values measured for $(\partial P/\partial X)_T$ were 2×10^{-2} and 3×10^{-2} atm per mole fraction of gallium. The average of these values, corrected by means of the Clausius-Clapeyron equation to 1203°K, is 1.0×10^{-2} atm per mole fraction of gallium. Values for all quantities in Eq. 2 have now been determined except the limit X_2; we calculate $X_2 - X_1 = 0.002$ for the change in the mole fraction of gallium along the h-Ga_2S_3 phase boundary when the temperature changes from 1228° to 1203°K, compared to a measured change of 0.001 for the ℓ-Ga_2S_3 phase boundary. We calculate from the phase boundary compositions a heat and an entropy of transition that are well within the ranges of values found for transitions between low- and high-temperature modifications of solid phases (*9*).

Our analysis depends on measurements of differences in temperatures, pressures, and compositions. These differences can be measured with higher precision than the absolute values. The composition changes are probably uncertain by no more than a factor of 2. Our measurements appear certainly precise enough to justify the conclusions that a decomposition reaction of a high-temperature form of Ga_2S_3 has a partial pressure which increases with decreasing temperature and that the increase is a consequence of changes in the compositions of the equilibrium solids.

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23 August 1976; revised 16 November 1976

Myasthenic Immunoglobulin Accelerates Acetylcholine Receptor Degradation

Abstract. *Degradation of acetylcholine receptors by cultured rat skeletal muscle cells was determined from the release of ^{125}I from bound ^{125}I-labeled α-bungarotoxin. Addition of immunoglobulin from patients with myasthenia gravis to the culture medium accelerated the degradation rate to a mean of 8.51 ± 0.44 percent per hour, compared with the mean control rate of 3.97 ± 0.14 percent per hour ($P << .001$). A similar mechanism may possibly be involved in the autoimmune pathogenesis of myasthenia gravis in man.*

Myasthenia gravis (MG) is a neuromuscular disorder characterized by weakness and fatigability of muscles. Recent evidence indicates that there is a decrease of available acetylcholine (ACh) receptors at the neuromuscular junctions of myasthenic patients (*1*), sufficient to account for the typical clinical and physiological manifestations (*2*). Several lines of evidence suggest that the pathogenesis of this abnormality involves an autoimmune attack directed against ACh receptors: (i) An experimental model analogous to MG has been produced by immunizing various animals with purified ACh receptor (*3*). (ii) The serums of patients with MG contain antibody for ACh receptor, which has been demonstrated by several different methods (*4*). (iii) Immunoglobulin G (IgG) from myasthenic patients has been shown, when passively transferred to experimental mice, to reproduce the typical features of myasthenia gravis (*5*). Thus, a humoral immune process appears to play an important role in the pathogenesis of the human disease. However, the mechanism by which the antibody affects the ACh receptors is not yet known. In this study, we have examined the effects of immunoglobulin from myasthenic patients on mammalian skeletal muscle cells in culture. The findings indicate that the immunoglobulin from MG patients produces an increase in the rate of degradation of ACh receptor by the cultured cells and reduces the total number of available receptor sites.

Cultures of rat skeletal muscle were prepared by conventional methods (*6*). The limb muscles of 16- to 18-day fetuses from Sprague-Dawley rats were minced, and trypsinized for 1 hour in Hanks balanced salt solution without divalent cations, containing 0.25 percent trypsin. The cells were strained through bolting silk, centrifuged, resuspended in culture medium, and plated for 30 minutes in a Falcon 75-cm² plastic flask for selective removal of the rapidly adhering fibroblasts. The nonadhering cells, predominantly myoblasts, were transferred to a second flask and cultured for 48 hours. The cultures were trypsinized and replated in gelatin-coated 35-mm Falcon plastic petri dishes. Cultures were grown at 37°C in Eagle's minimum essential medium (MEM) with Earle's salts, supplemented with 10 percent fetal calf serum, under an atmosphere of 10 percent CO_2. Large numbers of dishes were prepared simultaneously, so that the cultures would be at the same stage of development and have closely comparable numbers of ACh receptors.

