EXPERIMENTAL AUTOIMMUNE MYASTHENIA: A MODEL OF MYASTHENIA GRAVIS IN RATS AND GUINEA PIGS*

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Patrick and Lindstrom (1) reported that rabbits developed weakness, reversible by anticholinesterases, after repeated immunization with acetylcholine receptor (AChR)[1] protein purified from electric eel. This observation suggested a potential experimental animal model for myasthenia gravis (MG) and provided the first experimental support for the hypothesis proposed by Simpson (2), and Lennon and Carnegie (3), that defective neuromuscular transmission in MG may be caused by antibody blocking AChR at motor end plates.

We report in this paper the establishment in rats and guinea pigs of experimental autoimmune myasthenia gravis (EAMG), a reproducible model disease based on the Patrick-Lindstrom observation. We will describe its clinical picture and preliminary immunological and electrophysiological observations. The effects of blockade of receptors in vivo by specific toxin will be compared with findings in EAMG, and similarities between EAMG and MG will be detailed.

Materials and Methods

Preparation of AChR. AChR protein from the main electric organs of *Electrophorus electricus* and *Torpedo californica* were purified by a slight modification of the method of Lindstrom and Patrick (4) employing affinity chromatography on a conjugate of cobra toxin (*Naja naja siamensis*) coupled to agarose. The concentration of AChR protein is expressed as moles of toxin-binding sites per liter. AChR is a multimer containing two to three toxin-binding sites (4). A crude extract of rat muscle AChR was prepared for antibody studies by extraction of a membrane pellet with Triton X-100 solutions as described elsewhere.[2]

Inoculation of Animals. 74 female Lewis rats (obtained from Microbiological Associates, Inc., Bethesda, Md.) and 30 outbred female albino guinea pigs, both aged approximately 10 wk, were injected with graded doses of eel AChR. Seven guinea pigs received 600 pmol of *Torpedo* AChR. Both noninoculated animals and animals inoculated only with adjuvants were used as controls. All rats

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[1] *Abbreviations used in this paper:* AChR, acetylcholine receptor, CFA, complete Freund's adjuvant; EAMG, experimental autoimmune myasthenia gravis; EMG, electromyograph; MG, myasthenia gravis.

[2] Lindstrom, J. M., V. A. Lennon, M. E. Seybold, and S. Wittingham. Antibodies specific for human muscle acetylcholine receptors found in serum from myasthenia gravis patients. Manuscript submitted for publication.

were premedicated with Tylosin (Eli Lilly & Co., Indianapolis, Ind.) for 1 wk before inoculation because of endemic chronic respiratory disease.

Because difficulties were encountered in emulsifying the AChR protein, especially with doses greater than 110 pmol, several methods of preparing both antigen and complete Freund's adjuvant (CFA) were employed in different experiments. In early studies (all guinea pig experiments and approximately one-third of the total rat experiments) stock solutions of AChR were diluted in phosphate-buffered saline (pH 7.4). In later studies emulsification was improved considerably by aggregating AChR by dialysis against water, allowing removal of excess Triton from the supernate before dilutions were made. Emulsions were prepared with equal volumes of CFA. For all experiments with guinea pigs and in initial experiments with rats, CFA was obtained from Difco Laboratories, Detroit, Mich., containing 0.5 mg/ml *Mycobacterium butyricum*. This was supplemented with *M. tuberculosis*, H37Rv (Eli Lilly & Co.), 2 mg/ml for guinea pigs and 0.5 mg/ml for rats (CFA-A). In later rat experiments the formula employed more emulsifer and different combinations of mycobacteria (2 parts Bayol F to 1 part Aquaphor (Duke Laboratories, Inc., South Norwalk, Conn.) with 2.7 mg/ml *M. butyricum* (CFA-B), or with 0.5 mg/ml *M. butyricum* plus *M. tuberculosis*, H37Rv, 0.5 mg/ml (CFA-C). Total vol of 0.1–0.2 ml of emulsion were divided intradermally among multiple sites. Rats received as an additional adjuvant 10^{10} *Bordetella pertussis* organisms (special vaccine product of Eli Lilly & Co.) subcutaneously into the dorsum of each hind foot.

