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Accelerated degradation of acetylcholine receptor from cultured rat myotubes with myasthenia gravis sera and globulins

(acetylcholine receptor/muscle culture/receptor antibody/membrane protein turnover)

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ABSTRACT **Altered geometry of the neuromuscular junction and a decreased number of acetylcholine receptors appear responsible for the defect of neuromuscular transmission in myasthenia gravis. We have used cultured rat myotubes as a model to study *in vitro* the potential role of myasthenic globulins in the pathological process. Acetylcholine receptor content was assayed by the extent of ^{125}I-labeled α-bungarotoxin binding, and acetylcholine receptor function was assayed by the sensitivity to acetylcholine iontophoresis. The half-life of the acetylcholine receptor was 18.5 hr in the presence or absence of control sera. Myasthenic sera and globulins produced a gradual reduction in acetylcholine receptors, as assessed by biochemical and electrophysiological techniques. The half-life in the presence of myasthenic sera was 6 hr. The accelerated turnover was unaffected by puromycin but was slowed by lowered temperature (18–20°), interference with energy metabolism (2,4-dinitrophenol), and interference with cytoskeletal structures (colchicine and cytochalasin B). We found no electrophysiological evidence to suggest globulin blockade of acetylcholine access to the acetylcholine receptor. Our observations suggest that circulating globulins in myasthenia gravis may contribute to the functional defects of neuromuscular transmission by accelerating the rate of internalization and degradation of surface membrane acetylcholine receptors.**

Myasthenia gravis (MG) is a remitting and relapsing neuromuscular disease of human beings characterized by muscle fatigability that increases with exertion and improves with rest. Recent studies have demonstrated altered morphology (1), decreased acetylcholine sensitivity (2, 3), and decreased number of acetylcholine receptors at the motor endplate of myasthenic muscle (4), suggesting that abnormalities of the postsynaptic membrane are responsible for the defect in neuromuscular transmission. The immune response to purified acetylcholine receptors in animals produces an experimental disease, experimental autoimmune myasthenia gravis (5), which is similar to human MG by morphologic, electrophysiologic, and clinical criteria (6–8). This animal model suggests that autoimmune factors involving the acetylcholine receptor may play a major role in the pathogenesis of human MG. Further evidence for an immune origin of human MG is provided by the presence of acetylcholine receptor antibodies in the sera of most myasthenic patients (9, 10).

The specific role of antireceptor antibodies in the pathogenesis of MG is not known. The simplest hypothesis would be that the myasthenic globulin directly blocks the acetylcholine site on the acetylcholine receptor. However, no evidence exists to support this hypothesis. Evidence does suggest that serum globulins from a limited number of patients with MG may block α-bungarotoxin binding to the acetylcholine receptor (11, 12), but no data demonstrate that these globulins block acetylcholine access to the receptor or acetylcholine sensitivity of the receptor. In fact, myasthenic globulin appears to bind to sites on the acetylcholine receptor other than the acetylcholine binding site (13); and myasthenic globulin applied *in vitro* does not decrease the acetylcholine sensitivity of junctional or extrajunctional receptors in human, rat, or frog muscle (2).

Nevertheless, myasthenic globulin appears to have a definite effect since intraperitoneal injections into mice over a 2-week period result in reduced miniature endplate potentials (14), and sera from animals with experimental autoimmune MG transfer many of the functional alterations of that disease to previously unaffected animals (15). Thus, the antibodies may indeed play an etiologic role in the neuromuscular defect in MG, not by masking the acetylcholine site of the acetylcholine receptor, but by some other mechanism.

The present studies were undertaken to develop a model to explore *in vitro* the way in which myasthenic antibody might reduce the number of acetylcholine receptors and the sensitivity to acetylcholine. Our results are compatible with an antibody-induced acceleration of internalization and/or degradation of surface membrane acetylcholine receptors and may help to explain the mechanism by which immunologic factors contribute to the functional defects in MG.

Abbreviation: MG, myasthenia gravis.

