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Antibodies from myasthenic patients that compete with cholinergic agents for binding to nicotinic receptors

(myasthenia gravis/acetylcholine receptor/chicken embryo skeletal muscle cells/antibodies against acetylcholine receptor/IgG_3/cholinergic ligands)

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ABSTRACT **We have purified immunoglobulins from sera of myasthenic patients and have identified antibodies directed against the cholinergic ligand-binding site of the nicotinic acetylcholine receptor. In the serum of one patient analyzed in detail these antibodies belonged to the IgG_3 class, and their effects were as follows: (*i*) In chicken embryo myogenic cultures, antibody binding was both competitive with ^{125}I-labeled α-bungarotoxin and irreversible on a time scale of hours. (*ii*) ^{125}I-Labeled α-bungarotoxin was not displaced by antibody from preformed complexes and, conversely, antibody was not displaced by toxin. (*iii*) Antibody binding was competitive with some, but not all, nicotinic agents. Thus, acetylcholine, carbamoylcholine, and dimethyltubocurarine competed effectively whereas decamethonium and hexamethonium did not, suggesting that the two classes of nicotinic ligands probably interact at different, nonoverlapping receptor subsites. (*iv*) There was no competitive binding between these antibodies and the muscarinic antagonist atropine. (*v*) Both this class of myasthenic immunoglobulins and rabbit antibodies raised against *Torpedo* acetylcholine receptors increased the rate of receptor degradation. However, synthesis and degradation remained coupled and there was a compensating increase in receptor synthesis. We propose that immunoglobulins directed against the ligand-binding site of acetylcholine receptors may account for the characteristic curare-like symptoms of early myasthenia and their response to cholinesterase inhibition, for the apparent decrease in receptors measurable by ^{125}I-labeled α-bungarotoxin binding, and for initiating localized complement activation at the postsynaptic membrane.**

The outstanding features of myasthenia gravis (MG) are weakness and abnormal fatiguability of voluntary muscles. Circulating antibodies directed against neuromuscular nicotinic acetylcholine (AcCho) receptors have been detected in about 90% of MG patients (1), and it is generally held that these immunoglobulins give rise to the neuromuscular deficiency by decreasing the concentration of postsynaptic AcCho receptors. Complement-mediated membrane destruction and antigenic modulation of AcCho receptor metabolism are considered to be the mechanisms responsible for reduction of receptor density (2). Although this version of the pathogenesis of MG is consistent with a large body of evidence (for a review, see ref. 3), it is not fully satisfying, first, because circulating antibody titers do not correlate well with clinical severity, and second, because it does not entirely rationalize the predominant early symptoms in some forms of the disease for which no structural damage in the synapse has been demonstrated. These symptoms, and their prompt reversal by cholinergic agents, resemble a transient curare-like neuromuscular block.

One implication of such a pharmacologic blockade is the possible existence of immunoglobulins directed against the ligand-binding site of the AcCho receptor, a potentially operative mechanism already envisioned for MG (4). Such molecules would be expected to compete with cholinergic ligands and elapid neurotoxins for receptor binding; they would escape detection by the usual AcCho receptor immunoassays, which depend on complex formation with radioactive α-bungarotoxin (α-BuTx) and accordingly identify only antibodies directed against sites external to the ligand-binding region (5). Thus, antibodies directed against the cholinergic binding site (ABS) should be detected by their presumed ability to compete with ^{125}I-labeled α-BuTx (^{125}I-α-BuTx) binding to cellular, membrane-bound, or soluble AcCho receptors (5). The ability of Ig fractions from some myasthenic patients to inhibit α-BuTx binding has been noted in several reports; however, the inhibition was only partial, irrespective of serum concentration, and the dose–response relationships expected for competitive binding were not observed (1, 6, 7). Lefvert and Bergstrom (7) showed that these effects were, in fact, produced by IgG molecules, specifically by the IgG_3 subclass, but the cholinergic variables of this reaction remained to be explored.

