Effect of Myasthenic Immunoglobulin on Acetylcholine Receptors of Intact Mammalian Neuromuscular Junctions

Abstract. *Degradation of acetylcholine receptors of intact mouse neuromuscular junctions was determined in vivo and in vitro by the release of radioactivity from mouse diaphragms labeled with ^{125}I-α-bungarotoxin. Treatment of mice with immunoglobulin from myasthenic patients accelerated the degradation rate to approximately three times normal, in both intact animals and organ cultures. The released radioactivity was in the form of [^{125}I]tyrosine, confirming the nature of the degradative process. Accelerated degradation of acetylcholine receptors at neuromuscular junctions may represent an important antibody-mediated mechanism in myasthenia gravis.*

Myasthenia gravis is a neuromuscular disorder of humans characterized by muscle weakness and a tendency to fatigue. The established decrease in the number of acetylcholine (ACh) receptors at neuromuscular junctions in myasthenic patients (*1–5*) is sufficient to account for typical clinical and physiological manifestations of the disorder (*6*). The pathogenesis of this abnormality involves an autoimmune attack against ACh receptors (*7*). A humoral immune mechanism has been implicated in myasthenia by the discovery of circulating antibodies against ACh receptors (*8–11*), capable of reproducing the basic features of the disease after passive transfer to experimental mice (*12, 13*).

Antibody to the ACh receptor may act by increasing the rate of degradation of ACh receptors, as was observed in cultured skeletal muscle (*14–16*). However, important differences exist between the extrajunctional ACh receptors of cultured or denervated muscle and receptors at intact neuromuscular junctions with respect to physical properties, pharmacology, and kinetics of turnover (*17, 18*). The applicability of results in tissue culture experiments to the situation in intact human neuromuscular junctions has been questioned (*15, 19*). We have therefore studied the effect of myasthenic immunoglobulin on the turnover of ACh receptors at intact mammalian neuromuscular junctions, both in vivo and in vitro. Our results show that myasthenic immunoglobulin (Ig) produces an accelerated degradation rate for junctional ACh receptors, similar to that seen in tissue culture.

Immunoglobulin fractions were prepared from individual patients' serums, previously shown to contain significantly elevated titers of antibody to ACh receptor (*11, 13*), and from pooled control serums derived from 15 normal individuals or patients with diseases other than myasthenia gravis (*12, 13, 20*). The serums were precipitated by adding ammonium sulfate to 33 percent saturation; the precipitates were redissolved and dialyzed against mammalian Ringer solution, producing a final IgG concentration of approximately 15 mg/ml.

To measure the turnover of ACh receptors in vivo, the diaphragms of intact mice were labeled with ^{125}I-α-bungarotoxin (^{125}I-α-BuTx), a purified snake toxin that binds specifically, quantitatively, and essentially irreversibly to ACh receptors (*21*). Adult female (C57B1/6 × DBA2)F_1 mice were anesthetized with chloral hydrate (0.6 mg per gram of body weight), and were given a single intrapleural injection of ^{125}I-α-BuTx (0.1 μg per gram of body weight, in a total volume of 140 μl of Ringer solution). The mice were maintained in vertical supports for 1 to 2 hours throughout the labeling procedure to facilitate contact of the ^{125}I-α-BuTx solution with the diaphragm. This resulted in binding of ^{125}I-α-BuTx to an average of 88.3 percent of the available binding sites, as determined by subsequent in vitro saturation of diaphragms with the labeled compound. Virtually all the radioactivity was bound to the diaphragm segments containing the neuromuscular junctions, as has been reported by others (*22, 23*).

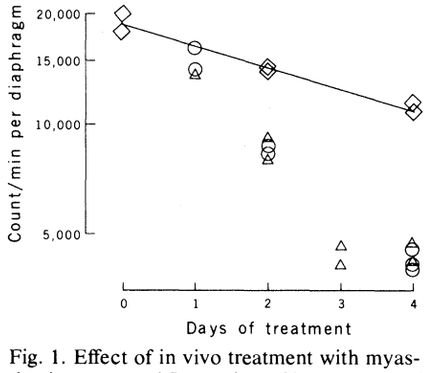

Fig. 1. Effect of in vivo treatment with myasthenic or control Ig on the ACh receptors of mouse diaphragms. Diaphragms were labeled by an intrathoracic injection of ^{125}I-α-BuTx. The mice were given daily doses of Ig from pooled serums of control patients (◇) or from two different myasthenic patients (○, △). The diaphragms were removed at intervals after the beginning of treatment (time zero), and the radioactivity remaining was counted. Each point represents the number of counts per minute recorded from a whole diaphragm of a mouse killed at the time indicated after the first Ig treatment. The rate of loss of radioactivity in the diaphragms of mice treated with myasthenic Ig is increased, as compared to that of the controls.