METHODS

Cultures

Skeletal muscle cells from newborn rats were cultured on collagen-coated, 60-mm petri dishes by the technique of Yaffe (16). The cells were dissociated with 0.05% trypsin. Cultures were enriched in myoblasts by differential adhesion. The final plating density was 5×10^6 cells per plate. Culture medium was Dulbecco's modified Eagle's medium with 8 g of glucose per liter, 9% (vol/vol) fetal calf serum, 0.5% (vol/vol) embryo extract (GIBCO), and 50 mg of gentamycin per liter. Cultures were maintained at 37° in 5% CO_2/95% air. Myoblasts began to fuse at 3 days and experiments were performed on 5- to 6-day-old myotube cultures.

Pooled sera from two patients with MG demonstrated to have high titers for acetylcholine receptor antibody by radioimmunoassay and pooled sera from two normal individuals were used in most experiments. When globulins were used, they were isolated in a 33% ammonium sulfate precipitate, and IgG was further purified by DEAE-cellulose chromatography as described by Flier *et al.* (17). ^{125}I-Labeled α-bungarotoxin was purified and labeled as described (13). The specific activity was approximately 6000 cpm/pmol.

Assays for acetylcholine receptor content

Binding of α-Bungarotoxin after Incubation with Sera (Postlabeling Technique). Aliquots of normal or myasthenic sera were added directly to the culture media and incubations were continued for varying time periods. Thereafter, cultures

were washed twice with carbonate-buffered saline (pH 7.5) at room temperature. Acetylcholine receptors were labeled by the technique of Devreotes and Fambrough (18) with slight modification. ^{125}I-Labeled α-bungarotoxin, 50 nM, in 2 ml of carbonate-buffered saline was added to the cultures, which were incubated for 60 min at 37° except when otherwise specified. The cultures were washed with carbonate-buffered saline until the supernatants were free of radioactivity. Acetylcholine receptors were extracted from the cultures by incubation with 1.5 ml of 1% Triton X-100 in 10 mM Tris·HCl (pH 9.5) for 90 min at room temperature. The extracted material was centrifuged at 16,500 × *g* for 15 min and the supernatant was assayed for radioactivity in a Packard autogamma spectrometer. As described by Devreotes and Fambrough (18), greater than 95% of the radioactivity was in the supernatant.

Binding of α-Bungarotoxin before Incubation with Sera (Prelabeling Technique). Myotube cultures were incubated with 50 nM ^{125}I-labeled α-bungarotoxin in carbonate-buffered saline for 60 min at 37°. The cultures were washed until the supernatant was free of radioactivity and then incubated with normal or myasthenic sera at 37° for varying periods of time. In some cases, the incubations with sera were at 18–20°. After incubation with sera, the cultures were washed twice with carbonate-buffered saline and extracted. The extracted material was then washed in an identical manner as in the postlabeling experiments.

Two different assays were used to determine whether radioactivity in the Triton X-100 extract was complexed to acetylcholine receptors. (*i*) Aliquots of the extracts were used in a previously described radioimmunoassay for acetylcholine receptor (9, 13). Extracts, whether previously exposed to normal or myasthenic sera, were incubated with myasthenic sera for 30 min at 37° and an additional 14 hr at 4°. Thereafter, rabbit antihuman IgG was added and the incubation continued for 3 hr at 37°. The precipitate was sedimented, the pellet was washed three times, and the radioactivity in the pellet and supernatant was determined.

(*ii*) Additional aliquots of the detergent extracts were used to determine whether radioactivity sedimented as free toxin or as a macromolecular complex. The aliquots of extract were applied to a 5–20% sucrose gradient containing 100 mM NaCl/50 mM Tris·HCl, pH 7.4/1% Triton X-100. Centrifugation was in a Beckman SW41T rotor at 40,000 rpm for 14 hr.

Assays for acetylcholine receptor function

Intracellular recording was performed with KCl-filled microelectrodes of 20–40 Mohm resistance, and the acetylcholine sensitivity was measured iontophoretically using microelectrodes filled with 2 M acetylcholine with a resistance of 50–200 Mohms. In the cultured cells acetylcholine receptors were located over the entire surface of the myotubes. The acetylcholine sensitivity was measured at several points and the average sensitivity was calculated. The sensitivity was expressed in mV/nC; and corrected for variation of membrane potential. Recordings were made at 23° in culture medium.