Clinical Observations. Animals were weighed three times weekly and observed daily for signs of muscular weakness which was graded according to the following score: 0, no definite weakness; +, weak grip or cry with fatigability; ++, hunched posture with head down, movements uncoordinated, and forelimb digits flexed; and +++, severe generalized weakness, no cry or grip, tremulous, and moribund.

Electrophysiological Observations. Electromyographs (EMG's) were recorded on a TECA-4 EMG instrument under Nembutal anesthesia (Abbott Laboratories, Chicago, Ill.) (35 mg/kg, intraperitoneally [i.p.]) with percutaneously implanted stainless steel scalp electrodes. Supramaximal stimulation was applied to the sciatic nerve with recording from posterior distal hind limb muscles and, when axillary stimulation was used in addition, from distal forelimb flexor muscles. A single supramaximal response was measured and then followed by repetitive stimuli. Brief trains of 2, 5, 10, and 20 responses/s were recorded superimposed, and then trains of 2, 5, 10, 20, and 50 responses/s were recorded by continuous write out. At stimulation rates of 50/s movement artifacts were frequent and apparent decrements were often not reproducible. Only decrements found at all rates of stimulation, on repeated recordings, and measuring at least 10% at stimulation rates of 2 or 5 /s, were considered significant. When a decrement of 10% or more was found (first compared with fifth response), neostigmine methylsulfate (50–75 μg) was given i.p., and serial studies were done at 1-min intervals thereafter.

Antibody Assays. Rat sera were tested for antibodies to both eel and syngeneic rat skeletal muscle AChR by immunoprecipitation using [^{125}I]toxin-lableled AChR in a modification of the method of Patrick et al. (5). This assay, employing precipitation by rabbit antirat IgG of rat antibody bound to [^{125}I]toxin-labeled AChR, measures antibody directed against sites on the AChR molecule other than the ACh-binding site. Control assays employed [^{125}I]toxin without AChR. Guinea pig sera were assayed by the Ouchterlony gel diffusion test.

Studies with Cobra Toxin. Cobra toxin purified by the method of Karlsson et al. (6) was administered to rats to determine its clinical and electrophysiological effects. An intravenous dose of 200 μg/kg in saline (twice the lethal dose [LD_{100}] for mice) induced a reproducible time-dependent lethal response and was selected for electrophysiological studies. Three rats injected with toxin were studied by repetitive stimulations at 2.5- to 5.0-min intervals until death, and a control rat injected with saline was similarly studied for 20 min.

Results

Clinical Observations. Within 4 wk after a single injection of eel AChR protein with adjuvant both rats and guinea pigs developed weakness characterized by easy fatigability. The response to neostigmine (i.p.) was usually one of partial motor improvement within 4 min. Relief was transient and some severely weak animals failed to respond.

Table I summarizes the dose response data from a single guinea pig experiment and data pooled from two separate experiments with rats (using CFA-A and -C) plus the experiment illustrated in Table II and Fig. 1 (using CFA-B). The incidence and severity of disease induced with the highest dose was lower in both species than that induced by doses one-half log lower, and probably reflects the relative instability of the emulsion with highly concentrated AChR. Rechallenge induced a higher incidence and greater severity of EAMG in both species, e.g.,

TABLE I
Incidence and Severity of EAMG in Guinea Pigs and Rats after a Single Challenge with Eel AChR