After the ACh receptors of the diaphragms were labeled, the mice were given daily intraperitoneal injections of 0.7 ml of Ig from an individual myasthenic patient or from the control pool. At daily intervals of 0 to 4 days after the beginning of Ig injections, two or three mice from each treatment group were killed, and their diaphragms were removed in one piece. The diaphragms were washed until radioactivity could no longer be detected in the washing medium, and the amount of radioactivity remaining bound to each diaphragm was determined in a gamma counter (Fig. 1).

Control Ig-treated diaphragms showed a gradual decrease of remaining bound radioactivity, with a mean half-life of 5.65 days and a rate of loss of radioactivity (*24*) of 11.5 percent per day, in close agreement with previously reported values (*22, 23*). The loss of bound radioactivity from the diaphragms of mice treated with myasthenic Ig was significantly more rapid ($P < .001$), with a mean half-life of 2.92 to 1.64 days, and rates of loss of radioactivity from 21.2 to 34.5 percent per day, varying with the individual myasthenic patient's Ig used (Table 1) (*24*).

The loss of radioactivity could represent degradation of ACh receptors, dissociation of ^{125}I-α-BuTx from receptors, or shedding of ACh receptor–α-BuTx complexes. To distinguish among these possibilities, the radioactive material released from the diaphragms was collected from organ cultures of mouse diaphragms after in vivo labeling and treatment with Ig. In these experiments, the diaphragms were labeled by intrapleural injections of ^{125}I-α-BuTx; the mice subsequently received two injections of myasthenic or pooled control Ig at 0 and 24 hours. Six hours after the final injection, whole diaphragms were removed, washed twice in oxygenated medium, and pinned to a horseshoe-shaped Silastic platform in a 35-mm petri dish containing 1.5 ml of Trowell's T8 medium. The cultures were maintained at 37°C in a humidified atmosphere of 95 percent O_2 and 5 percent CO_2. They remained in good condition at the end of the culture period, as demonstrated by mean resting potentials of 62.5 ± 0.87 mV in a control diaphragm, and 62.4 ± 1.33 mV in a myasthenic Ig-treated diaphragm. The medium was completely withdrawn and

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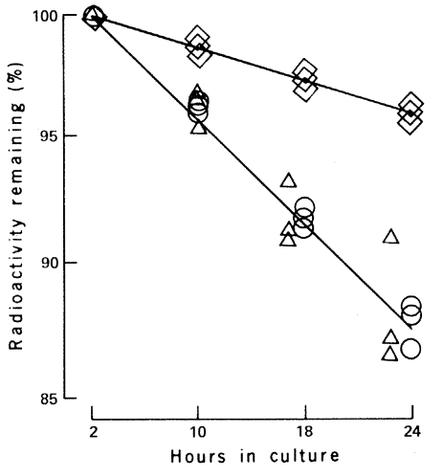

Fig. 2. Effect of myasthenic and control Ig on ACh receptor degradation in whole diaphragm cultures. Receptors were labeled with ^{125}I-α-BuTx in vivo, and the mice received two injections of Ig from control (◇) or myasthenic patients (○, △). The diaphragms were removed and placed in culture, and medium was removed at intervals as indicated. The results are plotted as the fraction of the total bound radioactivity that remained at the end of each culture period. (The 2-hour time point was considered to be time zero, as explained in the text.) Note the threefold increase of degradation rate in the diaphragms treated with myasthenic Ig compared with the controls.

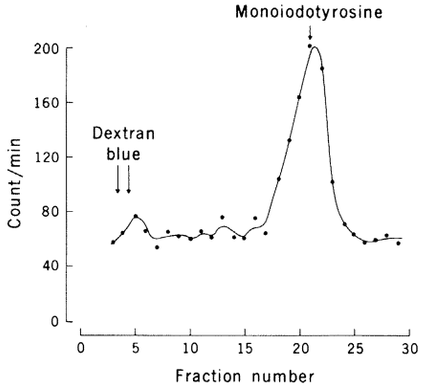

Fig. 3. Chromatogram of culture medium collected from a diaphragm following an 8-hour culture period. The diaphragm was labeled in vivo with ^{125}I-α-BuTx and treated with Ig from a myasthenic patient. Each point represents the radioactivity recorded in a 1.25-ml fraction eluted with tris buffer (0.05*M*, *p*H 7.4) from a Sephadex G-15 column (1 by 13 cm). More than 90 percent of the radioactivity was eluted at the same position as carrier monoiodotyrosine (unlabeled). Less than 5.0 percent appeared in the void volume, marked by the high-molecular-weight dye dextran blue.

