EXPERIMENTAL MYASTHENIA GRAVIS

A Murine System*

By PHILLIP W. BERMAN‡ AND JIM PATRICK

From the Neurobiology Laboratory, The Salk Institute for Biological Studies, San Diego, California 92112

The process by which an animal is able to elaborate an immune response against its own tissues is thought to underlie a number of degenerative disorders. One system which may provide insight into this phenomenon is experimental myasthenia gravis (EMG)[1] (1). Laboratory animals immunized with purified nicotinic acetylcholine receptor (AChR) produce anti-receptor antibodies and often develop myasthenia (2). Characteristics shared by human myasthenics and myasthenic animals include: muscular weakness and flaccid paralysis which is aggravated by exercise and relieved by acetylcholinesterase inhibitors, hypersensitivity to curare, low amplitude miniature endplate potentials, decrementing compound muscle action potentials in electromyographs, simplification of the postsynaptic membrane at the neuromuscular junction, and the presence of circulating antibodies reactive with autologous AChR (3–6).

Although many studies have detailed the consequences of anti-AChR antibodies binding at the neuromuscular junction, less attention has been focused on the mechanisms regulating the induction, maintenance, and suppression of the anti-AChR immune response. The present investigation describes murine EMG which, in principle, will permit these mechanisms to be examined. Mice have several distinct advantages over animals previously studied (rabbit [1], rat [7, 8], guinea pig [7], and monkey [9]). The immune system of the mouse is better characterized than that of any other animal, and the availability of inbred, congenic, and recombinant strains permit genetic analysis. Different functional subclasses of lymphocytes can be distinguished by antisera against cell surface antigens. Finally methodology exists for the selective manipulation of the mouse immune system.

Another advantage of the mouse EMG system derives from the availability of several mouse muscle cell lines. In the studies described, use is made of the mouse nonfusing muscle cell line BC_3H-1 (10). This cell line is a rich source of mouse AChR (11, 12) and is well suited for use as a target tissue in in vitro studies of the mouse anti-AChR immune response.

Materials and Methods

Snake Venom Neurotoxins. α-neurotoxin from the venom of the cobra *Naja naja kaothia* (Biologicals Unlimited) and α-bungarotoxin (αBT) from the venom of the krait *Bungarus multicinctus* (Ross Allen's Reptile Institute. Inc., Silver Springs, Fla.), were purified as previously

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[1] Abbreviations used in this paper: AChR, acetylcholine receptor(s); αBT, α-bungarotoxin; DME, Dulbecco's modified Eagle's medium; EMG, experimental myasthenia gravis; FCS, fetal calf serum; PBS, phosphate-buffered saline; TXB, 10 mM Tris, 100 mM NaCl, 50 μM phenylmethylsulfonyl fluoride, 1 mM NaN_3, 1 mM EDTA, pH 7.0.

described (11). α-BT was iodinated to sp act of 1×10^8 and 10^9 cpm/nmol by the method of Vogel et al. (13).

Purification of AChR from Torpedo californica. The electric organs of freshly killed *T. californica* were removed at 4°C, cut into cubes (~1 × 1 in), and quick-frozen on dry ice. The frozen tissue were stored at −70°C until needed. In a typical preparation, AChR-rich membranes obtained from 600 g of electroplax were solubilized in Triton X-100 and applied to an α-neurotoxin affinity column in a manner similar to that previously described (14). The column was then washed sequentially with 500 ml of 10 mM Tris, 100 mM NaCl, 50 μM phenylmethylsulfonyl fluoride, 1 mM NaN_3, 1 mM EDTA, pH 7.0 (TXB), 0.5% in Triton X-100; 120 ml TXB, 0.5% in Triton X-100, 1 M in NaCl; 120 ml TXB 0.5% in Triton X-100; 120 ml TXB 0.1% in Brij-35 (Calbiochem-Behring Corp, American Hoechst Corp., San Diego, Calif.). AChR was then eluted by incubating the column bed with 70 ml of TXB, 0.1% in Brij-35, 1 M in carbamylcholine for 3 h at 4°C. The first 130 ml of carbamylcholine eluate was collected and dialyzed overnight against three changes (1 liter/change) of TXB, 0.1% in Brij-35, concentrated by ultrafiltration to a final vol of 2 ml, and 500 μl was loaded onto each of four sucrose gradients (5–20% sucrose in TXB, 0.1% Brij-35, 100 mM in carbamylcholine). The gradients were centrifuged in a swinging bucket rotor (SW41-Ti, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 20 h at 125,000 *g* and fractionated with a bottom punch device (Hoefer Scientific Instruments, San Francisco, Calif.). Analogous fractions from each tube were pooled. The absorbance at 280 nm of each fraction was measured and the protein-containing peaks were pooled to give four fractions corresponding to varying proportions of monomeric and dimeric receptor species. The pooled fractions were then dialyzed against multiple changes of phosphate-buffered saline (PBS), 0.1% Brij-35, divided into aliquots, and stored under liquid nitrogen until needed.

Mouse AChR from BC_3H-1 Cell Line. The nonfusing muscle cell line BC_3H-1 was used as a source of mouse AChR (10). Cells were grown in Dulbecco's modified Eagle's medium (DME) containing 20% fetal calf serum (FCS), at 37°C in 12% CO_2, 88% air. Cells were harvested 9–12 d after passage and solubilized in PBS containing 6% Triton X-100. ^{125}I-αBT-AChR complexes were formed by incubating the Triton extract with excess ^{125}I-αBT (~10 pmol ^{125}I-αBT per pmol toxin-binding sites) for 2 h at 4°C. The mixture was then centrifuged at 12,000 *g* for 2 min and the supernates were pooled. Unbound ^{125}I-αBT was separated from ^{125}I-αBT-AChR complexes by gel filtration chromatography on a Sephadex G-100 (Pharmacia Fine Chemicals Div. of Pharmacia, Inc., Piscataway, N. J.) column (2 × 40 cm) equilibrated with PBS, 0.1% Triton X-100, 1 mM NaN_3. Fractions of 0.5 ml were collected and fractions containing radioactivity and appearing in the void volume were pooled. Toxin-AChR complexes were used within 24 h of preparation.

Mice. All mice used were females 8–12 wk of age weighing between 20 and 30 g. BALB/cke mice were obtained from a colony maintained at The Salk Institute. AKR/Cu mice were obtained from Cumberland View Farms (Clinton, Tenn.) C3H/St mice were obtained from Strong Laboratories (Del Mar, Calif.). All other mice used were obtained from The Jackson Laboratory (Bar Harbor, Maine).

Immunizations. Mice were immunized with *T. californica* AChR emulsified in complete Freund's adjuvant (one part aqueous antigen:two parts adjuvant). The amount of detergent (Brij-35) in the emulsion was kept to a minimum with the final concentration ranging from 0.025 to 0.075 mg/ml. In the primary sensitization, 200 μl of antigen emulsion containing 15 μg of *T. californica* AChR was distributed among six intradermal sites along the back (20 μl/site), the hind footpads (20 μl/footpad), and the base of the tail (40 μl). Mice were boosted 5–8 wk after the primary sensitization with 100 μl of antigen emulsion containing 15 μg of AChR. The antigen was injected at three intradermal sites along the back (20 μl/site) and at the base of the tail (40 μl). In most cases the blood was harvested by tail bleeding 7–14 d after the secondary immunization, depending on the physical status of individual mice.

Radioimmunoassays. The binding of antibodies to AChR was measured in a double-layer radioimmunoassay technique similar to that described by Patrick et al. (15) using a 10- to 25-fold molar excess of ^{125}I-αBT-AChR complexes over antibody. In assays using *T. californica* AChR, each tube contained 1 pmol of toxin-receptor complexes (0.25 μg of purified AChR protein), whereas in assays of mouse AChR, each tube contained 0.25–0.50 pmol of toxin-receptor complexes.