Species	Dose in CFA	Number of animals with clinical EAMG			Maximum severity		
		Total	Early*	Late*	+	++	+++
	pmol						
Guinea pig	350	4/5	1	3(4)‡	2	2	0
	110	5/5	0	5	0	1	4
	35	1/5	0	1	1	0	0
	11	1/5	0	1	1	0	0
	3.5	1/5	0	1	1	0	0
	1.1	1/5	0	1	1	0	0
	0	0/4	0	0	0	0	0
Rat	350	21/23	21	15(15)	2	2	17
	110	10/10	9	9(9)	0	2	8
	55	2/2	2	2	0	1	1
	35	8/9	6	6(8)	1	1	6
	11	6/11	5	4	3	2	1
	3.5	7/9	6	4	2	5	0
	1.1	0/10	0	0	0	0	0
	0	0/10	0	0	0	0	0

Guinea pigs were observed for 30 days and rats up to 80 days. All rats received *B. pertussis* and CFA-A or -C except the nine indicated in Fig. 1 which received 350 pmol AChR in CFA-B.
* Early, before day 16; late, generally after day 20.
‡ Parentheses indicate numbers surviving beyond 20 days; some accidental deaths occurred.

EAMG of +/++ severity was induced in three of five guinea pigs by a repeated dose of 1.1 pmol.

Weakness predominately affected the upper limb, head and neck, and laryngeal and respiratory muscles. A characteristic posture was noted in both species: the animal's head tended to be low, often with its chin and elbows on the floor; the back was exaggeratedly humped with the thighs partially abducted (Fig. 2). Jerky movement of the head and forelimbs on attempted ambulation was observed with severe EAMG. Incontinence of urine, inability to swallow, and, in guinea pigs, tachypnea and cyanosis were sometimes observed as weakness became more severe; severe weakness of the hind limbs was uncommon. Early signs of weakness were loss of cry and hypotonia associated with weakness of the neck and forelimbs. In rats, the ability to grasp the bars of a cage lid was rapidly fatigable, and guinea pigs often failed to retract their ears on painful stimulation.

TABLE II

Occurrence of Weakness and EMG Decrements, and Titers of Antibodies to Eel and Rat AChR in Rats Inoculated with Eel AChR and in Controls

Inoculum and rat no.	Day tested	Weakness score	EMG decrement at 5 cps	Titer of antibody to AChR*	
				eel $\times 10^{-7}$ M	rat $\times 10^{-10}$ M
			%		
AChR in CFA-A					
1	9	0	0	4.8	<1.0
	16	0	0	—	—
	24	0	0	14	1.6
	43	0	0	—	—
2	9	+	21	—	—
	15	0	11	—	—
	24	0	0	4.1	<1.0
	43	+	0	—	14
3	9	+	33	—	—
	16	0	0	—	—
	23	0	0	29	5.4
4‡	9	0	0	—	—
	15	0	0	—	—
AChR in CFA-B					
1	8	++	35	—	—
2	9	++	33	5.4	<1.0
	15	0	3	—	—
	23	0	0	56	11
	34	+++	—	—	13
3	9	0	9	6.3	<1.0
	10	+	11	—	—
	15	0	0	—	—
	23	+	41	—	—
4	8	+	14	8.7	<1.0
	15	0	0	—	—
	24	0	0	110	15
	43	++	0	—	—
	49	++	0	65	18
5	10	++	32	—	—
	15	0	0	—	—
	24	0	0	110	17
	43	+	0	74	17
6	8	+	33	4.0	1.0
	15	0	0	—	—
	24	0	0	80	13
	35	+	—	—	17
7	7	+++	—	—	—
8	9	++	38	—	—
	15	0	0	—	—
	24	0	0	—	—
9	9	+	11	6.0	<1.0
CFA-B alone					
1	10	0	3	<0.001	<1.0
	15	0	0	—	—
	27	0	—§	<0.001	<1.0
2	10	0	0	<0.001	<1.0
	15	0	0	—	—
	27	0	—	<0.001	<1.0
3	10	0	0	<0.001	<1.0
	15	0	0	<0.001	<1.0
	27	0	—	<0.001	<1.0
4	10	0	0	<0.001	<1.0
	15	0	0	<0.001	<1.0
	27	0	—	<0.001	<1.0

Further details are given in footnote of Fig. 1.