replaced at 2, 10, 18, and 26 hours. The 2-hour collection was discarded since it may have contained nonspecifically trapped radioactive material. The collections made at 10, 18, and 26 hours were retained for counting of released radioactivity and chromatographing the radioactive material. At the end of the experiment, the radioactivity remaining in each diaphragm was determined. The rate of release of radioactivity was calculated as the percentage of total radioactivity released per day. The mean release rate for four control Ig-treated diaphragms was 4.3 ± 0.4 percent per day; the mean release rate for six diaphragms treated with Ig from two different myasthenic patients was 12.7 ± 1.4 percent per day (Fig. 2). Thus the rate of release of ^{125}I was accelerated threefold by myasthenic Ig in vitro as it was in intact mice ($P < .001$). Both control and myasthenic Ig-treated diaphragms showed lower absolute values for the in vitro rates compared with those measured in vivo, presumably because of culture conditions.

The radioactive material released into the medium was examined by gel chromatography. Twelve different samples of collected culture media were chromatographed (Bio-Rad P4, Sephadex G-15, or Sephadex G-50 columns; 0.05*M* tris buffer, *p*H 7.4). The recovery of applied radioactivity averaged 85.7 percent. In media obtained from both control and myasthenic Ig-treated diaphragms, 70 to 95 percent of the recovered radioactivity appeared in a single peak at approximately the same position as monoiodotyrosine (Fig. 3). In most experiments, less than 10 percent of the radioactivity appeared in the void volume, and it was eluted from a G-50 column at the same rate as ^{125}I-α-BuTx. The fact that most of the radioactivity released into the culture medium was associated with a substance of low molecular weight indicated that the ^{125}I-α-BuTx had been degraded.

In our study we monitored the loss of radioactivity from bound ^{125}I-α-BuTx as an indirect measure of degradation of ACh receptors. Previous reports have established the validity of this approach in tissue culture (*14, 25–27*), and similar methods have been applied to whole skeletal muscles (*22, 23, 28*). Our demonstration of a low-molecular-weight radioactive material released from control and myasthenic Ig-treated mouse diaphragms provides strong supporting evidence that the loss of radioactivity is due to a degradative process (*29*). Dissociation of free ^{125}I-α-BuTx was minimal and could have led to only a slight overestimate of the degradation rates for both control and myasthenic Ig-treated preparations. These findings suggest that shedding of intact ACh receptors as proposed by others (*5, 30*) is not an important effect of myasthenic Ig, although the possibility that it may play a role in the more chronic clinical situation of human myasthenia gravis has not been completely excluded.

The normal degradation process for ACh receptors is thought to involve their internalization and proteolysis by muscle cells (*25, 28*). It is not known how the binding of the myasthenic antibody accelerates this process. Since the binding of other ligands such as α-BuTx to ACh receptor does not lead to accelerated degradation, the complex of antibody and receptor must have some special property that triggers the increase in degradation rate. Binding of antibody to lymphocyte surface antigens induces a similar process (*31*), while the action of certain hormones also results in a reduction of their surface receptors (*32*).

In conclusion, our observations indicate that Ig from myasthenic patients increases the rate of degradation of ACh receptors at intact neuromuscular junctions of mammalian muscles. Although this study could not be performed in humans, the striking similarities between the passive transfer model in the mouse and the disease in man (*12, 13*) suggest that humorally mediated processes may be the same in both. We therefore propose that acceleration of ACh receptor degradation may represent a major humoral mechanism in the pathogenesis of myasthenia gravis.

Table 1. Effect of Ig on loss of bound radioactivity from ACh receptors of diaphragms in vivo. Mouse diaphragms were labeled with ^{125}I-α-BuTx, and the mice subsequently received daily intraperitoneal injections of control or myasthenic patients' Ig. The rate of loss of bound radioactivity is expressed in three different ways: rate, half-life, and remainder. The loss of radioactivity (degradation of ACh receptors) produced by myasthenic Ig is enhanced.

Antiserum treatment	Mice per group	Loss per day (%)	Half-life	Remainder at day 4 (%)†
Control Ig	18	11.5	5.65	63.0 ± 6.8
Myasthenic Ig 1	10	21.2*	2.92*	42.6 ± 2.0*
Myasthenic Ig 2	7	34.5*	1.64*	21.5 ± 1.5*
Myasthenic Ig 3	7	32.5*	1.76*	22.9 ± 2.0*

*Significantly different ($P < .001$, Student's *t*-test) from control. †Mean ± standard deviation.

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Is Pump Stimulation Associated with Positive Inotropy of the Heart?