Degradation of ACh receptors was measured by an indirect method that depends on the specific labeling of the receptors with ^{125}I-labeled α-bungarotoxin (α-BuTx) and the release of radioactive material derived from degraded receptors into the culture medium (*7*). Sets of identical 7- to 8-day-old cultures were first saturated with ^{125}I-labeled α-BuTx for 30 minutes [0.05 μg (6.25×10^{-12} mole) in 1 ml; the specific activity was 3×10^4 to 5×10^4 c/mole], and the unbound toxin was removed by washing four times with culture medium (modified by omission of phenol red). At several intervals from 2 to 40 hours, the medium and a single washing were removed for counting of released radioactivity, and fresh medium was added to the culture dishes. At the end of the experimental period (usually 40 hours), the cultures were extracted with two washings of 1 ml of 1 percent Triton X-100 in phosphate-buffered saline, *p*H 7.2, to solubilize the remaining cell-bound complexes of ACh receptor and

Table 1. Degradation rate of ACh receptor in muscle cultures treated with MG or control immunoglobulin. Results are given as the means ± the standard error of the means. In these experiments, ACh receptors of cultured muscle fibers were first labeled with [^{125}I]α-BuTx. The cultures were then incubated with medium that contained immunoglobulin. The radioactivity released into the medium, representing degraded receptors, was determined at intervals up to 40 hours later, and degradation rates were calculated as described. Degradation rates of sets of cultures incubated with MG immunoglobulin from each patient were significantly different ($P << .001$) from degradation rates of simultaneously incubated control cultures containing pooled normal immunoglobulin.

Immunoglobulin	Degradation rate (% per hour)	Half degradation time (hours)
MG 1	11.09 ± 0.64	4.89 ± 0.63
MG 2	7.58 ± 0.21	7.71 ± 0.14
MG 3	6.28 ± 0.28	9.97 ± 0.50
MG 4	6.36 ± 0.01	10.22 ± 0.17
MG 5	8.83 ± 0.67	6.87 ± 0.69
MG 6	9.67 ± 0.04	5.89 ± 0.46
Control 1*	4.32 ± 0.06	15.15 ± 0.08
Control 2*	3.70 ± 0.10	15.56 ± 0.16

*Pooled.

^{125}I-labeled α-BuTx, and the radioactivity in the extracts was counted. The rate of degradation was expressed as the percentage of total radioactivity released into the medium per hour. The validity of this method for evaluating ACh receptor degradation has been established (*7*). The appearance of ^{125}I in the medium parallels the loss of receptor sites by the muscle. The ^{125}I released into the medium consists almost entirely of monoiodotyrosine, derived from the degradation of complexes of BuTx and ACh receptor (*8*). Moreover, ^{125}I-labeled α-BuTx itself does not alter the natural degradation of ACh receptors by the muscle cells.

In order to test the effect of MG patients' immunoglobulin on receptor degradation, a crude immunoglobulin fraction (hereafter referred to as "immunoglobulin") was prepared from the serums of six individual patients and from two pools of controls consisting of from 10 to 30 normal individuals or patients with diseases other than MG (*5*). The serums were first precipitated under sterile conditions with ammonium sulfate at 33 percent final saturation. The precipitate was redissolved and dialyzed against Ringer solution, giving a final IgG concentration of approximately 20 mg/ml. This fraction was heated to 56°C for 30 minutes to inactivate complement.

In the experimental cultures, the usual culture medium was replaced with 1 ml of MEM to which 0.1 ml of patient's or control immunoglobulin was added. Three or four culture dishes were used for testing each MG or control immunoglobulin.