* Titers expressed as moles [^{125}I]toxin-binding sites precipitated per liter of serum.

‡ Accidental death.

§ No significant decrement was found in any of four similar control rats tested between days 20 and 55.

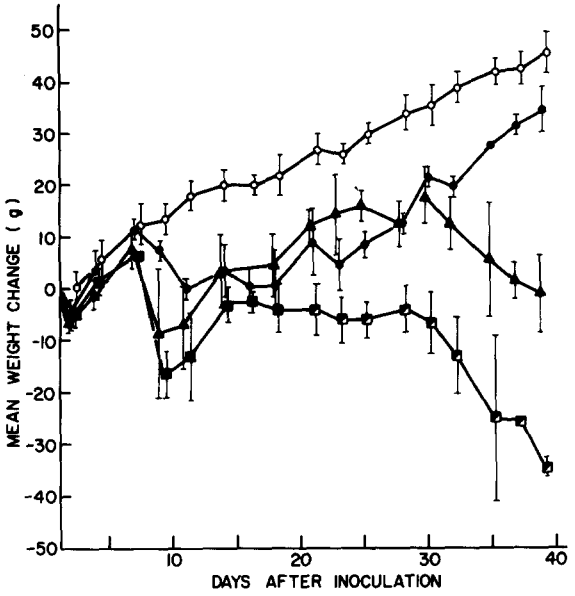

FIG. 1. Weight changes (±SD) recorded in 10 noninoculated rats (O—O) compared with rats inoculated with 350 pmol of eel AChR in two different CFA preparations (CFA-A, ▲—▲, four rats) and (CFA-B, ■—■, nine rats). Five rats received CFA-B without AChR (●—●), Two of the four recipients of AChR in CFA-A had transient early weakness (days 8 and 9) of mild degree (+); none were reinoculated. The more potent CFA-B caused adjuvant arthritis (commencing day 11, peaking days 17-23 and resolving by day 25) which may have accounted for some early weight loss. All recipients of AChR in CFA-B had early weakness (+/+++; mean onset day, 8); three died and the remaining six recovered completely (mean recovery day, 12). On day 19 the six survivors were reinoculated (◩—◩). All surviving rats in both groups which received AChR had a second episode of weakness commencing between days 23 and 35 and progressing to death. Table II documents the severity of weakness and electrophysiological and antibody data on these rats.

In rats the digits of the forelimbs became flexed and failed to extend when the rat was placed on a flat bench. Neck muscle weakness prevented the animals from reaching up for food or water, and excessive growth of the teeth, which occurred only in rats with EAMG, suggested impairment of the muscles of mastication. Weight loss at this stage could be delayed by placing soft food within easy reach of the animals. At the height of the severity of EAMG in rats, characteristic signs of chronic respiratory disease often became manifest as porphyrin staining of nasal secretions and tears. Loud respiratory noises, sometimes observed in the absence of overt respiratory distress or necropsy evidence of gross lung pathology, may have been due to laryngeal paralysis. Signs of chronic respiratory disease disappeared when muscular strength was regained.

Two distinct episodes of weakness, accompanied by weight loss, were observed in rats, especially with large doses of AChR (Table I and Fig. 1). The first was acute in onset, commencing most often between day 7 and 12, and was usually transient with apparent full recovery of strength by days 11–15. The second episode, one of progressive weakness, began most often between days 26 and 35 and was usually preceded 1–2 days by weight loss and signs of chronic respiratory disease which worsened with time. In guinea pigs, numerous relapses of weakness

