shown for a single rat sacrificed 14 days after immunization with 120 μg *Electrophorus* AChR assayed as described in Materials and Methods. Because of the high specific activity of the ^{125}I-α-bungarotoxin used, large numbers of cpm make possible accurate determinations despite the low amounts of AChR involved.

EAMG by sequential treatment with dithiothreitol and iodoacetamide (21). However, this treatment did not increase the yield of AChR extracted from the muscle (Table III). These data also are consistent with diminished yields of AChR from rats with EAMG being due to a decrease in AChR content of their muscles.

TABLE III

Effect of Reduction and Alkylation on Yield of Extracted AChR

	Before reduction and alkylation		After reduction and alkylation	
	AChR $\times 10^{-11}$ mol/rat	AbAChR $\times 10^{-11}$ mol/rat	AChR $+ 10^{-11}$ mol/rat	AbAChR $\times 10^{-11}$ mol/rat
Chronic EAMG rats	1.20	0.752	1.13	0.714
Chronic EAMG rats, more intense reduction and alkylation	0.708	0.260	0.760	0.266

Homogenates of the two rats in each group were divided in half. One aliquot was extracted normally, whereas the other was first reduced and alkylated as described in Materials and Methods.

All six rats immunized with syngeneic muscle AChR showed biochemical evidence of EAMG (Table I). As in rats immunized with AChR from eel, the amount of AChR extracted from muscle of rats immunized with rat AChR was greatly decreased. Reduction in the amount of AChR was more pronounced in rats immunized with AChR alone than in rats immunized with antibody-AChR complexes. In rats immunized with muscle AChR alone, 50% of the AChR extracted was complexed with antibody, whereas in those immunized with antibody-AChR complexes, only 17% was complexed with antibody.

Changes in Muscle AChR Content During the Course of EAMG.   The results so far have established that an autoimmune response to muscle AChR, whether induced by immunization with syngeneic or xenogeneic AChR, results in humoral antibodies to AChR being bound in vivo to AChR at the motor end-plate. As a result of the immune response to AChR, and perhaps through mechanisms mediated by these antibodies, the amount of AChR in muscle is greatly reduced. In order to ascertain the mechanisms involved in decreasing AChR content and the importance for neuromuscular transmission of the amount of AChR in muscle, we measured the changes in AChR content after immunization. Fig. 2 illustrates for individual rats, total body contents of muscle AChR, anti-muscle AChR antibody, and antibody-AChR complexes at intervals after immunization with purified eel AChR. These values are plotted in the same units (equivalent moles ^{125}I-toxin-binding sites) to emphasize the quantitative relationship between the three factors during the development of EAMG. Antibodies with specificity for syngeneic muscle AChR were first detected in the serum 3 days after immunization. The amount of anti-AChR antibody in serum increased slowly until day 20 and then increased very rapidly. The amount of anti-muscle AChR antibody in the serum of some rats with chronic EAMG exceeded their body content of AChR by a factor greater than 50. The amount of antibody to eel AChR was at all times 20–100 times greater than the amount of anti-muscle AChR antibody. AChR extracted from rat muscle was first found to be complexed with antibody 6 days after immunization. The percentage of AChRs complexed in situ with antibodies increased gradually, from 1.4% at day 6 to
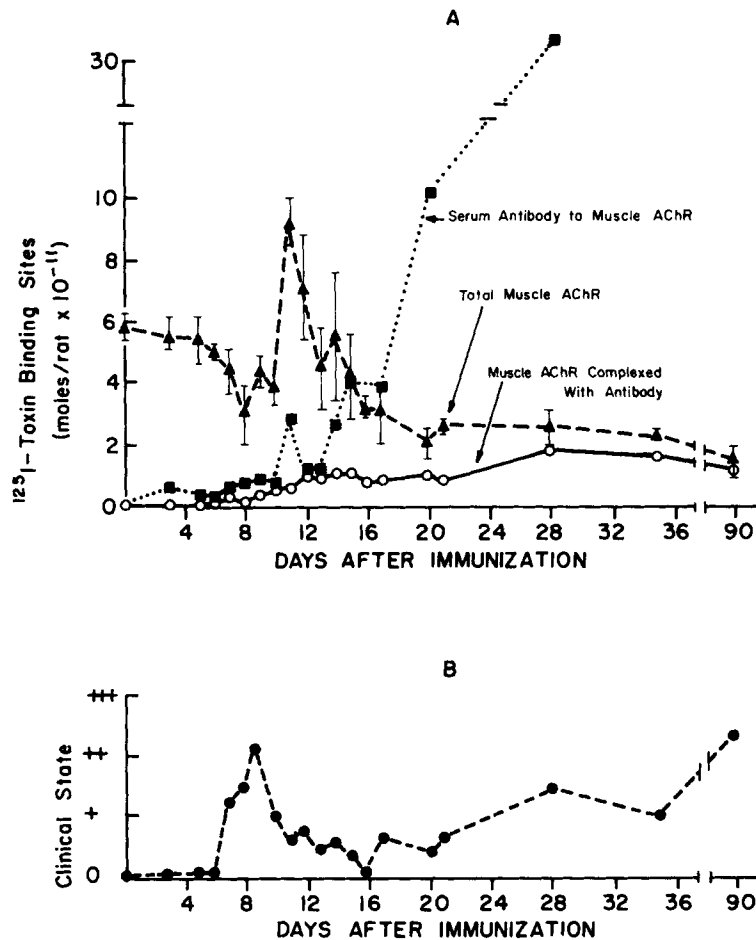
A

