Myasthenia Gravis: Passive Transfer from Man to Mouse

Abstract. *Daily injections into mice of an ammonium sulfate–precipitated immunoglobulin fraction of serum from patients with myasthenia gravis were carried out for up to 14 days. The mice showed reduced amplitudes of miniature endplate potentials and reduced numbers of acetylcholine receptors at the neuromuscular junctions. Some mice showed typical decremental responses on repetitive nerve stimulation, with reversal by neostigmine. This represents the first evidence of a circulating factor in the serum of patients with myasthenia gravis which on passive transfer reproduces features of the disease in experimental animals.*

Myasthenia gravis is a neuromuscular disorder manifested by weakness and fatigability of muscle. The abnormality involves the neuromuscular junction, and the site of the defect has recently been defined as the acetylcholine (ACh) receptors. By means of techniques which utilize iodine-labeled α-bungarotoxin (^{125}I-α-BuTx) binding to receptors, we determined that the number of available ACh receptors was reduced in myasthenic junctions, averaging 80 percent below normal (*1*). Furthermore, we demonstrated that experimental blockade of a proportion of ACh receptors by the specific α-toxin of the Formosan cobra (*Naja naja atra*) reproduced all of the physiological features of myasthenia gravis (*2*). This confirmed the concept that a receptor abnormality per se could account for the pathophysiology of myasthenia gravis.

How the receptor abnormality is brought about in human myasthenia remains a key question. A factor (presumably an antibody) in the serum of many myasthenic patients, which is capable of binding to ACh receptors in vitro, has been demonstrated (*3*). Further, immunization of rabbits with isolated ACh receptor antigen produced an experimental model with myasthenia-like features (*4*), and this added support to the widely accepted concept of myasthenia gravis as an autoimmune disorder (*5*). However, the relation of a serum factor to the disease process in myasthenia gravis has remained controversial. The results of attempts to transfer myasthenia from humans to experimental animals or nerve-muscle preparations have generally been negative (*6*) or have been criticized (*7*) for certain inherent faults. Yet the possibility of such a factor seemed plausible because of the reported observations of neonatal myasthenia gravis in infants of mothers with the disease (*8*), and the evidence for an autoantibody to the receptor in myasthenic patients (*3*). In previous studies the animal or nerve-muscle test preparation was exposed to the myasthenic serum for a brief time—usually minutes, or occasionally hours.

Our study was designed to test the effects of more prolonged exposure to the putative serum factor. We therefore investigated the neuromuscular effects of repeated injections of an immunoglobulin fraction of myasthenic or control human serum in experimental mice.

Six patients with typical generalized myasthenia gravis (five females and one male, from 14 to 62 years of age) gave informed consent for the withdrawal of 200 ml of blood by venipuncture. In each case, the diagnosis was established by the typical history and physical findings of weakness and fatigue; the diagnosis was confirmed by decremental electrical responses of muscles to repetitive nerve stimulation (*9*) and by improvement of muscle strength with anticholinesterase medications. All patients were receiving the anticholinesterase agent pyridostigmine bromide as treatment for myasthenia, but none had had prior thymectomy or adrenal corticosteroid medication. Pooled blood from patients without myasthenia gravis was used in control experiments.

The blood was allowed to clot, and the serum was removed after centrifugation and stored at −70°C until further processing. The serum was then thawed, and precipitation of a fraction containing immunoglobulins was carried out under sterile conditions with an ammonium sulfate solution at 33 percent saturation (*10*). (This preparation will hereafter be referred to as the immunoglobulin fraction, although this term is not meant to imply that it was a pure fraction.) The precipitate was centrifuged and redissolved in approximately four volumes of Ringer solution (Abbott). This solution was then dialyzed against 20 to 30 volumes of Ringer solution with one change over the course of approximately 24 hours. The concentration of immunoglobulin G (IgG) in the immunoglobulin solution, as measured by a single radial immunodiffusion test (*11*), ranged from 1.2 to 2.6 g/100 ml in preparations from both myasthenic patients and controls.