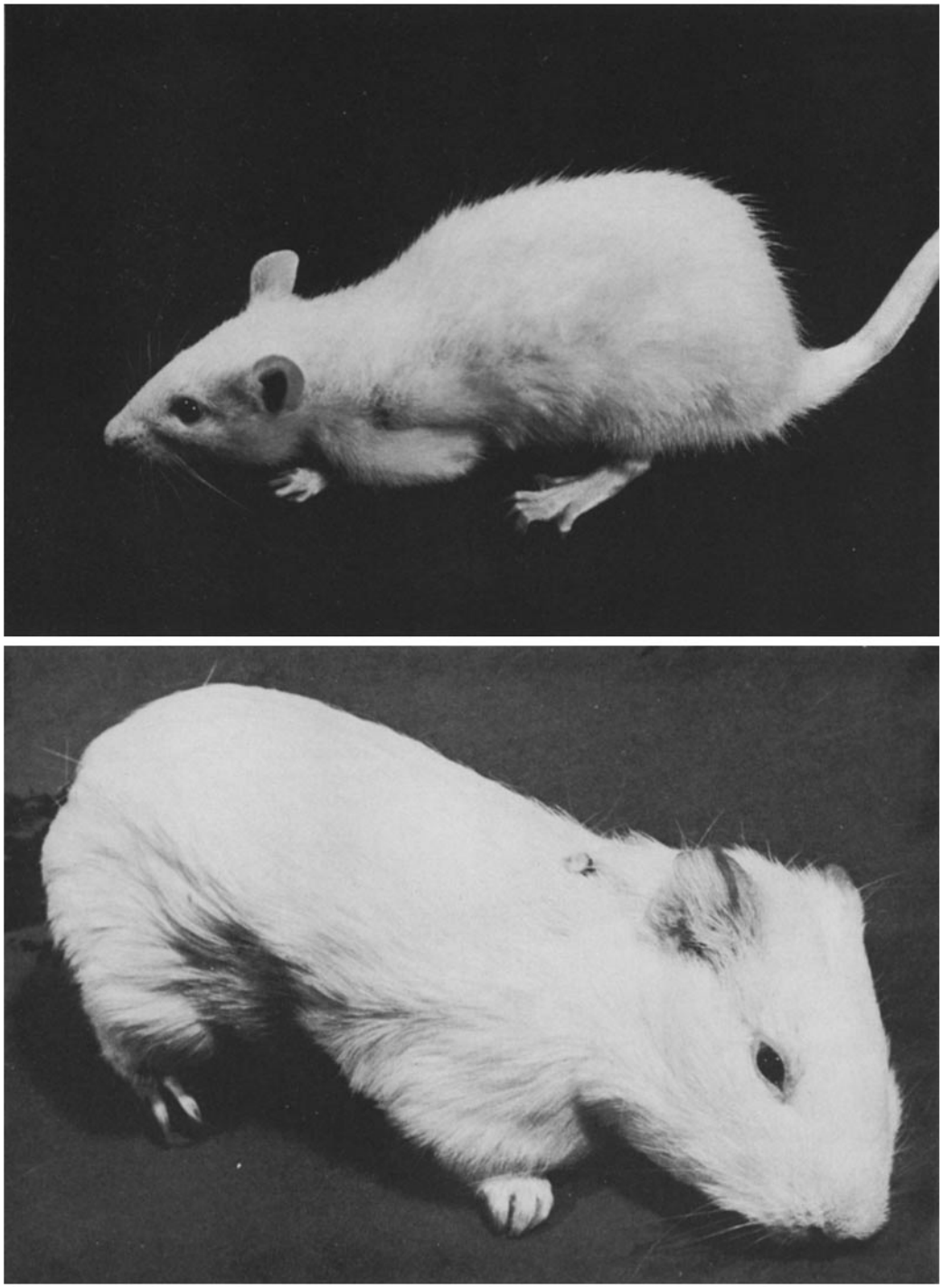

FIG. 2. Postures characteristic of EAMG in a rat and guinea pig. Note the low head with chin and elbows on the floor, hunched back, abducted thighs, and flexed digits.

were observed and often were associated with sudden stresses such as venesection or extensive shaving for skin testing. All seven guinea pigs inoculated with 600 pmol of *Torpedo californica* AChR developed relapsing signs of EAMG which became severe and progressive after day 42.