The preceding considerations prompted us to analyze Ig from myasthenic sera specifically for ABS antibodies, and we have found them in each of four patients so far examined. We have used membrane-bound rather than soluble AcCho receptors in chicken embryo myogenic cultures to reduce the possibility, suggested by some observations, that antibodies directed against remote portions of the receptor molecules may influence the properties of the ligand-binding site (8). The immunoglobulins purified from the plasma of one patient were studied in detail, and, apart from reducing ^{125}I-α-BuTx binding in a concentration-dependent manner, the ABS antibodies showed an interesting pattern of binding competition with small nicotinic ligands that may provide some insight into different AcCho receptor ligand-binding domains.

EXPERIMENTAL PROCEDURES

Materials. Chemicals were obtained from the following sources: protein A-Sepharose from Pharmacia; physostigmine salicylate from Merck; acetycholine chloride and atropine sulfate from Aldrich; hexamethonium chloride, decamethonium bromide, and dimethyltubocurarine iodide from K & K; and carbamoylcholine chloride from Nutritional Biochemicals.

Cell Cultures. Chicken embryo skeletal myogenic cultures were prepared and maintained as described (9). Cultures of different ages were used in different experiments. Because

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Abbreviations: AcCho, acetylcholine; ^{125}I-α-BuTx, ^{125}I-labeled α-bungarotoxin; MG, myasthenia gravis; ABS antibodies, antibodies directed against the cholinergic binding site.

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AcCho receptor metabolism changes systematically as a function of culture age (10), the absolute values for receptor half-life can be compared only within, but not between, experiments.

Labeling of Cell Surface AcCho Receptors by ^{125}I-α-BuTx. Surface AcCho receptor in myogenic cultures was labeled as follows: cultures were incubated for 45 min at 37°C in Dulbecco's modified Eagle's medium supplemented with heat-inactivated (56°C, 30 min) horse serum (10% vol/vol) and glutamine (1 mM) (complete medium) and containing ^{125}I-α-BuTx [7 nM; specific activity, 136,000 cpm/pmol, purified and radioiodinated as described elsewhere (11)]. Radioactive medium was aspirated and cultures were rinsed four times with 2.5 ml of ice-cold phosphate-buffered saline to remove nonspecifically bound radioactivity. Cells were collected twice in 2 ml of 0.1 M NaOH, and total radioactivity was measured in a Packard γ spectrometer. The results were corrected for radioactivity bound to culture dishes in the absence of cells (routinely less than 60 cpm).

Determination of Anti-AcCho Receptor Antibody. Titers for antibody against human or chicken AcCho receptor were measured by the method of Monnier and Fulpius (12).

Preparation of Human Ig. Plasma or serum was obtained from patients suffering from moderate to severe generalized MG. Immunoglobulins were precipitated at 40% of saturation with ammonium sulfate, further purified by chromatography on DEAE-Sephadex A50 according to Harboe and Ingild (13), lyophilized, and stored at −20°C. They were dissolved directly in the preincubation medium before use. Clear solutions were obtained for Ig concentrations of at least 3 mg/ml.

Preincubation of Muscle Cells with Human Ig. Under our conditions, myoblast fusion is complete within 3 days of plating, and in this work cultures 4–10 days old were used. They were rinsed twice with 2.5 ml of modified Eagle's medium and incubated for 90–120 min at selected temperatures in complete medium containing human Ig (0.25–10 mg/ml), then rinsed four times with 2.5 ml of modified Eagle's medium. Unless otherwise specified, 60-mm dishes were used. All determinations were performed in duplicate, but measurements of AcCho receptor half-life were in triplicate.

Chromatography of Human Ig on Protein A-Sepharose. Lyophilized immunoglobulins (60 mg) were dissolved in 20 ml of phosphate-buffered saline and sterilized by filtration through a 0.45-μm Millipore filter. A 12-ml aliquot was placed onto a 3-ml protein A-Sepharose column equilibrated with phosphate-buffered saline, and the effluent was collected and combined with a subsequent phosphate-buffered saline wash. The adsorbed immunoglobulins were eluted with 0.1 M glycine·HCl (pH 3.0), collected in 27-drop fractions in tubes containing three drops of Tris-OH (1.0 M), and finally pooled. Aliquots of the starting Ig solution and of both the unadsorbed and eluted fractions were dialyzed against modified Eagle's medium overnight at 4°C, filtered through 0.45-μm Millipore filters, supplemented with horse serum (10% vol/vol) and glutamine (1 mM), and used to preincubate myogenic cultures.