In the cultures treated with control immunoglobulin, the mean (± standard error of mean) ACh receptor degradation rate was 3.97 ± 0.14 percent per hour, giving a half-time of 15.38 ± 0.12 hours. These values are close to those reported for chick muscle cultures (*7*, *9*). By contrast, the mean degradation rates for cultures treated with MG patients' immunoglobulins ranged from 6.25 ± 0.28 percent to 11.09 ± 0.63 percent per hour, with half-times of 10.19 ± 0.17 to 4.96 ± 0.83 hours (Fig. 1 and Table 1). The differences between control and MG immunoglobulin-treated cultures were highly significant ($P << .001$) in all cases tested.

In order to determine whether the acceleration of degradation by MG immunoglobulin is temperature dependent, we incubated sets of culture dishes at 10°C. Phosphate buffer, *p*H 7.2, was substituted for bicarbonate buffer in the medium, since the cultures were kept in room air rather than in a 10 percent CO_2 atmosphere (*10*). Release of radioactive label into the culture medium was minimal for both control cultures (0.36 ± 0.02 percent per hour) and MG immunoglobulin-treated cultures (0.25 ± 0.01 percent per hour) (Fig. 1B).

Table 2. Acetylcholine receptor sites remaining after incubation with MG immunoglobulin. Cultures were first incubated with medium containing immunoglobulin for the indicated times. Immunoglobulin was removed by rinsing, and the remaining sites were labeled with [^{125}I]α-BuTx. Percentages were calculated as the mean number of BuTx binding sites for each set of MG immunoglobulin-treated cultures divided by the mean number of BuTx binding sites for simultaneously incubated set of control cultures.

Patient	Mean of control ACh receptor sites (%)	
	2 hours	20 hours
1	46*	7
2	35	11
3	44	30
4	82	38
5	31	12
6		8

*One hour after incubation.

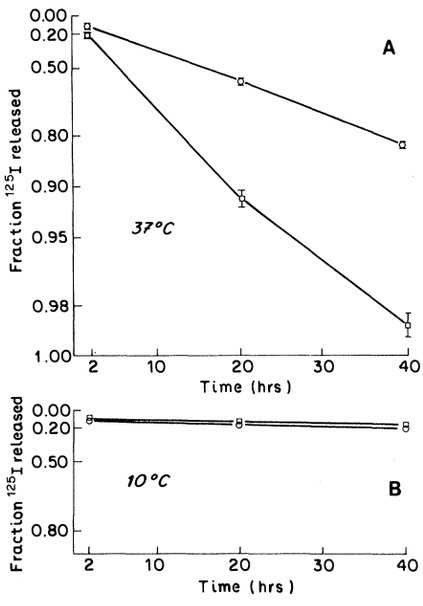
The number of available ACh receptors remaining after incubation with immunoglobulin for various time periods was also determined. In these experiments the cultures were first incubated with MG patients' or control immunoglobulin for 2 to 20 hours; at the end of the incubation period the remaining receptors were labeled with [^{125}I]α-BuTx. Washing and solubilization of ACh receptors were carried out after labeling, as described above. Exposure to MG immunoglobulin caused a progressive decrease of the number of BuTx-binding ACh receptor sites with time (Table 2). Control immunoglobulin did not affect ACh receptor sites.

Throughout these procedures, the cell cultures were examined repeatedly by phase microscopy. The cells continued to appear normal morphologically, even after more than 40 hours of exposure to MG immunoglobulin. As a further test of the integrity of the myogenic cells, resting membrane potentials (RMP's) were determined by standard microelectrode techniques in three cultures treated for 20 hours with normal immunoglobulin and three cultures treated for 20 hours with MG immunoglobulin. The mean RMP of muscle fibers in the control cultures was 45.45 ± 1.94 mv (S.E.M.), and the mean RMP of myofibers in cultures treated with MG immunoglobulin was 47.10 ± 2.37, a normal value for this stage of development (*11*).

Our results show that immunoglobulin from myasthenic patients produces a marked acceleration of ACh receptor degradation by cultured mammalian muscle cells. This effect cannot be attributed to cell damage, since the integrity of the cells, as indicated by morphology and membrane potentials, is well maintained despite prolonged exposure to the immunoglobulin. Indeed, any interference with the muscle cells would be expected to have an opposite effect, decreasing the degradation rate (*7*).