AChR Degradation Assays. The rates of AChR degradation in the cell line BC_3H-1 were measured in the presence of mouse anti-AChR sera by a method similar to that described by Heinemann et al. (16). The cells were incubated with 10^{-8} M ^{125}I-αBT for 2 h at 37°C in DME-10% FCS, and then washed four times with fresh medium. The medium in each culture was replaced with 1 ml of DME-10% FCS containing 2% immune or preimmune mouse serum and the cultures were then incubated at room temperature (21–23°C) for 1 h in an atmosphere of 12% CO_2 and 88% air. The vol of the cultures was increased to 2 ml with DME-10% FCS and the cultures were returned to an incubator and maintained at 37°C. 100-μl samples of the culture medium were removed at various times over an 8- to 10-h period and the radioactivity in each aliquot was measured in a gamma counter. After the last aliquot, the radioactivity remaining associated with the cells in each culture was measured in a filter assay (11). The average rate of release of radioactivity into the medium from duplicate cultures was determined by linear regression analysis using the method of least squares, assuming a single exponential process.

Exercise of Mice. Mice were subjected to forced exercise by one or more of the following methods: (*a*) repetitive grasping, (*b*) forced running, (*c*) inverted hang, or (*d*) free swimming. In the repetitive grasp exercise, a mouse is held by the base of the tail and allowed to grasp the steel meshwork of a cage top with its forepaws. The mouse is pulled by the base of the tail until it releases its grip. The procedure is repeated several times in rapid succession. The grip of a myasthenic animal weakens appreciably after a few grasps, while that of a normal animal remains comparatively constant. The forced running exercise entailed gently chasing a mouse for a distance of up to 4 m with a pencil eraser. Severely myasthenic and moderately myasthenic mice cannot run at all, whereas weakly myasthenic mice exhibited paralysis and stopped running after 2–3 m. In the inverted hang exercise, a mouse is allowed to stand on a piece of heavy steel screening, which is then inverted. A normal mouse moves freely around the screen and can maintain the inverted position, while myasthenic mice have difficulty moving and soon fall off the screen. In the free-swimming exercise, a mouse is placed in a container of water filled to a depth of ~10 cm and equilibrated to room temperature. The mouse is allowed to swim freely for 2 min. A normal mouse swims freely without apparent fatigue. Severely myasthenic and moderately myasthenic mice can swim only for a few seconds and have to be rescued. Weakly myasthenic mice can swim for the entire 2-min period; however, their movements tend to be jerky and uncoordinated and when returned to a solid surface they exhibit obvious weakness and paralysis.

Thermosensitivity Test. Some mice which did not display behavioral characteristics of EMG at room temperature (21–23°C) did so after warming under a heat lamp (air temperature 30–35°C) for <2 min. When returned to room temperature, such animals exhibited obvious weakness and paralysis.

Electromyography. Mice were anesthetized by inhalation of methoxyflurane (Pittman-Moore Inc., Washington Crossing, N. J.) and were restrained on a cork dissecting board. Monopolar stimulating and recording electrodes were fashioned from stainless steel dissecting pins. Paired stimulating electrodes separated by ~2–3 mm were inserted intramuscularly near the sciatic notch. Recording electrodes were inserted in the medial compartment of the gastrocnemius and near the insertion of the Achilles tendon. To isolate stimulus artifacts, a ground electrode was placed at a variable position between the stimulus and recording electrodes. Pulses were applied with a square-wave stimulator (model SD9, Grass Instrument Co., Quincy, Mass.). Electromyographic responses were recorded without additional amplification using a storage oscilloscope (model R5 103N, Tektronix, Inc., Beaverton, Oreg.).

Neostigmine Treatment. Neostigmine bromide (0.0375 mg per kg) was given in the presence of atropine sulfate (0.015 mg per kg) by intraperitoneal injection. A typical mouse weighing 20 g received 50 μl of a freshly prepared solution containing 0.015 mg per ml of neostigmine bromide and 0.006 mg per ml of atropine sulfate in PBS.

Curare Sensitivity. The LD_{50} of curare was determined by the method of Litchfield and Wilcoxin (17). Dose-response curves were constructed with 5–10 experimental points using 10 mice of each strain tested per dosage. The drug was dissolved in PBS and given by intraperitoneal injection. The volume in which the drug was administered was normalized to 5 μl per g of bodyweight.

Results

The studies below describe humoral immune responses against *T. californica* AChR in inbred strains of mice and their association with the development of EMG. All animals were immunized with monomeric AChR from a single preparation. The subunit composition of this preparation is similar to that described by Raftery et al. (18) with the α, β, γ, and δ subunits corresponding to 43,500, 50,000, 60,000, and 63,500 mol wt, respectively.

In preliminary studies mice were immunized in *T. californica* AChR to determine whether or not mice do in fact develop EMG. In one experiment, five BALB/c mice were immunized with receptor, and one mouse subsequently developed weakness and flaccid paralysis which could be relieved by treatment with neostigmine or eserine. When the experiment was repeated with a group of 20 BALB/c mice, not a single animal exhibited any signs of EMG. From these studies it was clear that although BALB/c mice may develop EMG, the frequency is low. Other inbred strains of mice were immunized with AChR to determine if they might be more responsive. The frequency of myasthenia was found to be higher in C57BL/6 (5 of 5), and SJL/J (4 of 5) mice than in BALB/c (1 of 25) and or C3H/St (0 of 5) mice. To explore this apparent difference in susceptibility, a more extensive survey was undertaken and assay procedures were developed.

Characteristics of EMG. The primary criteria used to distinguish myasthenic from nonmyasthenic animals is extreme muscular weakness, flaccid paralysis, and increased fatigability which can be relieved by acetylcholinesterase inhibitors. Severely affected and moderately affected mice exhibited obvious weakness without forced exercise. The posture assumed by moderately myasthenic mice is distinctive and is illustrated in Fig. 1 A. There is a drooping of the head, neck, and tail; weakness of the forelimbs such that the animal cannot support its body weight, exaggerated arching of the back, and sprawled hind limbs. Walking is also abnormal and characteristic of the disorder. Mice are able to use their forelimbs only with great difficulty and were propelled primarily by their hindlimbs. After exercise, their movements became progressively jerky and tremulous, and in some animals, the hindlimbs also became paralyzed.

As the condition progressed some animals developed severe paralysis. These animals could not right themselves and could not propel themselves if righted. They could neither eat nor groom and consequently lost weight, had a ruffled appearance and developed mucous deposits around the eyes. A severely myasthenic mouse is illustrated in Fig. 1 C.

Weakly myasthenic mice appeared normal until stressed by: (*a*) forced running, (*b*) repetitive graspings, (*c*) inverted hang, (*d*) free swimming, or (*e*) thermosensitivity test. Myasthenic mice developed obvious weakness during the course of the stresses and assumed a posture similar, if not identical, to that illustrated in Fig. 1 A upon completion of the tests.