RESULTS

The four myasthenic patients studied in this work were selected because plasma or serum was available in the quantities needed for obtaining the required amounts of purified Ig. The plasmas or sera were tested in the usual immunoassay procedure for antibodies against AcCho receptor by using both human and chicken AcCho receptor–^{125}I-α-BuTx complexes (12). They contained antibodies that reacted with the human AcCho receptor–^{125}I-α-BuTx complexes, whereas no antibodies were detectable with the corresponding chicken AcCho receptor complexes. The same characteristic pattern was found in 21 out of 22 other human myasthenic sera (S. Cuénoud, personal communication). Because this assay, by definition, reveals only antibodies directed against sites extrinsic to the cholinergic ligand-binding regions, it follows that the corresponding domains in human and chicken AcCho receptor did not crossreact immunologically, at least with these sera. All four plasmas and sera gave qualitatively identical results in blocking ^{125}I-α-BuTx binding to chicken myotubes (data not shown), but detailed experiments were performed with purified Ig from only a single patient (S. K.), a 23-year-old woman with severe generalized MG who had been treated by plasmapheresis. The three other sera remain to be studied in detail.

Interaction of Myasthenic Ig with Chicken Myogenic Cultures. When mouse or rat myogenic cultures are exposed to antisera obtained from rabbits immunized with purified *Torpedo* or *Electrophorus* AcCho receptor or to sera from myasthenic patients, the degradation rate of myogenic AcCho receptor–^{125}I-α-BuTx complex is accelerated (14–20). It is generally assumed that the modulation of AcCho receptor degradation by these sera is due to their content of anti-AcCho receptor antibodies. In the present report, the purified Ig preparation was initially tested in 4-day chicken embryo muscle cultures in which AcCho receptor was fully saturated with ^{125}I-α-BuTx. The Ig neither changed the half-life of receptor–toxin complexes nor reduced the amount of bound ^{125}I-α-BuTx. As shown below, the Ig competed for the α-BuTx-binding site; any discernible effect of the Ig on AcCho receptor turnover was blocked if the receptor was first saturated with toxin because the binding of antibody was thereby excluded. This failure to accelerate receptor degradation did not result from any defect in responsiveness of the system because the serum of a rabbit immunized against purified *T. marmorata* AcCho receptor (final concentration in culture medium, 1.25% vol/vol) reduced the half-life of AcCho receptor–^{125}I-α-BuTx complexes from 23 to 11 hr in 4-day cultures.

Although the myasthenic Ig studied neither modified AcCho receptor half-life nor displaced ^{125}I-α-BuTx from preformed complexes, it blocked subsequent ^{125}I-α-BuTx binding after a 90-min preincubation with the myogenic cultures. The dose–response curve is shown in Fig. 1. The observed inhibition was unaffected by repeated washing of the monolayer and was irreversible for at least 24 hr. Full inhibition also occurred in cultures pretreated with and maintained in dinitrophenol (5 mM) and NaF (2 mM), additions that block the decrease in surface AcCho receptor produced by anti-receptor antibodies

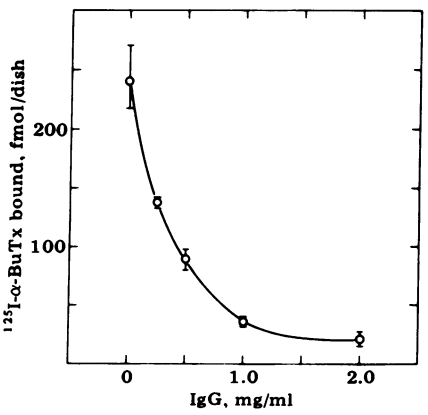

FIG. 1. Labeling of cell surface AcCho receptor by ^{125}I-α-BuTx after preincubation (90 min, 37°C) with different concentrations of myasthenic Ig. Eight-day cultures were used; each point is the mean of two determinations.