Twenty-six BDF_1 mice were used in these experiments; 15 were treated with the myasthenic (M.G.) immunoglobulin fractions and 11 with the control fractions. Each mouse received daily intraperitoneal injections of 0.4 to 0.65 ml of the immunoglobulin fractions for 10 to 14 days. This volume contained 10 to 11 mg of human IgG, which is about 50 percent more than the total amount of circulating native IgG for the mouse (*12*). Twenty-four hours after the first injection, each mouse was given cyclophosphamide (300 mg/kg) intraperitoneally in order to induce tolerance to the human serum proteins (*13*). The intraperitoneal injections did not cause visible inflammation or adhesions in any of the animals injected with M.G. or control immunoglobulin. The human IgG in the blood of the injected mice ranged from 500 to 940 mg/100 ml, when measured 24 hours after the last injection. This is equal to or more than the concentration of the mouse's native IgG (*12*). In addition, two mice received M.G. immunoglobulin for only 3 days and two others received immunoglobulin from human controls for 3 days.

At the end of the experimental period, the mice were anesthetized with pentobarbital (80 mg/kg), and three test procedures were performed as described below:

1) The left extensor digitorum longus (EDL) and soleus muscles were surgically removed for determination (by ^{125}I-α-BuTx binding) of the number of ACh receptors per neuromuscular junction. This procedure was carried out essentially as described (*14*), except that the radioactivity was measured in a gamma spectrometer with a counting efficiency of 82 percent.

2) The mice were prepared for recording of compound muscle action potentials evoked by stimulation of the right sciatic nerve. The method was the same as that described for rats (*2*) but was scaled down to the smaller proportions of mice.

3) The diaphragms were removed for

Table 1. Effect of immunoglobulin from myasthenic patients on mepps and endplate acetylcholine receptors (AChR) of mice. The results are means of the average values for individual muscles, with standard errors of the mean. The numbers in parentheses indicate the number of muscles tested in each group.

Treatment with immunoglobulin	Mean mepp amplitude (mv)	Mean AChR per endplate
10- to 14-day M.G.	0.19 ± 0.02 (7)	$1.5 \times 10^7 \pm 0.1 \times 10^7$ (29)
10- to 14-day control	0.62 ± 0.05 (6)	$2.6 \times 10^7 \pm 0.2 \times 10^7$ (22)
3-day M.G.	0.16 (2)	
3-day control	0.57 (2)	
Untreated controls		$2.4 \times 10^7 \pm 0.2 \times 10^7$ (14)

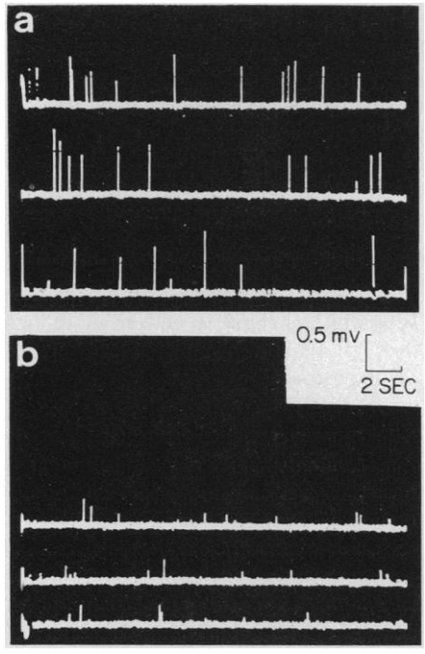

Fig. 1. Miniature endplate potentials (mepps) recorded from diaphragms of mice receiving injections of immunoglobulin fraction from (a) pooled normal serums, (b) serum of 14-year-old female myasthenia gravis patient J.L.H.

recording of miniature endplate potentials (mepps). Each hemidiaphragm was pinned at resting length in a 60-mm Falcon plastic petri dish lined with Silastic strips. The diaphragms were maintained in Liley's medium, gassed with a mixture of 95 percent O_2 and 5 percent CO_2 at 22°C. Intracellular recordings were made with conventional glass microelectrodes (7 to 15 megohms) filled with 2*M* KCl. The electrical signals were amplified with a Grass P-16 amplifier, displayed on a Tektronix storage oscilloscope, and photographed on Polaroid film. The resting membrane potential (RMP) of each cell was recorded, and only cells with RMP's of 60 mv or greater were used. All mepps with rise times of 2 msec or less (at 22°C) were measured and corrected (*15*) to an RMP of 75 mv.