All animals which exhibited myasthenia could be restored to nearly normal behavior by treatment with neostigmine. The drug was administered intraperitoneally and began to affect behavior after ~12 min. The improvement in muscle strength realized is illustrated in Fig. 1 B and D. The animals regain nearly normal posture, and typically begin feeding, drinking, and grooming behavior soon after treatment. The neostigmine-induced repair of weakness lasts from 2 to 12 h depending on the

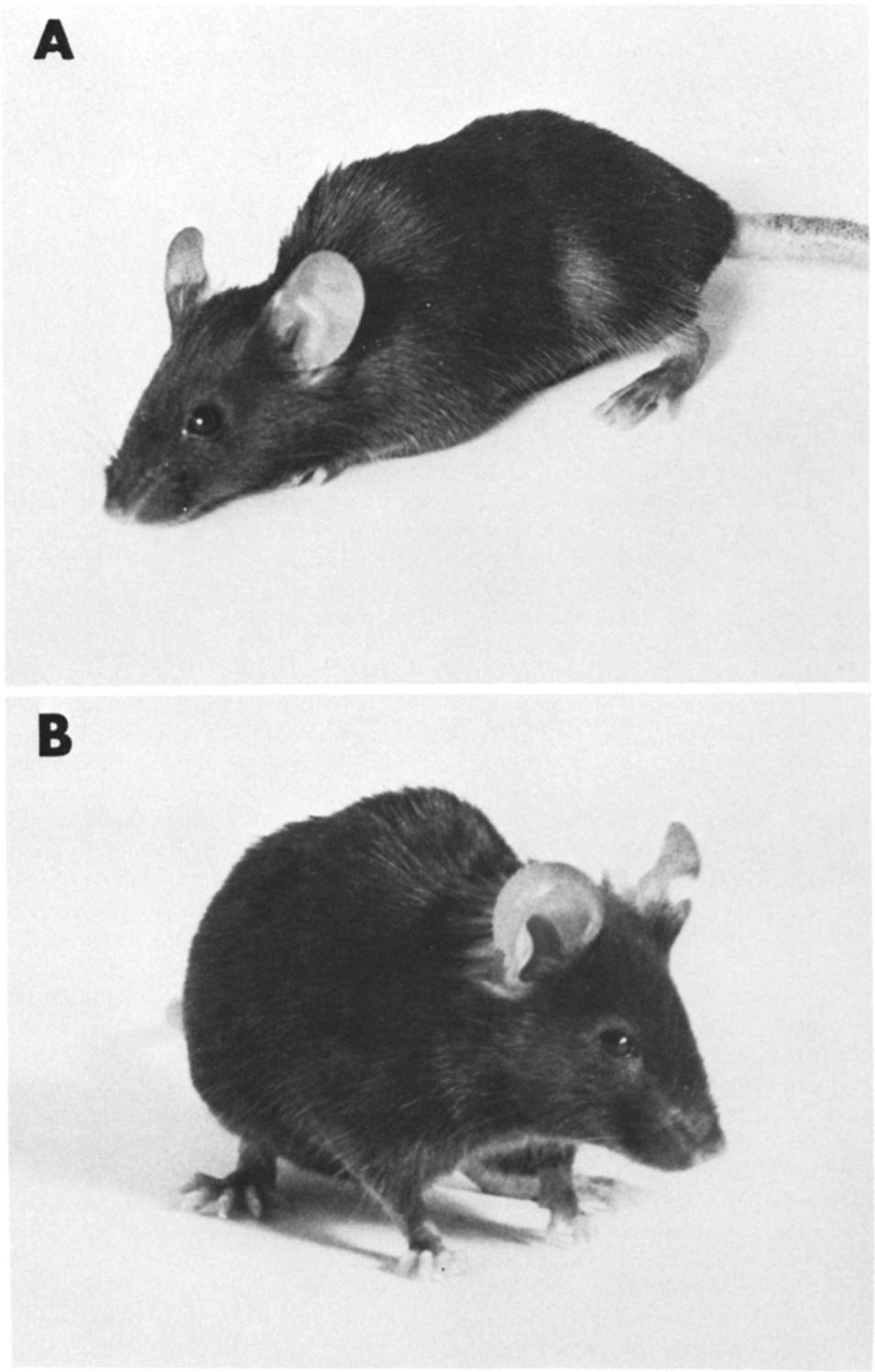

FIG. 1 A, B

FIG. 1. Posture of myasthenic mice before and after treatment with neostigmine bromide. These animals were immunized with 15 μg of *T. californica* AChR and boosted 45 d later with 15 μg receptor. (A) Moderately myasthenic C57BL/6J without forced exercise. (B) Same animal as in (A), 12 min after receiving an intraperitoneal injection of 37.5 μg/kg neostigmine bromide plus 15 μg/kg atropine sulfate. (C) Severely myasthenic BALB/cke. (D) Same mouse as in (C) but 6 min after receiving neostigmine and atropine as described in (B) above.

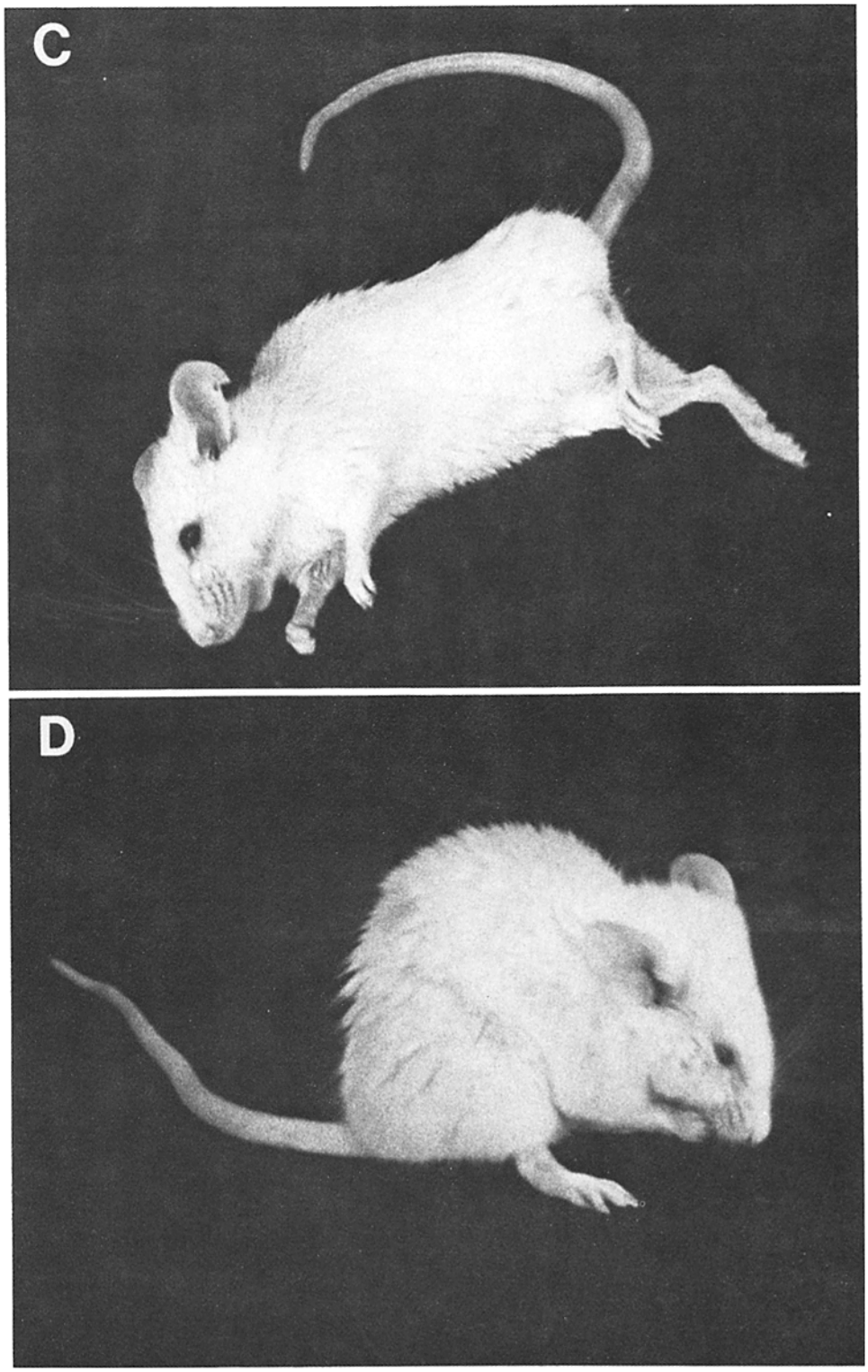

FIG. 1 C, D

severity of the paralysis. In blind studies, attempts were made to correlate the characteristics of EMG described above with weight loss, hypoactivity, ruffled coat and basal metabolic rate. None of these parameters in and of themselves were found to be reliable indicators.