Electrophysiological Observations. No significant decremental responses to repetitive stimulation were found in any control animal inoculated with adjuvant nor in any animal which had not developed clinical signs of EAMG. In guinea pigs clinically severe weakness was frequently observed without demonstrable EMG abnormalities. In rats, decremental EMG patterns were demonstrable at the peak of clinical weakness, and the decrement lessened with clinical signs of improvement (Table II). In the experiment illustrated in Fig. 1 and Table II, decrements were found in 10 of 12 rats tested after inoculation with AChR. Decrements of action potential, when present, were sometimes demonstrable only in the forelimbs, were always reproducible, and were repaired with 6–13 min after i.p. injection of neostigmine, the repair lasting less than 3 h (Fig. 3).

The smallest response generally occurred with the fourth or fifth stimulus and often increased slightly thereafter. Postactivation facilitation and exhaustion

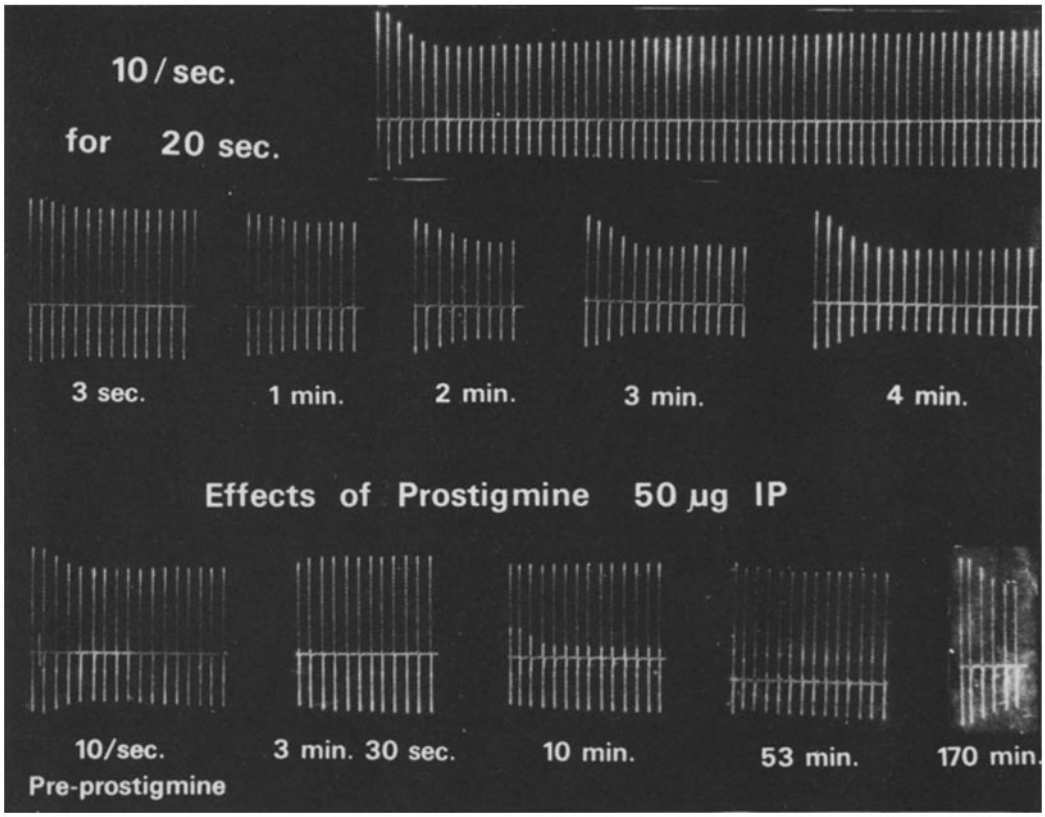

FIG. 3. (top) Typical decrementing EMG response of a rat with clinical EAMG. (middle) Post-activation facilitation lasted 1 min, followed by return of the decrement at 2 min and exhaustion at 4 min. (bottom) Repair of decrement by prostigmine lasted less than 3 h.

were demonstrable (Fig. 3). Only 1 of 10 surviving rats had a significant decrement (11%) when studied 15–16 days after inoculation at which time none was clinically weak. One rat developed a decrement on day 23, which was 4 days after a second inoculation (Fig. 2 and Table II). Three rats studied later, when weight loss and progressive weakness were present, showed no decrement.