B

FIG. 2. Changes in amount of antibody to AChR, antibody-AChR complexes, and AChR during the course of EAMG. Three groups of rats totaling 73 were immunized at day 0 with 120 μg of purified eel AChR. On the indicated days animals were sacrificed. Each point represents the average of two to six rats. (A) (▲---▲) shows the average content of AChR from each rat. Bars indicate one standard deviation. (■· · ·■) shows the average content of antibodies to rat AChR assuming a serum vol of 5 ml. (O—O) shows the average content of antibody-AChR complexes from each rat. (B) (●---●) indicates the average clinical state of the animals (ranging from 0 for normal to + + + for moribund).

more than 70% after day 28 (Fig. 2). By day 10 there were approximately equal amounts of antibody free in serum and complexed with muscle AChRs. From day 6 to 8, when antibodies were first detected on muscle AChRs, the total amount of AChR extractable was significantly decreased. However, by days 9 and 10 total amounts of AChR were in the normal range. The yield of AChR on days 11 and 12, and in some instances through day 14, was significantly greater than normal. This increase was followed by a progressive decrease in the total amount of AChR. Clinical weakness was first observed on day 7, with recovery in most animals by day 10, and recurrence starting approximately day 28.

Discussion

The antigenicity of mammalian skeletal muscle AChR has been established by specific interaction with antibodies derived from both patients with MG (8, 17, 22–24) and from animals immunized with AChR isolated from electric organs of fishes (2–8, 11, 14, 15). This paper presents the first evidence of the immunogenicity of mammalian muscle AChR. The immunogenicity of syngeneic muscle AChR was demonstrated in Lewis rats by its capacity to induce autoantibodies and clinical and electrophysiological signs of EAMG. Autoimmunity was induced by an amount of muscle AChR comparable to the amount of eel AChR required to induce EAMG (3). Complexing of muscle AChR with antibody reduced its immunogenicity. The immunogenicity of mammalian muscle AChR may be enhanced by alterations in its structure during purification or emulsification in adjuvant. The demonstration that EAMG can be induced with syngeneic muscle AChR suggests that small alterations of muscle AChR conformation or structure, e.g. associated with viral infections, could render this membrane protein immunogenic in man. For the "spontaneous" occurrence of autoimmunity, predisposing immunogenetic factors may also be of importance.

The method we have developed for quantitating the total amount of AChR extractable from a rat has provided a reproducible normal value for the AChR content of this inbred strain. The equation of "^{125}I-α-bungarotoxin-binding sites" with "muscle AChRs" is justified by the fact that the toxin-binding material, which is solubilized in Triton X-100, has in common with nicotinic AChR of muscle (a) pharmacological specificity (as judged by inhibition with benzoquinonium), (b) antigenic identity (as judged by its precipitability with antibodies to purified AChR), and (c) molecular size (as judged by sucrose gradient sedimentation). The striking decrease in amount of AChR extracted from rats with chronic EAMG appears related to diminished content rather than technique of extraction. Firstly, interaction of AChR with antibody (either in vitro or in vivo) does not prevent binding of ^{125}I-toxin to the isolated receptor (toxin labeled AChR is routinely used to detect AChR, and antibody-AChR complexes formed in vivo were shown to retain the ability to bind toxin). Secondly, if AChR were insoluble because of extensive cross-linking by antibodies in situ, reduction and alkylation would be expected to enhance its solubilization, as had been demonstrated in a model system in which antibody was allowed to interact in vitro with AChR in situ in eel electroplax (21).

The changes that occur in a rat's AChR content with time after immunization (Fig. 2) can be interpreted in terms of what is known about the acute and chronic phases of EAMG. The initial decrease in AChR during the early acute phase of EAMG is no doubt largely due to extensive phagocytosis of the postsynaptic membrane by macrophages, which occurs at that time (9, 10). The muscle fibers during this acute stage of EAMG were shown to be functionally denervated, since direct nerve stimulation failed to elicit muscle responses in 90% of the fibers (12). Therefore, the increased yield of AChR around days 10 and 12, by which time the inflammatory response at the motor end-plate is subsiding (9, 10), is presumably due to synthesis of extrajunctional AChR which is known to occur as a consequence of denervation (25). It is known that blockage of neuro-muscular transmission for only 3 days with curare or toxin causes large in-

creases in extrajunctional AChR content of rat muscle (26). Repair synthesis of junctional AChR probably contributes also to the increased yield of AChR. Reinnervation after subsidence of the macrophage invasion would be expected to terminate extrajunctional AChR synthesis. This probably accounts for the rapid return of AChR yields to normal around day 13.