Studies were conducted to determine whether or not myasthenic mice exhibit electromyographs similar to those seen in human myasthenics. Compound muscle action potentials were evoked by supramaximal stimulation of the sciatic nerve and were recorded in the gastrocnemius. The tests were complicated by the fact that myasthenic mice are more sensitive to anesthesia (methoxyflurane, ether, sodium pentobarbital) than normal mice and often died before reliable recordings could be obtained. In addition, considerable difficulty was encountered with movement artifacts; although the frequency of these artifacts can be reduced by restraining straps, normal mice were sometimes found which exhibited decrementing responses similar to those of myasthenics. To circumvent this difficulty, recordings were made from at least two and sometimes as many as four different recording electrode positions on every animal tested.

In Fig. 2 are recordings from a myasthenic mouse and a matched, adjuvant control mouse. The compound muscle action potentials of the myasthenic mouse decrement upon stimulation at 5 Hz without prior tetanic stimulation. The amplitude of the fifth pulse is 53% that of the first pulse. There is no decremental response in a matched adjuvant control mouse when stimulated at 10 Hz even after prior stimulation at 10 Hz for 5 s. It can be seen that treatment of the myasthenic mouse with neostigmine resulted in progressive repair of the decrement.

Electromyography alone could not be used as an assay to screen mice for EMG. In double blind studies consisting of nine myasthenic mice and seven adjuvant control mice, myasthenic mice could not always be distinguished from nonmyasthenic mice. Decremental responses were detected in all moderately myasthenic mice (exhibited obvious weakness and paralysis without forced exercise) and such responses were never seen in adjuvant control mice. Weakly myasthenic mice (paralyzed only after exercise), however, did not always give decremental responses, and in this respect, could not be distinguished from control animals. Although electromyography did not result in any normal animals being classified as myasthenic, they did result in myasthenic animals being classified as normals. Thus electromyography of hind leg muscles was found to be less reliable than behavioral tests in screening for myasthenia.

Another characteristic of human myasthenia is hypersensitivity to AChR antagonists. Studies were conducted to determine if myasthenic mice exhibit similar sensitivity to such drugs. A curare dosage of 100 μg per kg had no effect on normal mice, resulted in flaccid paralysis in weakly myasthenic mice, and often proved lethal for myasthenic mice which exhibited obvious weakness and paralysis. Similarly, myasthenic mice were found to be hypersensitive to ^{125}I-αBT. Although all normal mice tested ($n = 34$) could tolerate an intrathoracic dose of 24–40 μg per kg, a dosage of 0.5–8 μg per kg was lethal for 90% of the myasthenic mice ($n = 10$) tested. On the basis of these studies and the previously described studies of acetylcholinesterase inhibitors, it was concluded that myasthenic mice exhibit pharmacological characteristics similar to human myasthenics.

To study the strain dependence of EMG it was necessary to determine whether or not the time-course of the primary immune response to AChR differed between mice

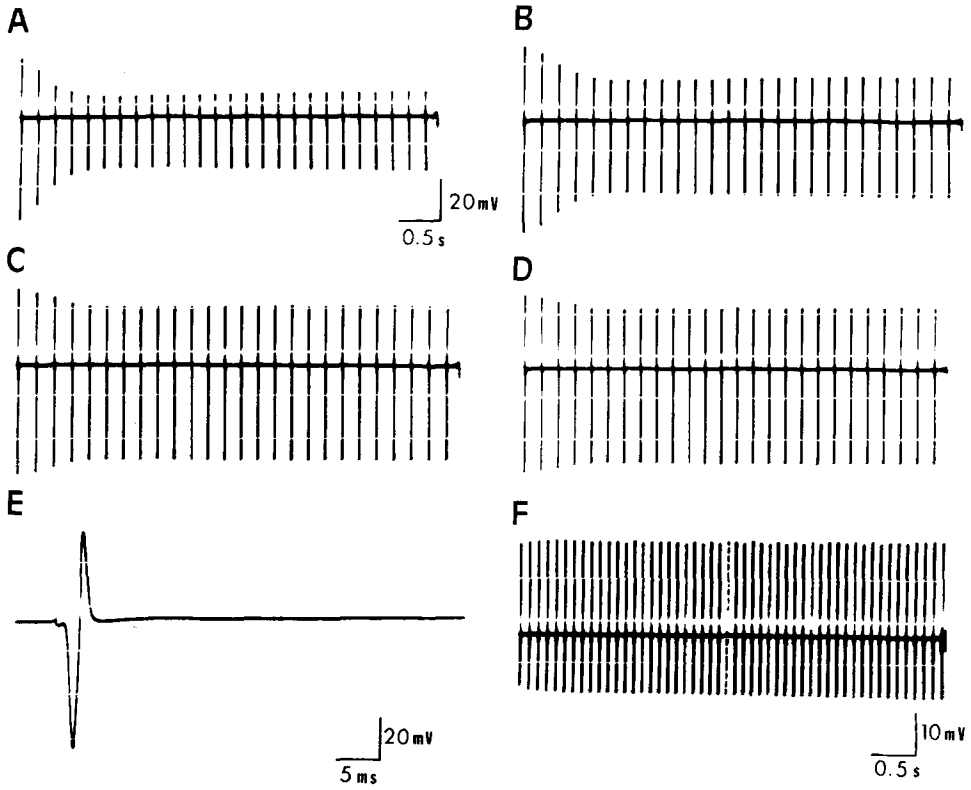

FIG. 2. Electromyograms obtained from a moderately myasthenic C57BL/6J mouse and from an adjuvant-immunized control C57BL/6J mouse. 30 mice were injected, one-half with adjuvant and one-half with adjuvant plus 15 μg *T. californica* receptor. 45 d after the first injection, the control mice were re-injected with adjuvant and the experimental mice boosted with adjuvant plus receptor. 70% of the experimental and none of the control mice developed myasthenic paralysis. Electromyograms were taken 17 d after the booster injection; (A) myasthenic mouse; (B–D) myasthenic mouse 6, 12, and 18 min, respectively, after injection with neostigmine plus atropine; (E) compound muscle action potential obtained between records (C) and (D); (F) control mouse. In (A–D) stimuli of 10 V and .035-ms duration were given at a rate of 5 per s without prior stimulation. The same stimulus was used to obtain the record in (E). The record in (F) was obtained with a stimulus of 9 V and 0.01-ms duration applied at a rate of 5 per s after a conditioning stimulation of 5-s duration at 10 stimuli per s.

with high and low susceptibility to myasthenia. The kinetics of the primary immune response to *T. californica* and BC_3H-1 AChR was monitored in three strains of mice: BALB/c, C57BL/6, and SJL. Antibody titers were measured for five mice of each strain at each time point. In Fig. 3 it can be seen that the primary response continues for at least 5 wk after the initial sensitization with maximal anti-AChR antibody concentration occurring near day 30. After boosting, the antibody concentration rises 3- to 30-fold over that at the peak of the primary response. The titer against mouse AChR ranges between 0.2 and 2% of that to *T. californica* AChR throughout. No significant difference was found in the kinetics of the primary immune response to *T. californica* or mouse AChR between the high-susceptibility strains C57BL/6 and SJL and the low-susceptibility strain BALB/c.