(16). Because the Ig preparation prevented the subsequent binding of ^{125}I-α-BuTx but did not displace toxin from preformed complexes, we conclude that the myasthenic Ig contained antibodies whose binding to AcCho receptor was mutually exclusive with that of α-BuTx and whose specificity might be directed against the receptor's ligand-binding site (ABS).

As a control for the specificity of the myasthenic Ig, we studied the properties of an identical Ig purified from the plasma of a normal healthy adult. The preparation was tested (at final concentrations of 1–10 mg/ml) in the experimental protocols described for myasthenic Ig, and in no case did it produce any reduction in or competition with α-BuTx binding.

Because mutually exclusive binding of antibody and α-BuTx could have accounted for the apparent failure of these ABS myasthenic Ig to alter AcCho receptor degradation, we performed further experiments designed to detect such modulation, had it occurred. The presence of multiple neurotoxin-binding sites (for a review, see ref. 21) suggested that under appropriate conditions myasthenic Ig and α-BuTx might be bound simultaneously to separate sites on individual receptor molecules. Accordingly, two groups of 10-day cultures were first treated with 1.75 nM ^{125}I-α-BuTx, a concentration that saturated about 50% of the available sites with toxin; one group was then further incubated with excess (2 mg/ml) myasthenic Ig to complex the remaining sites. The half-life of the AcCho receptor–^{125}I-α-BuTx complexes was reduced from 66 hr in the control to 46 hr in the Ig-treated cultures. On the other hand, when a group of cultures was first treated with 0.25 mg of myasthenic Ig per ml to saturate about 50% of the available sites with antibody then further incubated with excess (7 nM) ^{125}I-α-BuTx to complex the remaining sites, the half-life was not modified. Thus, the modulation of AcCho receptor half-life by ABS antibodies depended on the order of addition of the reagents. Accelerated degradation occurred under conditions that fostered the interaction of an antibody with two separate receptor molecules, but not when the ABS antibodies were free to form complexes at two sites within a single receptor. These results recall the finding that receptor degradation was accelerated by antibody-mediated crosslinking of AcCho receptor–^{125}I-α-BuTx complexes (22) and that apparently only receptors directly complexed with antibodies were affected (23).

In maturing myogenic cultures, the concentration of surface AcCho receptor reflects the coordinate regulation of synthesis and degradation such that increased destruction, stimulated by embryo extract or serum, is matched by a compensating change in receptor production (10). As seen in Table 1, rabbit antibodies against *Torpedo* AcCho receptor, which shortened AcCho receptor half-life, also increased receptor synthesis. Hence, if the same biosynthetic coupling exists *in vivo*, any measured decrease in AcCho receptor occurring in MG could not be explained simply by reduction of receptor half-life. Myasthenic Ig also increased receptor synthesis, but the effect was smaller than that found with rabbit antibodies against *Torpedo* AcCho receptor and can be considered only as suggestive pending further tests of other myasthenic and nonmyasthenic human Ig.

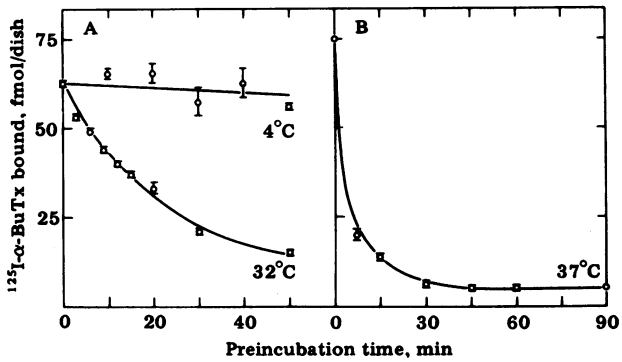

FIG. 2. Labeling of cell surface AcCho receptor by ^{125}I-α-BuTx after preincubation for different periods with myasthenic Ig (2 mg/ml). (*A*) Cells were incubated at 4°C and 32°C; (*B*) cells were incubated at 37°C. Five-day cultures were used. Initial values were measured in the absence of human Ig. Each point is the mean of two determinations.