The most striking abnormalities in the mice injected with M.G. immunoglobulin were the reduction in amplitude of mepps and the reduction of ACh receptor sites at the neuromuscular junctions. The mean mepp amplitude of the diaphragms of the seven mice injected with M.G. immunoglobulin was 0.19 mv, while that of six mice injected with control serum was 0.62 mv, which is normal. The difference was readily apparent (Fig. 1) in every diaphragm, and was statistically highly significant ($P << .001$) (*16*). The true mean mepp amplitude was probably even lower than stated since the smallest mepps were often obscured by baseline noise, which usually was 60 to 100 μv. This became apparent when particularly favorable electrodes with low noise characteristics were used, since many mepps of less than 100 μv could then be seen. The histograms showed a normal distribution of mepp amplitudes in the control mice, while the great majority of mepps were below 0.2 mv in amplitude in the mice injected with M.G. immunoglobulin (Fig. 2). In the two animals that were treated for 3 days with M.G. immunoglobulin, mepp amplitudes were reduced, as compared to the 3-day injected controls (Table 1). However, receptor determinations and tests for decremental responses have not yet been made in mice treated for 3 days.

The mean number of ACh receptors per neuromuscular junction was reduced by 42 percent in the leg muscles of the mice injected with M.G. immunoglobulin, as compared with the injected controls. This difference was highly significant ($P << .001$) (Table 1), although not observed in every muscle.

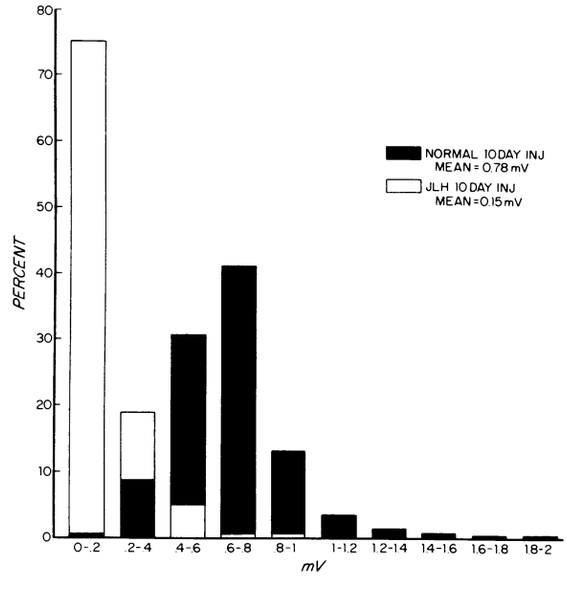

Fig. 2. Histograms of amplitudes of mepps recorded from mouse diaphragms. The distribution of mepps in the diaphragm from a mouse injected with control immunoglobulins for 10 days is normal, unlike the much smaller mepps in the diaphragm of the mouse treated for 10 days with immunoglobulins that were injected from a myasthenic patient (J.L.H.). The distribution of the mepps in the J.L.H.-injected mouse appears skewed, since most of the mepps fall in the smallest group, and those smaller than 60 μv were obscured by baseline noise.

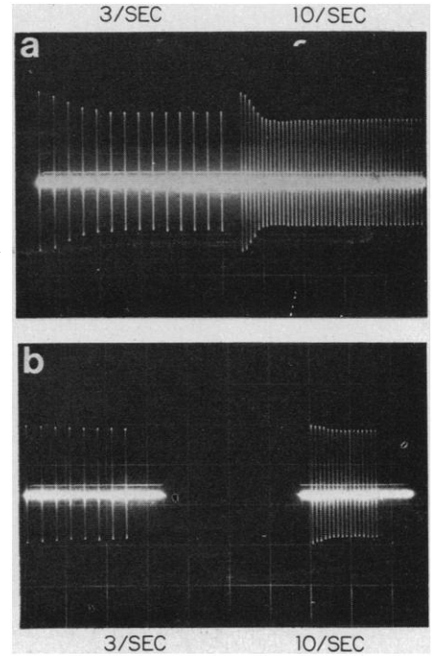

Fig. 3. Evoked potentials recorded over calf muscles with sciatic nerve stimulation at 3 sec^{-1} and 10 sec^{-1}. (a) A mouse that had received myasthenia gravis immunoglobulin (patient G.P., age 36 years, 10 day) injections for 9 days. (b) The same mouse after intravenous injection of 0.3 μg of neostigmine.

Decremental responses on repetitive nerve stimulation at a rate of 3 sec^{-1} occurred in four mice injected with M.G. immunoglobulin (two each with injections from two different severely affected patients). Only one of the mice with a decremental response was tested with intravenous neostigmine (0.3 μg), and it showed improvement from an original 28 percent decrement to 4 percent after neostigmine (Fig. 3). Ten other mice injected with the M.G. immunoglobulin fraction and 11 injected with the control fraction showed no decrement when stimulated at 3 sec^{-1}.