Having established that mice immunized with *T. californica* AChR do develop

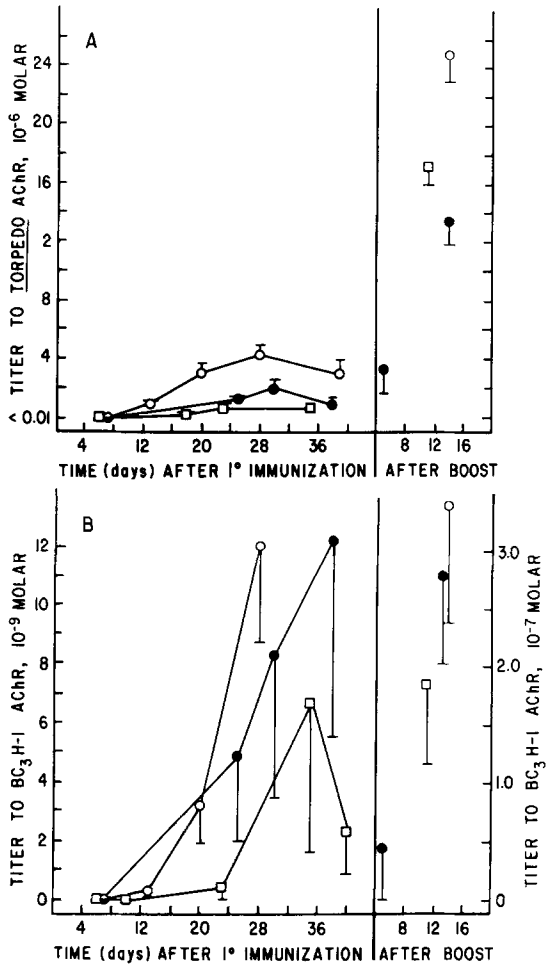

FIG. 3. Time-course of the humoral immune response to *T. californica* AChR assayed on *T. californica* and BC_3H-1 AChR. Mice from three strains were immunized on day 0 with 15 μg *T. californica* AChR as described in Materials and Methods. At the indicated times, five mice of each strain were bled and the concentration of antibodies reactive with *T. californica* and BC_3H-1 receptor determined. Mice were boosted between day 40 and 45. The figure shows the mean concentration of antibody from the five mice taken at each time as function of days after immunization. (○) SJL/J; (●) C57BL/6J; (□) BALB/cke.

characteristics which closely resemble myasthenia gravis, the strain dependence of EMG could be explored in detail. Mice from eight inbred strains were immunized with *T. californica* AChR and were scored for the behavioral characteristics of EMG. The results are indicated in Table I. At least one individual of each strain tested, with the exception of SWR, developed myasthenia. However, the frequency of myasthenia was much greater in some strains than in others. The strains C57BL/6, SJL, and AKR were high responders with frequencies between 50 and 70%. Other strains such as SWR, BALB/c, and C3H/He were low responders with frequencies between 0 and 15%. Additional studies were undertaken to determine whether or not animals which failed to exhibit weakness or flaccid paralysis might exhibit pharmacological or

TABLE I

Strain Dependence of Experimental Myasthenia Gravis

Strain	H-2	Number tested	Number paralyzed	Frequency of paralysis	Curare LD_{50}
					$\mu g/g$
1 A/J	a	15	2	0.13	—
2 C57BL/6J*	b	45	32	0.71*	0.450
3 BALB/c	d	56	4	0.07	0.445
4 AKR/cu*	k	10	5	0.50*	0.475
5 C3H/HeJ	k	35	4	0.11	0.415
6 DBA/1J	q	15	2	0.13	—
7 SWR/J	q	15	0	0	—
8 SJL/J*	s	45	29	0.64*	—

Eight strains of mice were immunized with *T. californica* AChR and boosted with the same antigen 40–45 d after the primary injections. The mice were then examined for paralysis using the assays described in Materials and Methods. The strains, along with their histocompatibility type, and frequency of paralysis are listed above. Nonimmunized mice from the strains indicated were tested for sensitivity to curare. LD_{50} curves were constructed using 5–10 experimental points per strain using 10 mice per point.

* We have designated these strains as high-responder strains.

electrophysiological characteristics of myasthenia. Nonmyasthenic animals from the strains A/J ($n = 10$), SWR ($n = 11$), and DBA/1 ($n = 8$) were tested for hypersensitivity to curare. None of the animals tested exhibited paralysis or weakness at dosages of 140–180 μg per kg which results in flaccid paralysis or death in myasthenic animals. 10 nonparalyzed BALB/c mice were additionally tested for electromyographic characteristics of myasthenia, none of these animals studied exhibited decrementing responses.

Mice typically developed symptoms of EMG 7–14 d after boosting. In high responder strains (i.e., C57BL/6 and SJL) several individuals developed weakness and paralysis 3–4 wk after the primary sensitization, without boosting. These animals progressively deteriorated until death. Approximately 90% of the animals that developed EMG did so within 15 d after boosting. Unlike rats immunized with *T. californica* AChR (7), mice do not appear to exhibit distinct acute and chronic phases of myasthenia. In addition, sensitization to *T. californica* AChR in the presence of *Bordetella pertussis* vaccine did not alter the frequency of myasthenia in BALB/c mice ($n = 20$).

High- and low-susceptibility strains were examined for characteristics which might correlate with susceptibility. Because the immune response to a number of defined antigens is known to segregate with H-2 alleles (19, 20), EMG might also segregate with particular H-2 haplotypes. It can be seen in Table I that animals of all H-2 haplotypes tested developed EMG. It was also found that high and low EMG-susceptibility strains can share a given H-2 allele. Thus both AKR and C3H/He mice possess the $H\text{-}2^k$ allele, with AKR being a high-susceptibility strain and C3H/He being a low-susceptibility strain. These studies give no indication that the H-2 locus alone determines susceptibility to EMG.

At the simplest level there are two alternative explanations to account for the differences in susceptibility which we observe. In one case there could be a difference in the anti-AChR immune response between high and low responders. In the second case the immune response may be the same but neuromuscular transmission may be more sensitive to a reduction in receptor density in high responders than in low