RESULTS

α-Bungarotoxin interacts specifically and in a slowly reversible manner with the nicotinic acetylcholine receptor. In the present experiments, after exposure of 5- to 6-day-old myogenic cell cultures to ^{125}I -labeled bungarotoxin and thorough washing of the cultures and detergent extraction, virtually all radioactive toxin appeared to be in macromolecular complexes with characteristics of the acetylcholine receptor. On sucrose gradients, the macromolecular toxin complexes sedimented as 10–11S material. In all experiments, less than 10% of the total radioactivity remained at the top of the gradient and was recoverable either as free ^{125}I-labeled α-bungarotoxin or breakdown products. Furthermore, greater than 84% of the bound radioactivity was precipitated by the addition of myasthenic sera followed by rabbit antihuman IgG serum. For all subsequent experiments assessing the effects of normal or myasthenic sera on surface receptors of cultured rat myotubes, the specificity of ^{125}I-labeled α-bungarotoxin binding was checked with sucrose gradient velocity sedimentation and immunological precipitation. In all instances there appeared to be good agreement between the total bungarotoxin radioactivity in the detergent extracts and the radioactivity migrating as macromolecular complexes on sucrose density gradients or recoverable in precipitates in the radioimmunoassay.

In the first experiments, cultures were incubated with normal or myasthenic sera, washed, and exposed to toxin. With this postlabeling technique, myasthenic sera produced a dramatic decrease in the number of acetylcholine receptors. However, it was difficult to differentiate between an effect of antibody blockade on bungarotoxin binding and a direct effect of acetylcholine receptor internalization and degradation. Further experiments demonstrated that this postlabeling technique was monitoring both effects. Incubations were carried out with myasthenic sera for varying time periods (from 0 to 8 hr). The decrease in α-bungarotoxin binding followed a first-order exponential decay with a half-life of 5.5 hr. However, extrapolation of the exponential decay back to a theoretical zero time yielded values 25–35% less than the actual zero time suggesting two processes: (*i*) a partial (25–35%) immediate blockade of α-bungarotoxin binding, and (*ii*) a more gradual decrease of α-bungarotoxin binding sites with a half-life of 5–6 hr.

In order to monitor the effects of myasthenic sera independent of any effects on blockade of α-bungarotoxin binding, a prelabeling technique was used (18). With this technique, incubation with ^{125}I-labeled toxin prior to incubation with sera prevents simple masking of blockade from interfering with estimates of the number of remaining toxin binding sites. In the absence of sera, the disappearance of bound radioactivity followed a first-order exponential decay. In the presence of normal human sera, the half-life averaged 18.5 hr (Table 1 and Fig. 1) and no significant difference was noted in the presence or absence of normal sera. When rat myotube cultures were prelabeled and exposed to myasthenic sera for equivalent time periods, the disappearance of bound toxin was significantly increased. The half-life in the presence of myasthenic sera averaged 6 hr, and in all situations the disappearance of bound toxin fit an exponential decay function. Extrapolation of the exponential curve back to zero time gave a calculated zero time essentially identical to the experimental zero time. Thus, the major effect of the myasthenic sera appeared to be an acceleration of the disappearance of acetylcholine receptor, and in all subsequent experiments the prelabeling technique was used.

A 1:10 dilution of the myasthenic sera still yielded a more rapid disappearance than noted with normal sera. For example, in experiment 7 the half-life for acetylcholine receptors was 10.7 hr with undiluted myasthenic sera and 14.1 hr for the diluted myasthenic sera, compared to 19.3 hr for normal sera.

When the exponential decay rates were calculated from the results of the radioimmunoassay, similar half-lives for the acetylcholine receptor were achieved (Table 2).