Our findings demonstrate that many of the basic features of myasthenia gravis can be transferred from human patients to animals by injection of a serum factor. We believe that the consistent changes in mepp amplitude and junctional receptors in the animals, as well as the decremental responses in some, are indicative of myasthenia gravis, although only one of the animals was clinically weak. The lack of weakness in spite of marked reduction of mepp amplitudes is consistent with the greater safety factor of neuromuscular transmission in rodents as compared to humans, apparently due to a larger amount (number of quanta) of ACh released per

impulse (*17*). Our results cannot be attributed to the experimental procedure itself, since identically treated control animals that received immunoglobulins from nonmyasthenic humans showed none of the myasthenic features. Furthermore, dialysis performed during preparation of the immunoglobulin fraction would remove any residual anticholinesterase medication from the patients' serums.

Our study differs from the many previous attempts to transfer myasthenia gravis to animals over the past three decades (*6*) in at least two important respects which contributed to the present positive results: (i) We exposed the test animals to the serum factor for a relatively prolonged time, in contrast to the minutes or hours previously attempted. (ii) We used more sensitive electrophysiological and radiometric methods to detect the myasthenic abnormalities rather than relying on clinical weakness or decremental responses, which are often absent.

The nature of the myasthenia-producing serum factor has not yet been elucidated, but the 33 percent ammonium sulfate fraction used in these experiments contains the immunoglobulins as well as other serum proteins (*18*). Whether the antibody that binds to ACh receptor (*3*) is itself the transferable serum factor in myasthenic patients remains to be determined. Our experiments may provide the critical link between such an antibody and the pathogenesis of myasthenia gravis as a humorally mediated autoimmune disease.

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References and Notes

1. D. M. Fambrough, D. B. Drachman, S. Satyamurti, *Science* **182**, 293 (1973).
2. S. Satyamurti, D. B. Drachman, F. Slone, *ibid.* **187**, 955 (1975).
3. R. R. Almon, C. G. Andrew, S. H. Appel, *ibid.* **186**, 55 (1974); A. N. Bender, S. P. Ringel, W. K. Engel, M. P. Daniels, Z. Vogel, *Lancet* **1975-II**, 607 (1975); J. Lindstrom, V. Lennon, M. Seybold, S. Whittingham, *Ann. N.Y. Acad. Sci.*, in press.
4. J. Patrick and J. Lindstrom, *Science* **180**, 871 (1973).
5. J. A. Simpson, *Scott. Med. J.* **5**, 419 (1960); A. J. L. Strauss, C. W. Smith, G. W. Cage, H. W. R. Vander Geld, D. E. McFarlin, M. Barlow, *Ann. N.Y. Acad. Sci.* **135**, 557 (1966).
6. N. P. Bergh, *Scand. J. Clin. Lab. Invest. Suppl.* **5**, 1 (1953); W. L. Nastuk, A. J. L. Strauss, K. E. Osserman, *Am. J. Med.* **26**, 394 (1959).
7. W. L. Nastuk and A. J. L. Strauss, in *Myasthenia Gravis*, H. R. Viets, Ed. (Thomas, Springfield, Ill., 1961), chap. 3.
8. K. E. Osserman, *Myasthenia Gravis* (Grune & Stratton, New York, 1958), chap. 17.
9. C. Ozdemir and R. R. Young, *Ann. N.Y. Acad. Sci.* **183**, 287 (1971).
10. K. Heide and H. G. Schwick, in *Handbook of Immunology*, D. M. Weir, Ed. (Blackwell Scientific Publications, Oxford, ed. 2, 1973), chap. 6.
11. Quantiplate, Kallestad Laboratories, Chaska, Minn.
12. J. L. Fahey and A. G. Robinson, *J. Exp. Med.* **118**, 845 (1963).
13. G. W. Santos, *Exp. Hematol.* **14**, 32 (1967).
14. D. M. Fambrough, *J. Gen. Physiol.* **64**, 468 (1974).
15. B. Katz and S. Thesleff, *J. Physiol. (Lond.)* **137**, 267 (1957).
16. Student's two-sample *t*-test, comparing the means of mepp amplitudes for the M.G. immunoglobulin and control injected mouse diaphragms.
17. E. Lambert, Mayo Clinic, Rochester, Minn., personal communication.
18. S. Keller and R. J. Block, in *The Separation and Isolation of Proteins*, P. Alexander and R. J. Block, Eds. (Pergamon, New York, 1960), chap. 1. Electrophoretic analysis of this fraction in one case showed that the precipitate contained the following percentages of serum proteins: albumin, 12 percent; α_1-globulin, 30 percent; α_2-globulin, 30 percent; β-globulin, 65 percent; γ-globulin, 87 percent.
19. Supported by NIH grant HD01083 and fellowships from the Muscular Dystrophy Associations of America to K.V.T. and A.P. We thank Drs. H. McFarland, R. Humphrey, and I. Sensenbrenner for helpful discussions.