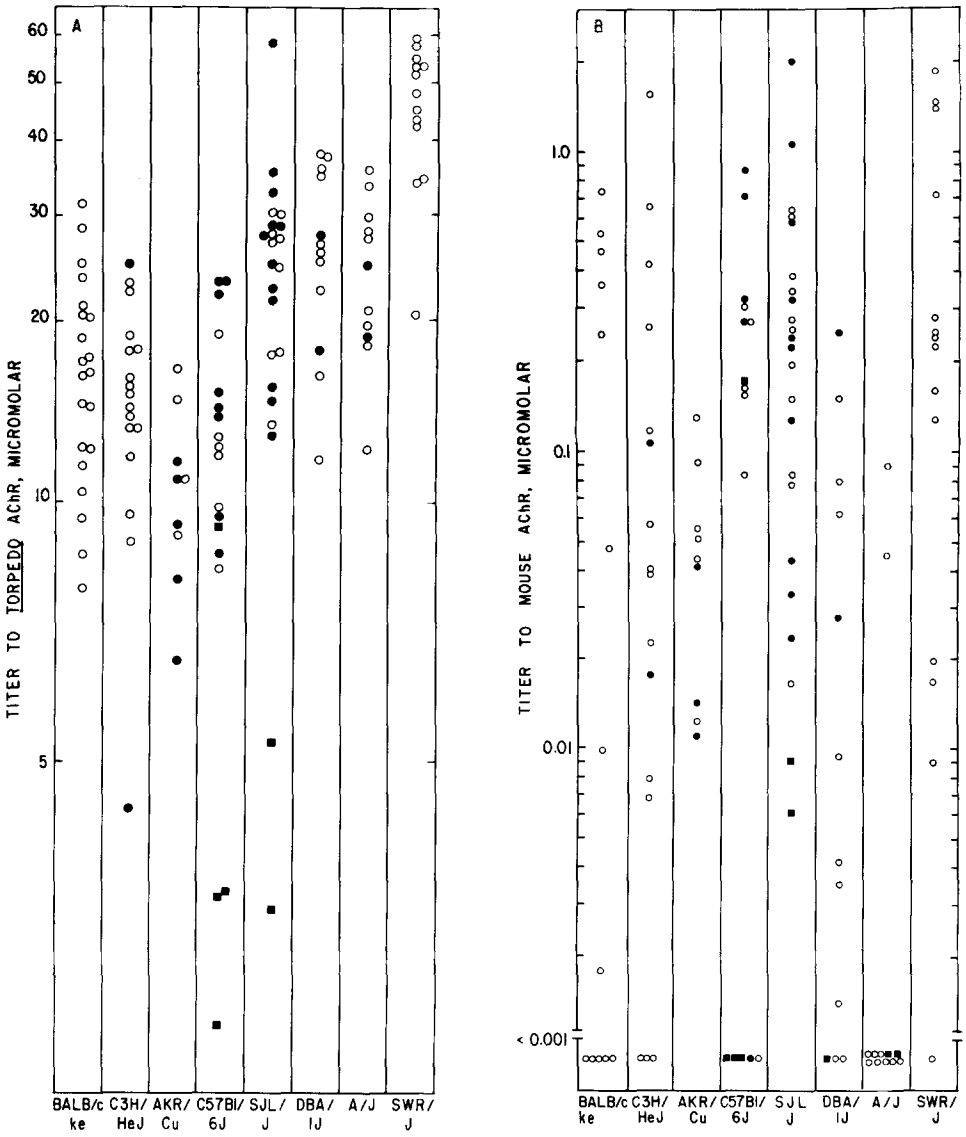

FIG. 4. Concentration of anti-AChR binding sites reactive with *T. californica* and mouse BC_3H-1 AChR. Each point represents the slope of a line obtained when picomoles of receptor-toxin complexes precipitated was plotted against microliters of mouse serum added. The slope of each binding curve was calculated by linear regression analysis using five experimentally determined points and zero. (A) titer to *T. californica* AChR; (B) titer to mouse BC_3H-1 AChR. (○) Mice not exhibiting weakness or paralysis; (●) mice exhibiting weakness or paralysis; (■) mice exhibiting weakness or paralysis 7 d before or 3 d after boosting.

responders. One way to address this distinction is to determine sensitivity to an AChR antagonist, such as curare, using nonimmunized mice. If neuromuscular transmission is more sensitive to a reduction in the number of functional receptors in some strains of mice than in others, then there should be a detectable difference in curare sensitivity. This possibility was tested by determining the curare LD_{50} curves for strains of mice with high and low susceptibility to myasthenia. The results obtained

are indicated in Table I and show that the LD_{50} of the high-susceptibility strains C57BL/6 and AKR did not differ significantly from the LD_{50} of the low susceptibility strains C3H/He and BALB/c, suggesting that high- and low-susceptibility strains do not differ with respect to the fraction of AChR that must remain active to insure synaptic transmission. This result focused attention on the first alternative, that a difference in the anti-AChR immune response might account for the existence of strains of high and low susceptibility.

We first examined the concentration of antibodies reactive with *T. californica* AChR using a radioimmunoassay procedure. The results in Fig. 4A show that all animals tested developed high titers of anti-receptor antibodies. Some strains such as SWR possessed very high concentrations of anti-receptor antibody but did not exhibit EMG, whereas other strains such as C57BL/6 possessed much lower concentrations of anti-receptor antibody and frequently exhibited EMG. Strains such as DBA/1 and SJL exhibited intermediate titers; with SJL frequently developing EMG and DBA/1 rarely developing EMG. Thus, there is no obvious correlation between the concentration of serum antibodies reactive with *T. californica* AChR and EMG.

We next considered whether or not the concentration of serum antibody reactive with mouse AChR correlated with myasthenia. Antibody concentrations were again measured using a radioimmunoassay technique, but in this instance the cell line BC_3H-1 was employed as the source of AChR. The results in Fig. 4B show that the immune response against mouse receptor differs markedly from that against *T. californica* receptor in that the range of titers to the mouse protein spans four orders of magnitude, whereas that to the elasmobranch protein spans less than two orders of magnitude. This increased variability is found within a given strain as well as between strains. Furthermore, some individuals of all strains tested had high concentrations of serum antibodies reactive with mouse AChR, but the presence of such antibodies does not correlate with myasthenia.

Again, some animals, such as SWR, were found to possess very high levels of anti-mouse receptor antibody but gave no indication of EMG. Other animals, particularly C57BL/6, possessed low levels of antibody reactive with autologous AChR but did develop EMG. Thus the presence of antibodies reactive with autologous AChR is not sufficient to ensure myasthenia. The finding that animals with very low levels of anti-AChR antibody seem to develop EMG which is as severe as that seen in individuals with high levels of anti-AChR antibody suggests that if indeed humoral antibodies are able to induce paralysis, then an antibody subpopulation of a particular structure and/or specificity is required.

We began a search for a correlating specificity by testing whether or not nonmyasthenic mice which possess high concentrations of antibody against autologous AChR also make antibodies which react with determinants on the receptor which are exposed on the cell surface. This question was answered by measuring the ability of immune serum to increase the rate of AChR degradation on cultured muscle cells. A number of studies have demonstrated that antibody binding to AChR on cultured muscle cells results in an increase in the rate of AChR degradation (16, 21, 22). If antibodies from nonparalyzed mice increase the rate of AChR degradation they must contain populations which react with cell-surface determinants. Fig. 5 shows that the rates of AChR degradation measured without added mouse serum and in the presence of adjuvant control mouse serum are identical and correspond to an AChR half-life of 9.3 h. The rate of AChR degradation in the presence of serum from a mouse

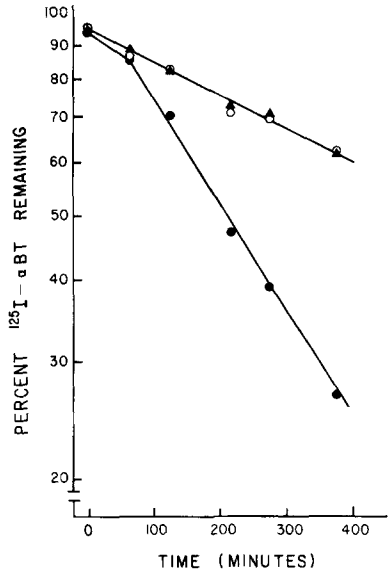

FIG. 5. Effect of serum from receptor-immunized and adjuvant-immunized control mice on the rate of degradation of BC_3H-1 AChR. Cultures of BC_3H-1 were labeled with ^{125}I-αBT, washed, and covered with 1 ml of growth medium containing 10% FCS. Mouse sera were then added to give a 2% solution and the cultures returned to the incubator, and the growth medium sampled at the times indicated. (○) control; (●) serum from paralyzed SJL/J; (▲) serum from control immunized SJL/J.

immunized with *T. californica* AChR increases the rate of AChR degradation such that the receptor half-life is reduced to 3.5 h. Degradation rates measured in the presence of sera from strains highly susceptible to myasthenia, i.e., C57BL/6 and SJL are indicated in Table II. 9 out of the 11 sera tested increased the rate of AChR degradation at the single serum concentration (2%) tested. The AChR half-life does not strictly correlate with the concentration of anti-receptor antibodies. Thus, serum 249 contains the highest concentration of antibodies reactive with mouse AChR and increases the rate of receptor degradation by a factor of 1.6. Another serum, 262, contains one-third the concentration of antibody reactive with mouse AChR, yet increases the rate of degradation by a factor of 2.7. This result suggests that there are either anti-receptor antibody subpopulations which are more effective than others in increasing the rate of AChR degradation, or that the concentration of the species that increases the rate is higher in 262 than in 249. When sera from mice without detectable titers against mouse AChR were tested, the rates of AChR degradation remained at control values. Serum from nonparalyzed animals with high concentrations of antibody reactive with autologous receptor were tested for AChR degradation activity and results in Table II were obtained. 8 of the 10 sera tested increase the rate of AChR degradation and therefore must contain antibodies which bind to exposed antigenic determinants. By this criterion then, antibodies from paralyzed animals could not be distinguished from nonparalyzed animals. This result demonstrates that the presence of antibodies reactive with exposed cell surface antigenic determinants on AChR is not sufficient to result in the induction of myasthenia. Furthermore, the presence of antibodies which are capable of increasing the rate of AChR degradation