The effect appears to be triggered by antibody alone, without the necessity for other cellular or humoral components of the immune system. The culture conditions were such as to exclude the actions of complement, lymphocytes, or macrophages, although the possibility that these elements might play a role under

Fig. 1. (A) Degradation of ACh receptors in skeletal muscle cultures incubated at 37°C with medium containing immunoglobulin from a myasthenic patient (□) and pooled control patients (○). After the receptors were saturated with [^{125}I]α-BuTx, the immunoglobulin preparation was added to each culture (0.1 ml in 1 ml of medium per 35-mm plate). The medium was collected at 2, 20, and 40 hours for counting of released radioactivity and replaced with fresh medium. At the end of the final period, the remaining ACh receptor–αBuTx complexes were extracted from muscle with 1 percent Triton solution. The rate of degradation is calculated from the fraction of the originally bound ^{125}I released into the medium at each interval. Four sets of cultures were used for each experiment. The rate of receptor degradation was 4.32 ± 0.06 percent per hour for the control cultures, and 11.09 ± 0.64 percent for cultures incubated with the MG immunoglobulin. (B) Degradation of ACh receptors at 10°C. The experiments were carried out as in (A), except for the incubation temperatures. Degradation was inhibited at this temperature.



other conditions has not been ruled out.

Both the normal receptor degradation process and the acceleration produced by MG immunoglobulin are temperature-dependent, suggesting that they involve the active participation of the muscle cells. Devreotes and Fambrough have proposed that the normal degradative process is energy-dependent and involves internalization and proteolysis of ACh receptors by muscle cells (*12*). Binding of antibody to surface antigens of lymphocytes has been shown to induce a similar process in lymphocytes (*13*), while the action of certain hormones (*14*) also results in the reduction of their surface receptors. It seems likely that the effect of antibody from MG patients on ACh receptors may be of the same nature, at least in the muscle tissue culture system. Perhaps the binding of this antibody alters the ACh receptor in some way, so that it is selected for degradation. Since binding of other ligands such as BuTx to ACh receptors does not lead to accelerated degradation, the complex of antibody and receptor must have some special property that triggers the increase in rate of degradation.

It appears that the degradation process alone does not account fully for the loss of available ACh receptor sites, but rather that some additional mechanism plays a role. Our data (Tables 1 and 2) indicate that more receptor sites are lost, or unavailable, after exposure to immunoglobulin than can be explained by the radioactivity released into the medium. This difference is most marked early and then diminishes, as a result of the cumulative effect of degradation. Evidently a substantial proportion of receptors becomes unable to bind BuTx before being degraded. Theoretically, this effect might be due to blocking of ACh receptor sites by the antibody or, perhaps, to prolonged detainment of internalized receptors within the muscle cells. However, the accelerated rate of degradation is sustained for at least the 40-hour period of the experiments.

Although we have demonstrated that MG immunoglobulin accelerates degradation of ACh receptors in skeletal muscle cells in an in vitro system, a similar mechanism may be involved in the autoimmune pathogenesis of myasthenia gravis in man. This hypothesis might explain several features of MG which have heretofore been puzzling, such as (i) the relatively prolonged exposure required for passive transfer of myasthenic features to animals by injection of serum immunoglobulins from MG patients (*5*); (ii) the lack of effect of MG serum on neuromuscular transmission in vitro, after relatively brief periods of exposure (*15*); (iii) the difficulty in demonstrating the presence of antibody at neuromuscular junctions of patients (*16*); and (iv) the incompleteness or lack of blockade of ACh receptors in vitro by serum from myasthenic patients (*4, 17*). Further studies will be needed to evaluate the role of receptor degradation in the autoimmune pathogenesis of human myasthenia gravis.

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18 January 1977; revised 23 February 1977