The kinetics of myasthenic ABS antibody interaction with AcCho receptor at three different temperatures are presented in Fig. 2. After incubation with Ig for the indicated times at the respective temperatures, the cultures were washed and assayed for residual ^{125}I-α-BuTx binding under standard conditions at 37°C. The inhibitory interaction of Ig with AcCho receptor was strongly temperature dependent; the rate was rapid at 37°C ($t_{1/2}$ < 2.5 min), somewhat slower at 32°C, and very slow at 4°C. As expected for such reactions, in significant antibody excess the inactivation of AcCho receptor observed at 32°C was clearly first order (k_1 = 43.4 M^{-1} sec^{-1}), with a half-time of 20 min. This leads to an estimate of at least 1 ABS antibody for each 40,000 Ig molecules in the patient's plasma, assuming no selective loss or enrichment during purification.

Competition of Cholinergic Ligands with Myasthenic Ig for AcCho Receptor. We studied the effect of cholinergic drugs to test the inference that the myasthenic Ig was blocking ^{125}I-α-BuTx binding by occupying a portion of the ligand-

Table 1. Effect of antibodies against AcCho receptor on incorporation of new surface receptors

Supplement	^{125}I-α-BuTx bound after incubation for: 6 hr fmol/dish	%	10 hr fmol/dish	%	Synthesis during interval (6–10 hr) fmol/dish	%
Control rabbit serum	38.0	100	49.4	100	11.4	100
Rabbit serum against *Torpedo* AcCho receptor	41.0	108	60.7	123	19.7	173
Myasthenic Ig	37.4	98	52.1	105	14.7	129

Seven-day muscle cultures were incubated for 90 min with 1.2 ml of modified Eagle's medium containing control rabbit serum (25 μl/ml), rabbit antiserum against *Torpedo* AcCho receptor (25 μl/ml), or myasthenic Ig (10 mg/ml). Cultures were rinsed four times with modified Eagle's medium, incubated for 45 min with 2 ml of fresh complete medium containing 20 nM α-BuTx to block all of the surface AcCho receptors, again rinsed four times, and finally reincubated with fresh complete medium. The amount of newly incorporated surface AcCho receptor was determined by ^{125}I-α-BuTx binding performed under standard conditions between the 5th and 6th and 9th and 10th hours of incubation. The level of free surface receptor 1 hr after incubation with unlabeled toxin was 4.7 fmol/dish; that in control cultures not treated with α-BuTx was 165.6 fmol/dish.

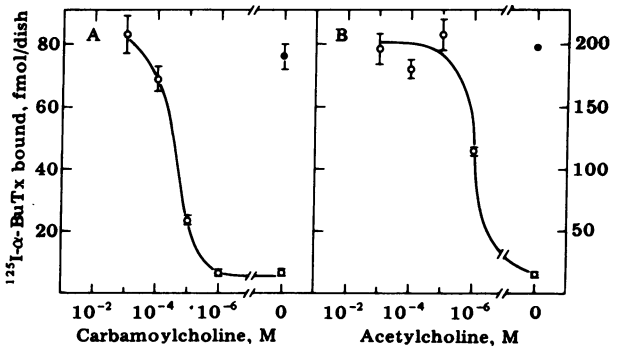

FIG. 3. Effect of two cholinergic agonists, (*A*) carbamoylcholine and (*B*) acetylcholine, on the inhibition of cell surface AcCho receptor labeling by myasthenic Ig (2 mg/ml). Eight-day (*A*) and 5-day (*B*) cultures were preincubated for 120 min at 37°C. The controls (●) were measured in the absence of human Ig. (*B*) The experiment was performed in the presence of physostigmine (0.1 mM). Each point is the mean of two determinations.

binding region of AcCho receptor; three nicotinic agonists (acetylcholine, carbamoylcholine, and decamethonium), two nicotinic antagonists (dimethyltubocurarine and hexamethonium), and the muscarinic antagonist atropine were examined. The cultures were exposed for 2 hr to the Ig in the presence of various drug concentrations, washed, and then assayed as usual for residual ^{125}I-α-BuTx binding activity. As seen in Figs. 3 and 4, low concentrations of the nicotinic drugs (carbamoylcholine, acetylcholine, and dimethyltubocurarine) protected the AcCho receptor in a dose-dependent manner against inactivation by Ig, whereas high concentrations of atropine, a muscarinic antagonist, gave little protection.