TABLE II

Effect of Sera from Immunized and Control Mice on the Degradation Rate of BC_3H-1 AChR

	Strain	Mouse	Paralysis	Titer to BC_3H-1 AChR	$t_{\frac{1}{2}}$ AChR turnover	$t_{\frac{1}{2}}$ control/$t_{\frac{1}{2}}$ anti-AChR
				μM	*h*	
1	C57BL/6J	202	+	0.28	7.5	1.23
2		204	+	0.72	6.2	1.30
3		210	+	0.88	9.0	1.04
4		211	+	0.17	9.5	0.93
5		213	+	0.32	8.3	1.12
6		215	+	0.16	4.5	2.06
7	SJL/J	249	+	1.10	5.0	1.62
8		256	+	0.23	4.3	2.16
9		260	+	0.64	3.2	2.95
10		262	+	0.39	3.5	2.69
11		264	+	0.59	3.5	2.69
12	BALB/cke	22	−	<0.001	8.8	1.00
13		24	−	<0.001	8.8	1.00
14		26	−	<0.001	7.3	1.20
15		43	−	<0.001	8.3	1.00
16	A/J	123	−	<0.001	9.1	0.97
17		124	−	<0.001	9.5	0.93
18		126	−	<0.001	9.5	0.93
19		136	−	<0.001	9.5	0.93
20		137	−	<0.001	9.1	0.97
21	SWR/J	142	−	<0.001	8.5	0.94
22		143	−	<0.001	8.5	0.94
23		141	−	0.28	9.0	0.89
24		145	−	1.60	6.9	1.16
25		146	−	1.90	4.0	2.00
26		148	−	1.50	3.2	2.50
27		149	−	0.23	6.1	1.30
28	C3H/HeJ	59	−	1.60	9.8	0.95
29		62	−	0.12	5.1	1.82
30		64	−	0.26	6.0	1.55
31		65	−	0.10	4.5	2.05
32		66	−	0.66	4.7	1.72

Sera from six strains of mice were tested for their ability to increase the rate of degradation of BC_3H-1 AChR. Two high-responder strains (C57BL/6J and SJL/J) and four low-responder strains (BALB/cke, A/J, C3H/HeJ, and SWR/J) were used. The degradation rates were determined as described in Materials and Methods. Each serum was tested at a concentration of 2% in the presence of 10% FCS. The results from each experimental serum are given relative to results obtained with serum from nonimmunized animals of the same strain. Also included are titers to mouse BC_3H-1 AChR and whether or not that particular animal developed myasthenic paralysis.

on cultured muscle cells does not correlate with weakness or flaccid paralysis characteristic of myasthenia.

Another antigenic specificity which we considered concerned the possibility that antibodies elaborated against mouse AChR but not *T. californica* AChR correlated

with myasthenia. Such antibodies could only arise as a consequence of an autostimulatory immune response against endogenous AChR. The concentration of serum antibody uniquely reactive with mouse AChR was estimated by comparing the binding of anti-receptor sera to mouse ^{125}I-αBT-AChR complexes alone, and in the presence of a 100-fold excess of unlabeled *T. californica* αBT-AChR complexes. If all of the antibody which binds to mouse AChR was actually elicited against antigenic determinants on *T. californica* AChR, then 1% or less of the mouse ^{125}I-αBT-AChR complexes precipitated in the absence of *T. californica* AChR should be precipitated in the presence of *T. californica* AChR. If antibodies preferentially bind to mouse AChR rather than *T. californica* AChR, then 1% or more of the mouse ^{125}I-αBT-AChR complexes precipitated in the absence of *T. californica* AChR should be precipitated in the presence of *T. californica* AChR.

The results obtained are indicated in Table III. In 60% of the mice tested, all of the antibodies reactive with mouse AChR also react with antigenic determinants on *T. californica* AChR. 40% of the animals tested possessed antibodies which appear to be directed against antigenic determinants present on mouse AChR but not on *T. californica* AChR. In 85% of these mice, the antibody-binding activity unique to mouse AChR accounts for 10% or less of the total binding to mouse AChR. The occurrence of antibodies against determinants unique to mouse AChR does not appear to correlate with experimental myasthenia because approximately equal percentages of both myasthenic and nonmyasthenic mice possessed antibodies with this specificity.

Discussion

Mice from eight inbred strains were immunized with *T. californica* AChR. Individuals from all strains tested, with the exception of SWR, developed behavioral, electrophysiological, and pharmacological features of myasthenia gravis. The frequency of myasthenia-like muscular weakness and flaccid paralysis varied considerably among strains. Mice of the C57BL/6, AKR, and SJL strains exhibited high susceptibility with 50–70% of the injected animals becoming paralyzed. All other strains tested exhibited lower susceptibilities with frequencies of myasthenia ranging from 0 to 15%. These results suggest that mice from all strains can develop myasthenia, but that susceptibility to EMG is a heritable trait characteristic of some strains and not others. This conclusion is also supported by studies of susceptibility to EMG in the F_1 offspring from matings of the high-susceptibility strain C57BL/6 with the low susceptibility strain BALB/c where F_1 hybrids appear to be at least three times more susceptible (95% confidence interval) to EMG than the low susceptibility parental type, BALB/cke (P. W. Berman and J. Patrick. Manuscript in preparation.).

In these studies, all mice immunized developed antibody to *T. californica* AChR (mean concentration: 22.3 μM, $n = 124$). Individuals of all strains tested developed serum antibodies reactive with mouse AChR (mean concentration: 0.34 μM, $n = 127$). The occurrence of antibodies reactive with mouse AChR did not correlate with the myasthenic phenotype in that many individuals were found to possess high concentrations of antibody reactive with autologous AChR, yet were not myasthenic. Other mice were found to possess very low concentrations of antibody to autologous AChR and exhibited all the features normally associated with myasthenia. The main point from these studies is that mice are able to tolerate high concentrations of antibodies reactive with autologous AChR without exhibiting paralysis.

TABLE III

Percentage of Antibody Binding to BC_3H-1 AChR Not Reactive with T. californica AChR

Strain	Mouse No.	Titer to BC_3H-1 AChR	EMG	Percent residual binding
		μM		%
1 BALB/cke	23	0.54	−	1.2
2	27	0.25	−	<0.5
3	30	0.47	−	<0.5
4	39	0.36	−	<0.5
5 C3H/HeJ	52	0.42	−	1.1
6	59	1.60	−	<0.5
7	62	0.12	−	<0.5
8	63	0.11	+	<0.5
9	64	0.26	−	<0.5
10 DBA/1J	101	0.02	−	22.6
11	104	0.08	−	8.0
12	112	0.15	−	<0.5
13 SWR/J	145	1.06	−	<0.5
14	146	1.08	−	1.0
15	147	0.26	−	12.6
16	148	1.40	−	17.6
17	150	0.24	−	3.7
18	151	1.40	−	<0.5
19	152	0.73	−	1.5
20 C57BL/6J	202	0.28	+	1.0
21	203	0.30	−	8.9
22	204	0.72	+	3.1
23	205	0.16	−	4.8
24	210	0.88	+	4.9
25	211	0.17	+	<0.5
26	213	0.32	+	1.0
27	215	0.16	+	3.4
28	216	0.27	−	4.2
29 SJL/J	247	0.32	+	2.7
30	249	1.10	+	<0.5
31	254	0.61	−	0.7
32	255	0.28	−	20.3
33	256	0.23	+	2.9
34	257	0.26	−	1.3
35	258	0.24	+	5.0
36	259	0.24	+	<0.5
37	260	0.64	+	1.0
38	261	2.00	+	0.8
39	262	0.39	+	<0.5
40	263	0.34	−	3.4
41	264	0.59	+	<0.5

Competitive binding of anti-AChR antibodies to *T. californica* receptor and receptor from the mouse muscle cell line BC_3H-1. For each serum, antibody binding to BC_3H-1 receptor-^{125}I-αBT complexes was determined in the presence and absence of a 100-fold molar excess of unlabeled *T. californica* receptor-αBT complexes. If all the antibodies reactive with BC_3H-1 receptor are equally reactive with *T. californica* receptor, then the binding to BC_3H-1 receptor-^{125}I-αBT complexes in the presence of *T. californica* receptor-αBT complexes should be reduced to 1% of the value determined in the absence of the *T. californica* protein. This value is indicated on the column on the far right as the percent residual binding. A residual binding value >1% indicates that the antibodies have a higher affinity for the BC_3H-1 receptor than the *T. californica* receptor. Also indicated are the mice which developed paralysis after immunization.