Abstract. *A purified sodium and potassium dependent adenosinetriphosphatase isolated from cat heart was not stimulated by any concentration of ouabain that produced positive inotropy of cat papillary muscle. Only inhibition of enzyme activity was observed. Concentrations of ouabain used ranged from 3.3 × 10^{-10} molar to 5 × 10^{-7} molar and produced an increased force of contraction without any evidence of toxicity. The results are inconsistent with a concept that stimulation of sodium pump activity is associated with positive inotropy.*

Cardiac glycosides, in concentrations that are presumed to be nontoxic, produce a positive inotropic effect on the heart. The mechanisms that produce this improvement in cardiac muscle function are investigated at many levels including intact heart preparations, subcellular organelle function, ionic fluxes across membranes, and electrophysiological studies (*1*). In 1956, Solomon *et al.* (*1*) provided the first quantitative data on K^+ transport and cardiac glycoside action, thus implicating the Na^+- and K^+-dependent adenosinetriphosphatase (Na^+, K^+-ATPase; E.C. 3.6.1.3) pump system in the mechanism of digitalis action. Recent work has focused on the effect of ouabain on membrane currents altered by extracellular potassium ($[K]_o$) and on the coincident inotropic effect as a consequence of a supposed electrogenic pump stimulation or inhibition. For example, Cohen *et al.* (*2*) studied membrane currents in sheep Purkinje fibers. At high values of $[K]_o$, that is, 5.4 to 8 m*M*, ouabain (5×10^{-7}*M*) shifted the reversal potential (E_{rev}) for the pacemaker current (I_{k2}) in a negative direction; at low values of $[K]_o$, that is, 2.7 or 4 m*M*, this dose of ouabain shifted E_{rev} in a positive direction. In another experiment, 1×10^{-7}*M* ouabain at $[K]_o$ of 5.4 m*M* caused a transient negative shift in E_{rev} during the first 5 minutes of drug perfusion followed by a positive E_{rev} shift in the succeeding 30 minutes. A positive shift in E_{rev} was interpreted by these authors as an inhibition of the Na^+,K^+-pump and a buildup of potassium, $[K]_e$, in a restricted extracellular space, whereas a negative shift in E_{rev} was taken to imply Na^+,K^+-pump stimulation with depletion of $[K]_e$. Changes in $[K]_o$ might indicate corresponding changes in outward current, i_{k1}, and pacemaker current, i_{k2}, or the currents could be the same but, more or less, K^+ would be pumped back. The assumption by Cohen *et al.* (*2*) that E_{rev} may simply represent E_K may be incorrect. Voltage clamp techniques may have sources of error that obviate definitive conclusions about Na^+,K^+-pump activity. Very recently, Kass *et al.* (*3*) reported that 10^{-6}*M* strophanthidin increased a slow inward current (i_{Si}) in short Purkinje fibers from the calf, concomitant with an augmented twitch tension. This occurred at an early stage of drug action.

Blood (*4*) reported that doses of ouabain in the range of 10^{-10} to 10^{-7}*M* produced positive inotropic effects in sheep Purkinje fibers bathed with 5.4 m*M* $[K]_o$ Tyrodes solution. He indicated that the effects of low concentrations of ouabain could be mimicked by reducing $[K]_o$; a reduction from 5.4 m*M* to 4.05 m*M* produced a positive inotropic response similar to that resulting from 5×10^{-8}*M* ouabain, but no values for the degree of inotropy were given. Anderson *et al.* (*5*) reported that $[K]_o$ raised from 2.7 to 5.4 m*M* caused a decrease in the maximum diastolic potential from 96 to 89 mV, respectively. In the presence of 2.1×10^{-7}*M* ouabain, an increase in $[K]_o$ from 2.7 to 5.4 m*M* caused an increase in maximum diastolic potential from −52 to −65 mV. Using amphibian atrial trabeculae, Loh (*6*) observed a positive inotropic effect in response to the cardiac glycoside desacetyl-lanatoside C, 7.4×10^{-7}*M*; simultaneously, there was an increase in $[K]_i$ at 10 m*M* $[K]_o$, no increase or decrease at 4 m*M* $[K]_o$, and a decrease at 1.25 to 3.5 m*M* $[K]_o$.

Some years ago it was suggested (*7*) that stimulation of a Na^+,K^+-ATPase exchange pump may produce a positive inotropic state, although this concept is not now generally held (*1*). Since recent electrophysiological data have again raised the possibility, we reexamined this hypothesis. Cats were anesthetized with sodium pentobarbital (25 mg/kg) and the hearts were quickly excised. Isolated right ventricular papillary muscles were placed in an isometric myograph and stimulated to contract at a rate of 0.25 Hz with pulses 20 percent above

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