The continued gradual loss of AChR after the acute phase of EAMG may involve a process analogous to antibody-induced modulation of surface antigens, which has been extensively studied in other systems such as the lymphocyte (19). Normally, AChR in the motor end-plate appears to turn over very slowly (27), whereas extrajunctional AChR synthesized before innervation or after denervation turns over with a half-time of around 24 h (25, 28). Binding of antibody to AChR at the neuromuscular junction may trigger the removal of junctional AChR by mechanisms normally reserved for extrajunctional AChR. Rapid synthesis of junctional AChR in the chronic stage of EAMG would be a mechanism enabling a rat to survive in the chronic phase of EAMG and would also account for the constant small percentage of AChR which is not complexed with antibody despite high serum titers of antibody. The simplified morphology of the postsynaptic membrane in chronic EAMG may be the result of the dynamic balance between antibody-induced turnover of AChR and increased synthesis of new AChR.

In chronic EAMG a large proportion of the remaining AChR is complexed with antibody. In support of the direct evidence for this, which we have provided in this paper by extraction of antibody-AChR complexes from rat muscle, we have also been able to localize antibody in the end-plates of rats with EAMG using peroxidase-coupled anti-rat gamma globulin as a stain in light and electron microscopic studies (29). Because antibody to AChR applied to muscle (5, 11) or electroplax (4, 8, 14) in vitro inhibits AChR activity, it seems reasonable to expect that the functioning of these AChR bound with antibody in vivo may be partially or completely impaired. It is probable that there are several antigenic sites on AChR, and antibodies bound to different determinants on AChR may result in effects on AChR activity ranging from negligible to complete block. Our results suggest that one site to which antibody is not bound is the toxin-binding site (i.e., acetylcholine-binding site). Nonetheless, in the membrane, antibody bound at other sites might allosterically reduce or abolish binding of ACh. Antibody binding could leave ACh binding completely intact, but partially inhibit AChR activity by decreasing the duration of ionophore opening or its conductance when open. It is known that ACh analogues of differing affinity and efficacy differ from ACh in both the duration of channel opening which they induce (30) and in the conductance of the open channel (31). Since AChR function is effected by structural differences in these small ligands, and even by membrane potential (32), it seems likely that binding of a large molecule-like antibody should alter AChR function. Thus it will be important to study the properties of AChR ionophores in these muscles.

Because of the similarities between EAMG and human MG, the methods described in this paper for examination of rat AChR have been applied to muscle from humans with MG. We have found greatly reduced amounts of AChR and found that much of the remaining AChR is complexed with antibody (Lindstrom

and Lambert, manuscript in preparation). In further support of these results, we have recently shown extensive reduction in the area of postsynaptic membrane which can be stained with α-bungarotoxin coupled to peroxidase, both in humans with MG and rats with EAMG (29). These results constitute critical similarities between rats with chronic EAMG and humans with MG, suggesting the critical importance of decreased AChR content to impairment of neuromuscular transmission in both diseases.

Summary

Immunization of Lewis rats with acetylcholine receptor (AChR) purified from either *Electrophorus electricus* electric organ or syngeneic rat muscle induced experimental autoimmune myasthenia gravis (EAMG). This was demonstrated by clinical signs of weakness and by electromyographic evidence of impaired neuromuscular transmission. The amount of rat AChR required to induce an autoimmune response was comparable to the amount of eel AChR required. In vitro complexing of rat AChR with antibody reduced its immunogenicity. Autoantibody to muscle AChR was present in serum and complexed with AChR in muscle. Antibody was not bound to the ACh binding site of AChR, since antibody-AChR complexes extracted from muscle could still bind ^{125}I-α-bungarotoxin. The amount of AChR extracted from muscle of rats with EAMG was diminished. The amount of AChR and antibody-AChR complexes in muscle was measured at intervals after immunization with eel AChR. The amount of AChR decreased in rats with acute EAMG, then transiently increased to more than normal amounts during remission, and finally decreased to only about 20% of normal in rats with chronic EAMG. At least half of the AChR remaining in animals with chronic EAMG was complexed with antibody. Thus, both a decrease in amount of AChR and the formation of antibody-AChR complexes contribute to impairment of neuromuscular transmission in rats with EAMG. The possible mechanisms involved in the changes in AChR content are discussed.

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