Two alternative explanations could account for the lack of correspondence between the concentration of anti-AChR antibodies and paralysis. One possibility is that the immune response to AChR differs between myasthenic and nonmyasthenic mice. The other possibility is that there is no difference in response to AChR but that there is a

difference in the neuromuscular junction. The latter possibility seems unlikely. No difference in curare sensitivity could be detected between high- and low-susceptibility strains, suggesting that there is no great disparity in the numbers of AChR which must be blocked to impair neuromuscular transmission. In addition, antibody reactive with autologous AChR is often found in nonparalyzed individuals of strains highly susceptible to myasthenia. If there is no difference in the anti-AChR immune response, then this finding could only be explained by polymorphism in neuromuscular transmission within an isogenic strain; this possibility also seems unlikely.

A difference in the anti-AChR immune response between myasthenic and nonmyasthenic animals appears to be a more tenable hypothesis. The simplest interpretation would be that an antibody subpopulation of a particular structure and/or specificity is required for the induction of paralysis in mice. Because mice with very low concentrations of anti-AChR antibody were paralyzed, large free concentrations of the particular antibodies are not required. The occurrence of high concentrations of anti-AChR antibody might be because the mice were immunized with solubilized receptor. AChR is an integral membrane protein and a considerable portion of its structure may be hidden by the plasma membrane. It would be expected that mice make antibodies against antigenic determinants on solubilized AChR which are sequestered, *in situ*. Such antibodies could yield significant titers in radioimmunoassays, but would not be expected to bind receptor in vivo.

The possibility that myasthenia might correlate with the presence of antibodies reactive with antigenic determinants on AChR which are exposed on the cell surface was considered. Both paralyzed and nonparalyzed mice made antibodies which bind to cell surface determinants and increase the rate of AChR degradation. This result demonstrates that the presence of antibodies reactive with autologous AChR *in situ* is not sufficient to insure paralysis; which again suggests that an anti-receptor subpopulation of a particular structure and specificity is required. It has been suggested that antibody-induced AChR degradation at the neuromuscular junction might be a major pathological mechanism in myasthenia gravis (23–25). In these studies we found that both paralyzed and nonparalyzed mice possessed antibodies which can increase the rate of AChR degradation on cultured muscle cells. This result does not rule out the role of antibody-induced AChR degradation in myasthenia gravis. It should be noted that the AChR present on cultured muscle cells is of the extrajunctional type. The electrophysiological, metabolic, and biochemical, properties of extrajunctional AChR are known to differ from those of junctional AChR (26, 27) and recent studies suggest that there is an immunological difference between the two receptor types (28). It is not known whether antibody from nonparalyzed mice which accelerates the rate of extrajunctional AChR degradation can similarly increase the rate of junctional AChR degradation. Studies are, however, in progress to resolve this question (P. W. Berman and S. Heinemann. Unpublished results.).

Immunization of mice with *T. californica* AChR can result in the formation of antibodies against antigenic determinants present on solubilized mouse AChR but not solubilized *T. californica* receptor. Such antibodies can only arise as a consequence of an immune response stimulated by endogenous receptor. Because such antibodies were found in myasthenic and nonmyasthenic animals alike, they do not appear to play a dominant role in the expression of the myasthenic phenotype. It is possible that the production of antibodies unique to mouse AChR is characteristic of some

strains and not others. Further studies employing more animals will be required to resolve this point.

Previous attempts to induce EMG in mice with *T. californica* AChR yielded equivocal results. Fulpius et al. (29) repeatedly immunized BALB/c, DBA/2, and NZB mice with receptor and found that although the mice made high levels of anti-AChR antibody, they did not develop EMG. Fuchs et al. (30), on the other hand, reported that mice immunized with *T. californica* AChR did develop EMG and that the response was strain dependent in that AKR mice seemed to be very susceptible to EMG, and other strains, particularly those possessing the H-2^q and H-2^s haplotypes, seemed to be resistant to EMG. Our studies are compatible with the results of Fulpius et al. (29) in that BALB/c mice appear to be a low EMG-susceptibility strain and exhibit a high serum concentration of anti-AChR antibodies. Our studies are consistent with those of Fuchs et al. (30) to the extent that susceptibility to EMG is strain dependent, but differ in that mice possessing the H-2^q and H-2^s haplotypes were not found to be resistant to the induction of EMG. On the contrary, SJL mice which possess the H-2^s haplotype appear to be a high-susceptibility strain. The observation that a high-susceptibility strain (AKR) and a low-susceptibility strain (C3H/HeJ) possess the same H-2 allele (H-2^k), suggests that H-2 haplotype alone does not determine susceptibility or resistance to myasthenia.

In conclusion, immunization of mice with AChR can result in paralysis. The probability with which any given mouse can be expected to become paralyzed can be estimated knowing the strain of the mouse but not by knowing its haplotype. Knowledge of the concentration of circulating antibodies directed against *T. californica* or mouse receptor does not allow an estimation of the probability of paralysis. Furthermore, animals containing antibodies which increase the rate of extrajunctional AChR degradation are not always paralyzed. These observations, plus the observation that the susceptibility to paralysis can be manipulated genetically (i.e., the use of F_1 hybrids), make the mouse system of myasthenia attractive from two points of view. On one hand it may identify the immunological mechanism that results in paralysis. In addition it may provide access to those elements of the immune system which malfunction during the initiation of an anti-self immune response.

Summary

Mice from eight inbred strains were immunized with acetylcholine receptor (AChR) purified from *Torpedo californica.* All mice developed high concentrations of serum antibodies (10^{-6} M) against the immunogen and ~80% possessed antibodies reactive with mouse nicotinic AChR. 33% of the mice immunized (n = 236) developed muscular weakness and flaccid paralysis. Behavioral, electrophysiological, and pharmacological similarities were found between the experimentally induced muscular weakness and the disease myasthenia gravis. Susceptibility to experimental myasthenia was found to be strain dependent in that the frequency of paralysis was much greater in some strains than others. The occurrence of muscular weakness and flaccid paralysis did not correlate with the concentration of antibodies reactive with *T. californica* or mouse AChR. Anti-receptor antibodies which increased the rate of AChR degradation on the mouse muscle cell line, BC_3H-1, were found in the serum of both myasthenic and nonmyasthenic mice. 40% of the mice tested possessed antibodies reactive with antigenic determinants present on mouse receptor but not *T. californica*

receptor. The occurrence of antibodies unique to mouse receptor did not correlate with myasthenia. Thus, myasthenia in the mouse does not occur simply as a consequence of the presence of antibodies directed against cell surface antigenic determinants of AChR. If anti-AChR antibodies are both necessary and sufficient for the induction of myasthenia, then these studies suggest that populations of a particular structure and/or specificity are required. It is anticipated that the mouse model of myasthenia gravis will permit the regulation of the anti-receptor immune response to be studied in detail.

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