Centrifugation of the detergent extracts in sucrose density gradients demonstrated that the majority of radioactive bun-

Table 1. Effect of myasthenic or normal sera on decay of labeled acetylcholine receptor

Experiment	Normal sera		Myasthenic sera	
	Rate constant (hr^{-1})	$T_{1/2}$ (hr)	Rate constant (hr^{-1})	$T_{1/2}$ (hr)
1	—	—	0.123	5.6
2 (postlabel)	—	—	0.087	8.0
3	0.033	21.0	0.156	4.4
4 (postlabel)	—	—	0.091	7.6
5	0.044	15.8	0.200	3.5
6	0.037	18.7	0.087	7.9
7	0.036	19.3	0.065	10.7
Mean ± SEM	0.0375 ± 0.002	18.5	0.115 ± 0.018	6

In each experiment, duplicate cultures were incubated for varying time periods (2, 4, 6, and 8 hr in experiments 1–5, and 1, 4, 7, and 24 hr in experiments 6 and 7) in the presence of 0.43 ml of pooled myasthenic or normal sera. Experiments 2 and 4 used the postlabeling techniques, other experiments used the prelabeling techniques. The radioactivity remaining in detergent extracts at each time point was used to calculate the rate constant for decay by fitting the data to the exponential function $Y = ae^{bx}$ by the method of least squares. In all cases the data fit the equation with a correlation coefficient >0.97, which yields a $P < 0.01$.

garotoxin migrated as macromolecular complexes regardless of whether the prelabeling or postlabeling technique was used (Fig. 2). The radioactive toxin migrated as 10–11S particles with a suggestion of some higher molecular weight aggregates. When myotubes were prelabeled and then incubated with myasthenic sera, the radioactivity migrated in a more heterogeneous fashion. The vast majority of radioactivity appeared to be greater than 9 S and was skewed toward the more dense regions of the gradient. This asymmetry is most probably related to the aggregation of myasthenic acetylcholine receptor antibodies associated with the receptor, although we cannot rule out the

Table 2. Comparison of receptor turnover estimated from total bungarotoxin binding and from radioimmunoassay

Time of incubation (hr)	Detergent extract (cpm)	Radioimmunoassay pellet (cpm × 10)
	Normal sera	
1	6076	1303
4	4919	1238
7	4050	848
24	2511	472
Rate constant	0.037 hr^{-1}	0.044 hr^{-1}
$T_{1/2}$	18.7 hr	15.8 hr
	Myasthenic sera	
1	5817	1086
4	4140	655
7	2382	382
24	727	156
Rate constant	0.087 hr^{-1}	0.077 hr^{-1}
$T_{1/2}$	7.9 hr	9 hr

Duplicate cultures were preincubated in ^{125}I-labeled bungarotoxin, washed, and then incubated in 0.43 ml of normal or myasthenic sera at 37° for specified time periods. Detergent extracts were prepared and radioactivity was determined. Aliquots were used in a radioimmunoassay as described in *Methods*. In these experiments approximately 85% of the radioactivity was precipitated by the addition of myasthenic sera and rabbit anti-human IgG sera. For each point, the duplicates differed by less than 5%. The least squares method was used to determine the exponential decay rate.

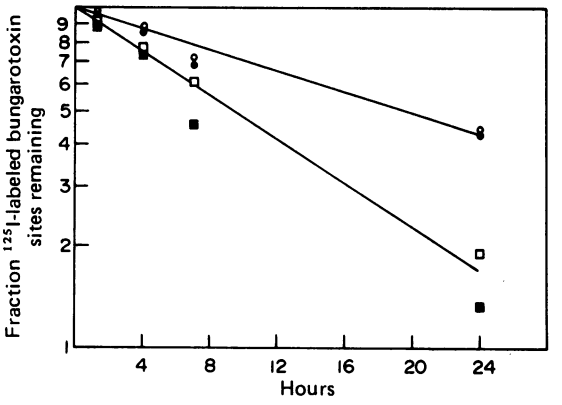

FIG. 1. Data points are the mean of duplicate cultures from experiments 6 and 7 presented in Table 1. The curves were fit to the exponential function by the method of least squares. The upper curve represents cultures incubated in normal sera; the lower curve represents cultures incubated in myasthenic sera.

contribution of receptor aggregation with minimal contribution from the antibodies.