Studies with Cobra Toxin. *Naja naja siamensis* toxin was chosen to examine in rats the clinical and electrophysiological effects of in vivo blockade of nicotinic AChR. All four nonanesthetized rats observed after intravenous injection of 200 μg toxin/kg showed signs of weakness 7–10 min after injection, namely: inability to raise the head, hunched posture, forelimb weakness, and inability to extend the forelimb digits. The rats were moribund at 20 min and complete respiratory paralysis ensued at approximately 30 min.

No significant decrement or change in amplitude of a single response occurred in the rat injected with saline. The three rats injected with toxin showed a progressive decline in amplitude of the response to stimulation. This decrease was first noted 10 min after injection (response amplitudes 84, 62, and 77% of the initial amplitude). Amplitudes were even lower at 20 min (17, 16, and 20% of initial values), and at death they were reduced to 10% of their initial values (12, 7, and 8%). Decremental responses were not found until 10–13 min after injection (11, 17, and 7% decrements). The smallest response occurred with the fourth or fifth stimulus and sometimes increased slightly thereafter. Significant decrements developed in all three rats, but the percent decrement did not progress with time (maximum percent decrement, 28).

Antibody Assays. Sera from rats inoculated with eel AChR contained antibody against AChR from both eel electric organ and rat skeletal muscle (Table II), but not against ^{125}I-labeled toxin. The titer of autoantibody was about 1/10,000 that of antibody to eel AChR. Antibody titers are expressed as moles of toxin-binding sites precipitated per liter of serum. Since AChR is a dimer or trimer, these titers are two- to threefold the molar concentration of antibody. Antibody to eel AChR was demonstrable by the relatively insensitive method of double diffusion in agar in γ-globulin fractions prepared from guinea pig sera 4 wk after inoculation.

Discussion

EAMG results from an autoimmune response to nicotinic AChR induced by heterologous AChR. To date EAMG has been induced in four species by inoculation with AChR prepared from the electric organ of the eel *Electrophorus electricus:* rabbit (1), goat (J. M. Lindstrom, unpublished observation), rat, and guinea pig, and in the latter also with AChR from *Torpedo californica*. Although remote phylogenetically, the electric organ AChR must retain sufficient antigenic homology to AChR of mammalian skeletal muscle to break immunological tolerance to the autologous antigen. The availability of inbred strains of rats and guinea pigs will allow detailed immunogenetic analysis of EAMG to be done, and these small species have the advantage of requiring only small quantities of AChR for disease induction.

All species in which EAMG has been induced have produced circulating antibody to AChR. The concept that anti-AChR antibody may cause defective

neuromuscular transmission in EAMG is supported by the fact that immune rabbit sera, but not normal rabbit sera, produced in vitro a functional block of ACh responses of both electric organ cells (5) and cultured rat myoblast cells (1). The presence of antibody against syngeneic rat muscle AChR in the serum of rats with EAMG documents the existence of autoimmunity in this experimental disease. The autoantibody measured in this study was directed at sites on the AChR molecule other than the ACh-binding site (which was occupied by radiolabeled toxin). Antibodies against the site on the AChR molecule which binds ACh comprise only a small fraction of the total antibody to AChR in the serum of animals with EAMG (5). If anti-AChR antibody is to be incriminated in the pathogenesis of EAMG, an explanation is required for its apparently minor specificity for the receptor's ACh-binding site. It might be envisaged that the presumed pathogenic species of antibody is directed against some functionally important part of the receptor molecule other than the ACh-binding site or that antisite antibody is selectively removed from the circulation by adsorption in vivo of ACh-binding sites in muscles.