18 June 1975

Hypothalamic Hyperphagia: Dissociation from Hyperphagia Following Destruction of Noradrenergic Neurons

Abstract. *Major differences were found between classical hypothalamic hyperphagia in rats and the recently discovered hyperphagia syndrome resulting from destruction of the ventral noradrenergic bundle. Traditional medial hypothalamic lesions produced no detectable loss of norepinephrine, and the rats overate both in the daytime and at night, whereas destruction of the noradrenergic bundles with 6-hydroxydopamine depleted norepinephrine to 6 percent of normal and caused overeating only at night. Moreover, the two procedures were additive, not substitutive, in their effects on eating. These results argue against recent suggestions that destruction of the ventral noradrenergic bundle mediates the classical hyperphagia syndrome associated with localized ventromedial hypothalamic lesions. However, damage to noradrenergic pathways may contribute to the hyperphagia after extensive hypothalamic lesions.*

One of the better known phenomena in the study of central nervous system function is the syndrome of overeating and obesity that follows destruction of the ventromedial region of the hypothalamus. We have demonstrated, in the rat, that hyperphagia frequently resulting in obesity also follows neurochemically selective lesions in the ventral noradrenergic bundle (*1*). This is the main noradrenergic pathway to the rat hypothalamus (*2*). Lesser numbers of epinephrine-containing fibers travel with the ventral bundle (*3*). Using somewhat different techniques, Kapatos and Gold (*4*) observed hyperphagia after knife cuts or electrolytic lesions that included the ascending noradrenergic systems within the area of destruction. More recently, Gold (*5*) has suggested that hypothalamic lesions that produce obesity do not appear to be associated with a particular nucleus but rather correspond to the diffuse hypothalamic distribution of ventral noradrenergic bundle; he therefore hypothesized that the destruction of this pathway is responsible for the classical syndrome of hypothalamic obesity.

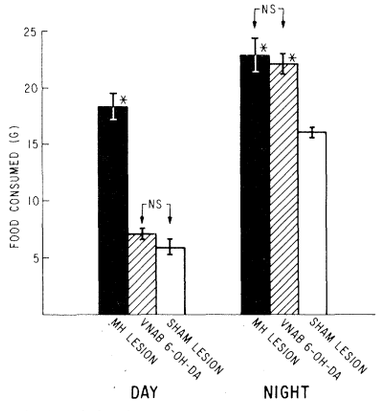

Fig. 1. At night the rats with ventral noradrenergic bundle, 6-hydroxydopamine lesions (VNAB 6-OH-DA) overate; their hyperphagia during the night matched that seen in the rats with medial hypothalamic (MH) lesions. During the day VNAB animals ate no more than the control rats, whereas the MH animals continued to overeat. Values represent 6 days and nights (* = $P < .001$ versus sham lesion group; NS = $P > .10$).

Our experiments suggest that hypothalamic hyperphagia and ventral bundle hyperphagia are actually two different syndromes. Diurnal feeding patterns were different; food intake was quantitatively different; one lesion did not substitute for the other; and rats with medial hypothalamic (MH) lesions became hyperphagic without norepinephrine loss.

Forty adult Sherman female rats were maintained in a room lighted from 8:30 a.m. to 11:00 p.m. They were housed in single cages and had constant access to food (Purina Laboratory pellets) and water. Assignment to one of the three surgical groups was random. (i) Eighteen animals received bilateral, intramesencephalic injections of 8.0 μg of 6-hydroxydopamine (6-OH-DA), an agent that destroys catecholamine neurons (*6*). The injections were made in the vicinity of the ventral noradrenergic bundle (VNAB) (*7*). Our previous studies (*1*) indicate that this procedure consistently destroys nearly all ascending noradrenergic input to the fore-