This finding established the nicotinic specificity of ligand competition with the receptor-inactivating myasthenic Ig. Of particular interest is the additional finding that the nicotinic bis-onium compounds, decamethonium (1 mM) and hexamethonium (1 μM–1 mM), respectively, an agonist and antagonist with distinctive pharmacologic properties, failed to protect the receptor against Ig inactivation.

Characterization of Myasthenic Ig That Inactivates Neurotoxin Binding to AcCho Receptor. The implication that circulating antibodies directed against the cholinergic binding region of AcCho receptor are present in myasthenic patients

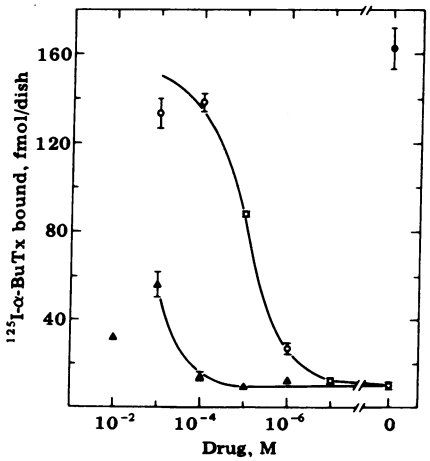

FIG. 4. Effect of two cholinergic antagonists, dimethyltubocurarine (nicotinic) (○) and atropine (muscarinic) (▲) on the inhibition of cell surface AcCho receptor labeling by myasthenic Ig (2 mg/ml). Six-day cultures were preincubated for 120 min at 37°C. The control (●) was measured in the absence of Ig. Each point is the mean of two determinations.

Table 2. Inhibition of the myasthenic IgG_3 effect by carbamoylcholine and rabbit anti-human IgG

Incubation conditions	Relative ^{125}I-α-BuTx binding
A. No Ig	1.0
B. Rabbit anti-human IgG	0.92
C. Myasthenic Ig	0.30
D. Myasthenic IgG_3	0.36
E. Myasthenic IgG_3 + carbamoylcholine	0.85
F. Myasthenic IgG_3 + rabbit anti-human IgG_3	0.91

Five-day cultures were incubated for 60 min at 37°C in phosphate-buffered saline supplemented with the following preparations (final concentration): (B) rabbit anti-human IgG (7.76 mg/ml); (C) myasthenic Ig (1 mg/ml); and (D) myasthenic patient's IgG_3 (0.115 mg/ml). IgG_3 were also incubated together with 1 mM carbamoylcholine (E) or preincubated with rabbit anti-human IgG (7.76 mg/ml) (F) for 30 min at 37°C and left on ice for 20 hr. The pellet was centrifuged (2000 × *g*, 4°C, 5 min) and the supernatant was added to the culture. In all cases cell surface AcCho receptor was measured by ^{125}I-α-BuTx binding in the standard way. ^{125}I-α-BuTx bound to control (A) was 170 fmol/dish.

depends critically on the proper identification of the responsible molecule as an Ig. The Ig preparation used in this work was strongly positive in immunodiffusion when assayed with goat anti-human IgG, but was negative in tests for IgA and IgM. A large sample (50 μg of protein), analyzed by $NaDodSO_4$/polyacrylamide electrophoresis under nonreducing conditions, showed a single Coomassie blue-stained band that migrated at a rate identical with that of an authentic IgG marker. When the myasthenic IgG preparations were fractionated on a column of protein A-Sepharose, all of the receptor-blocking activity was found in unabsorbed material that passed through the column. This suggests that the receptor-blocking antibody belonged to the IgG_3 subclass, consistent with the report of Lefvert and Bergstrom (7). The receptor-blocking activity of the IgG_3 preparation was precipitated by purified rabbit anti-human IgG (Table 2). These findings identify the responsible molecule as an IgG.