Both the entire globulin fraction and the gamma globulin fraction of the pooled myasthenic sera were capable of accelerating the half-life of acetylcholine receptor to the same extent as the pooled sera itself. Furthermore, gamma globulins from three additional myasthenic sera with high titers of acetylcholine receptor antibodies were also capable of accelerating the half-life of acetylcholine receptor.

Electrophysiologic experiments were done to confirm the accelerated disappearance of acetylcholine receptor in the

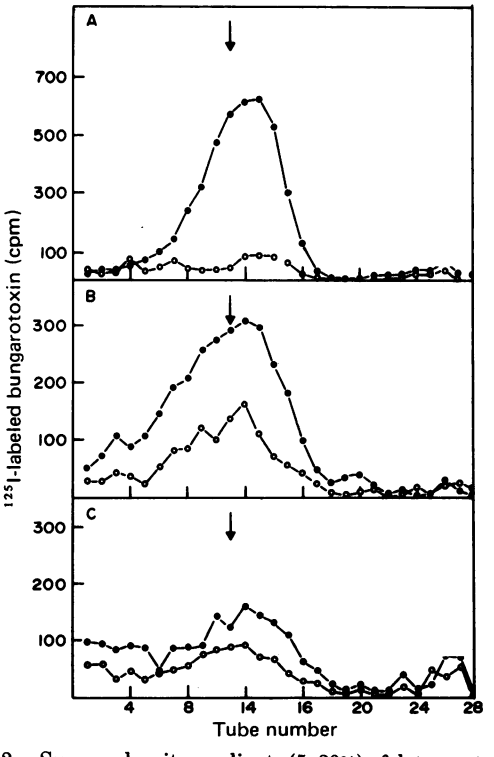

FIG. 2. Sucrose density gradients (5–20%) of detergent extracts of rat myotube cultures. The arrow indicates the peak of catalase migration. (*A*) Postlabeling technique. Aliquots of detergent extracts after incubation of cultures in normal (●) or myasthenic (○) serum for 24 hr. (*B*) Prelabeling technique. Aliquots of detergent extracts after incubation of cultures in normal sera for 1 (●) or 24 (○) hr. (*C*) Prelabeling technique. Aliquots of detergent extracts after incubation in myasthenic serum for 4 (●) or 7 (○) hr.

Table 3. Effects of various conditions on receptor turnover in normal or myasthenic sera

Condition	% of rate constant at 37°	
	With normal sera	With myasthenic sera
Temperature (18–20°)	30	22
Dinitrophenol (1 mM)	49	72
Puromycin (50 μg/ml)	83	82
Colchicine (100 μg/ml) + cytochalasin B (10 μg/ml) in 1% Me_2SO	88	57
Colchicine (100 μg/ml)	—	100
Cytochalasin B (10 μg/ml)	—	70

After labeling with radioactive toxin and extensive washing, the cultures were exposed to the varying conditions for 2 hr before addition of 0.43 ml of normal or myasthenic sera. They were then incubated for 1, 4, 7, and 24 hr at 18–20° or 37°, washed, and extracted with detergent. The rate constants of decay with the different conditions were compared to rate constants from duplicate cultures incubated for 1, 4, 7, and 24 hr in normal and myasthenic sera at 37°. The dimethylsulfoxide (Me_2SO) vehicle for solubilizing cytochalasin B had no effect *per se* on α-bungarotoxin binding or site turnover.

presence of myasthenic sera. Despite the partial blockade of α-bungarotoxin binding when pooled myasthenic sera were added first, we found no evidence to suggest that the myasthenic sera interfered with access of acetylcholine to the receptor. In the first hour, no effect could be demonstrated. Four hours after the myotubes were incubated with myasthenic sera at 37°, the average acetylcholine sensitivity had decreased by 40%. By 22 hr after the application of myasthenic sera, the acetylcholine sensitivity had been reduced to 5% of the control rates. In cultures exposed to normal sera for 22 hr, the acetylcholine sensitivity averaged 399 mV/nC, while in cultures exposed to myasthenic sera the acetylcholine sensitivity averaged 27 mV/nC. Under these same conditions, there was no change in resting membrane potential. No evidence was found to suggest an aggregation of acetylcholine receptors into "patches" or asymmetrically located "caps" before or during the exposure to myasthenic sera.