The occurrence of myasthenic-type weakness in some guinea pigs and rats without accompanying EMG changes is not readily explainable. In preliminary studies, direct measurement with microelectrodes has shown amplitudes of miniature end-plate potentials in rats with EAMG to be reduced in all stages of weakness (E. Lambert, S. Bevan, and S. Heinemann, unpublished observations). In order to directly investigate the clinical and electromyographic effects of in vivo blockade of muscle AChR, we treated rats with purified cobra toxin which is known to block nicotinic AChR (7). Both weakness and a decrementing EMG were induced. Toxin caused a progressive decrease in amplitude of the initial EMG response to supramaximal stimulation. This was possibly due to complete inactivation of many motor end-plates by the overwhelming amount of toxin used. The relatively few end-plates left with a marginal safety factor for firing would account for the decrementing responses noted. A similar but smaller decrease in amplitude was also recorded in EAMG.

Evidence implicating autoimmunity in MG has been well documented. Autoantibodies, especially to muscle striations (8) and thyroid (9), are more frequent in MG than would be expected by chance, as is the incidence of other putative autoimmune diseases (2). Thymic abnormalities in the form of hyperplasia with germinal centers and thymoma are common in patients with MG (10). Thymectomy (11) and immunosuppressive therapy (12) can be beneficial in treating MG, and newborns of myasthenic mothers sometimes exhibit transient signs of myasthenia which last for approximately as long as maternal antibody persists in the newborn (10). Reduction in the number of binding sites for ACh in biopsied nerve-muscle junctions from patients with MG (13) lends support to the hypothesis that antibody may be blocking AChR (2, 3). Almon and co-workers (14) reported that serum globulins from 5 of 15 patients with MG competed with radiolabeled toxin for the ACh-binding site on rat muscle AChR. Moreover, we recently have found antibody to human AChR in 72 of 74 sera tested from patients with MG.[2]

EAMG is similar to MG in many respects. Weakness in both experimentally induced myasthenia and spontaneous MG of humans and dogs (15) frequently affects muscles of the head and neck, upper limbs, and respiration; is charac-

terized by easy fatigue; and is relieved by anticholinesterases. Electrophysiologically, in both conditions a decrementing response is induced by fairly slow rates of stimulation (2–5/s), and impairment of electrophysiological activity does not uniformly involve all muscles even when weakness is quite severe (16). The amplitude of the response progressively diminishes to the fourth or fifth response and then plateaus or increases slightly. Rapid repetitive stimulation initially repairs the defect (postactivation facilitation), but after 2–6 min, the defect is accentuated (exhaustion). Repair of the electrical defect also is achieved with anticholinesterases. Reduction in amplitude of miniature end-plate potentials is an important point of similarity between EAMG (E. Lambert, S. Bevan, and S. Heinemann, unpublished observation) and MG (17). Finally, antibody to AChR is demonstrable in the serum of animals with EAMG and patients with MG (reference 14 and footnote 2). These data confirm the validity of EAMG as a model for the study of MG and suggest that a common immunopathogenic mechanism may underlie both diseases.

Summary

Immunization of animals with acetylcholine receptor (AChR) protein from the electric organs of *Electrophorus electricus* and *Torpedo californica* induces an autoimmune response to the AChR of mammalian skeletal muscle. Rats and guinea pigs develop experimental autoimmune myasthenia gravis (EAMG) after a single inoculation with small quantities of AChR and adjuvant. The incidence and severity of disease appears to depend on the dose of AChR and stability of the emulsion. EAMG is strikingly similar to myasthenia gravis (MG) of man in its clinical picture and its electrophysiological abnormalities. The presence of antibodies to syngeneic rat muscle AChR in the serum of rats with EAMG documents the existence of autoimmunity in the experimental disease. A common immunopathogenesis is suggested for both EAMG and MG.

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