DISCUSSION

The simplest explanation of our results is that the myasthenic serum studied contains an antibody directed specifically against the ligand- and neurotoxin-binding site of AcCho receptor. This conclusion is supported by the presumptive identification of the active principle as an IgG of the IgG_3 type whose interaction with AcCho receptor was both mutually exclusive with α-BuTx and competitive with low concentrations of small nicotinic, but not muscarinic, ligands. The alternative explanation (namely, that the nicotinic ligands and the antibody induced conformational changes that were responsible for the mutually exclusive binding) cannot be formally excluded, but it is much more complex and is not suggested by any available evidence. We have not tested Ig preparations with AcCho receptor other than in chicken myogenic cultures, and it remains to be shown that the ABS antibodies will produce the same effects on human AcCho receptor. Nonetheless, the evolutionary retention of nicotinic pharmacology and neurotoxin binding at vertebrate neuromuscular junctions points to an underlying conservation of protein structure, and the apparent immunopharmacologic blockade suggested by the symptoms in MG makes it reasonable to expect confirmation of our results with human AcCho receptor.

If that proves to be the case, what is the relevance for MG of the antibody directed against the ligand-binding site? First, to the extent that the ABS antibodies of the type described here

might be occupying a fraction of AcCho receptor sites at any time, they would block neurotoxin binding used in the receptor assay and thereby account for all or part of the apparent reduction in AcCho receptor concentration previously found in muscle biopsies from myasthenic patients (24). This explanation for apparent AcCho receptor reduction seems more plausible, at least at an early stage of the disease, than acceleration of turnover because the observed increase in AcCho receptor degradation rates (14–20) does not exceed the demonstrated capacity of muscle cells for compensatory increases in receptor synthesis (10) and because such an increased rate of receptor synthesis in fact accompanies the change in half-life induced by antibodies (Table 1). A further enhancement of AcCho receptor synthesis would be expected from the muscle response to the "denervating" action of antibodies because the neuromuscular block resulting from occupancy of the ligand-binding site should accelerate the production of receptor by the muscle. Second, these antibodies suggest a testable working hypothesis both for the pathogenesis of MG and for its clinical and serological evolution. If ABS antibodies appeared early in MG, perhaps by the neoplastic development of an antibody-producing clone, they could account for the characteristic curare-like blockade; likewise, the reduction of ABS antibodies binding at elevated acetylcholine concentration (Fig. 3) might explain part of the symptomatic relief produced by cholinesterase inhibition. Further, the antibodies should initiate the local activation of complement; the resulting attack on the postsynaptic membrane would liberate AcCho receptor-containing fragments into the surrounding tissue and circulation, thereby presenting receptor molecules to the immune system and generating additional antibody responses to normally unexposed or inaccessible AcCho receptor antigenic determinants. This hypothesis, which can be tested by surveying a larger patient population, implies that ABS antibodies should be generally associated with early myasthenia and their circulating titers should be correlated with clinical severity at this stage. We do not doubt that accelerated receptor degradation occurs in MG, merely that it accounts less satisfactorily than ABS for the curare-like symptoms and some apparent decreases in measurable AcCho receptors.

The chicken myogenic cultures used in this work may be generally useful for detecting myasthenic ABS antibodies, perhaps owing to the reduced immunological crossreactivity affecting other receptor regions and the muscle membrane components. However, the procedure required relatively large amounts of high-titer Ig and its sensitivity is relatively low. Hence, the development of a suitably specific immunodiagnostic method is desirable.

The ABS antibodies are likely to be valuable probes of AcCho receptor structure and pharmacological properties, especially if there are differences among individual MG immunoglobulins. The finding that the bis-onium compounds, decamethonium and hexamethonium, fail to compete with ABS antibodies contrasts with the effects of the other nicotinic agents; moreover, it is surprising because the bis-onium compounds, like the other nicotinic drugs, compete effectively with neurotoxins for AcCho receptor binding (25). This pattern suggests the existence of two separate and nonoverlapping ligand-binding subsites on AcCho receptor. One subsite would bind acetylcholine, carbamoylcholine, dimethyltubocurarine, and ABS antibodies and the second would interact with bis-onium compounds; and complexes with α-neurotoxins might block all or part of both.

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