The cultures were exposed to several conditions that had been reported to influence acetylcholine receptor turnover in rat myotubes (Table 3) (18). Decrease in the incubation temperature to 18–20° slowed the disappearance of bound toxin in the presence of normal and myasthenic sera. Puromycin had only minimal effects. 2,4-Dinitrophenol slowed receptor degradation with both normal and myasthenic sera, although the effect was less marked in the presence of myasthenic sera. Simultaneous addition of 100 μg of colchicine and 10 μg of cytochalasin B per ml had a small but reproducible effect on receptor turnover in rat myotubes exposed to normal human sera, similar to the results obtained by Devreotes and Fambrough in the presence of human sera (18). Colchicine and cytochalasin B had a greater influence on the accelerated turnover of acetylcholine receptor with myasthenic sera. When the effects of colchicine and cytochalasin B were examined separately in cultures exposed to myasthenic sera, only cytochalasin B significantly slowed acetylcholine receptor turnover.

The inhibitory effects of temperature, dinitrophenol, and colchicine and cytochalasin B suggest that the accelerated turnover of acetylcholine receptor in the presence of myasthenic sera is dependent upon the same mechanisms that appear to mediate receptor turnover under control conditions. Both situations seem to be influenced by the temperature of incubation, the levels of intracellular energy, and the integrity of microfilaments and possibly microtubules. The more accelerated half-life with myasthenic sera appeared more sensitive than the slower half-life with normal sera to perturbations of the membrane and its underlying cytoskeletal structure.

DISCUSSION

In the present experiments, myasthenic sera and their gamma globulins accelerated the disappearance of acetylcholine receptors from the surface of cultured rat myotubes monitored both electrophysiologically and biochemically. Throughout the incubation, the myotubes retained a healthy appearance morphologically and demonstrated no alteration in resting membrane potential or electrical excitability even after 22 hr of exposure to myasthenic sera. When the myasthenic sera were added before the toxin, a partial blockade of toxin binding was noted. However, no blockade was noted electrophysiologically. Thus, even if globulins from a small number of myasthenic patients sterically hinder access of an 8000-molecular-weight molecule (α-bungarotoxin), these same globulins do not hinder access of a 150-molecular-weight molecule (acetylcholine). The blockade does not contribute to the accelerated degradation of receptor and probably plays no role in the pathophysiology of MG.

The accelerated disappearance of acetylcholine receptor appears to result primarily from accelerated degradation rather than inhibited synthesis. Furthermore, those conditions known to inhibit lectin and antibody-induced internalization of lymphocyte surface constituents (decreased temperature, dinitrophenol, and cytochalasin B) (19–23) also inhibit acetylcholine receptor degradation (18). The receptors do not appear to be released extracellularly since no toxin bound to macromolecular complexes could be demonstrated in the incubation media.

The present tissue culture model supports the etiological role of circulating antibodies in the decreased content of acetylcholine receptor in human myasthenic muscle and suggests internalization and degradation as one of the important mechanisms that may decrease the number of receptors on postsynaptic folds *in vivo*. However, *in vivo* there is sequestration of injured junctional membrane into the synaptic space, and it is not clear that immune complexes attached to terminal expansions of junctional receptors that differ in cytoarchitecture and in receptor half-life (5–7 days) (24–26) would be handled in the same way as those attached to extrajunctional receptors *in vitro*. Nevertheless, the present model provides a sensitive system for assay of the specificity and etiological significance of acetylcholine receptor antibodies. It also offers the opportunity to study fused myotubes with minimal acetylcholine receptors and acetylcholine sensitivity yet normal resting membrane potentials and normal electrical excitability.

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