

ROLE OF COMPLEMENT IN THE PATHOGENESIS OF EXPERIMENTAL AUTOIMMUNE MYASTHENIA GRAVIS*

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Myasthenia gravis (MG)¹ is an organ-specific autoimmune disease of skeletal muscle. Both cellular and humoral immunity to nicotinic acetylcholine receptors (AChR) are demonstrable in patients with MG (1). Experimental autoimmune myasthenia gravis (EAMG) can be induced in animals by a single immunization with solubilized AChR and complete Freund's adjuvant (CFA). The chronic phase of EAMG in rats has proven an excellent animal model for MG (1). EAMG can be adoptively transferred to normal animals by living immune lymph node cells (2, 3) but an effector role has not been demonstrated for T cells in the pathogenesis of EAMG or MG. Helper T cells participate in the rat's autoantibody response to AChR (2, 4). Considerable evidence indicates that anti-AChR antibodies play a central role in impairing neuromuscular transmission both in EAMG and MG (5).

When *Bordetella pertussis* is used as a supplementary adjuvant, a transient acute phase of weakness occurs in Lewis rats 7–8 days after inoculation with AChR (6). Acute EAMG is characterized histologically by infiltration of motor end plates with mononuclear inflammatory cells which separate nerve terminals from the muscle's postsynaptic membrane (7). That membrane is selectively destroyed by intensely phagocytic cells. Acute EAMG can be passively transferred to normal rats by intravenous injection of the IgG fraction from sera of rats with chronic EAMG (8). Usually within 4 days of the onset of weakness, clinical recovery occurs and inflammatory cells disappear.

It is not known what preclinical events lead to simplification of muscle postsynaptic membranes and loss of AChR in spontaneously occurring MG (9). A human counterpart of acute EAMG has not yet been described but occasional association of polymyositis with MG (10) and occurrence of muscle "lymphor-

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¹ Abbreviations used in this paper: α -BT, α -bungarotoxin; AChR, nicotinic acetylcholine receptors; ADCC, antibody-dependent cell-mediated cytotoxicity; CoF, cobra venom factor; CFA, complete Freund's adjuvant; EAMG, experimental autoimmune myasthenia gravis; EMG, electromyogram; HEAE, hyperacute experimental autoimmune encephalomyelitis; MEPP, miniature end plate potential; MG, myasthenia gravis.

rhages" in MG may be evidence of a similar inflammatory cellular event in MG.

To investigate the role of complement as a potential mediator of the pathogenicity of anti-AChR antibodies in vivo, rats were depleted of complement by treatment with cobra venom factor before injection of anti-AChR antibodies. Other rats were depleted of complement after immunization with AChR to determine whether expression of the acute phase of EAMG was complement dependent.

Materials and Methods

AChR Preparation and Inoculation. AChR was prepared from the electric organs of *Electrophorus electricus* (11) and *Torpedo Californica* (12) as described previously. Female Lewis rats (obtained from Simonsen Laboratories, Gilroy, Calif.), aged approximately 10 wk, were inoculated on a single occasion in multiple intradermal sites either with AChR (5–10 μ g) plus CFA and *B. pertussis* vaccine (2), or with adjuvants only.

Antibody Preparation and Inoculation. Immunoglobulins were prepared by 40% ammonium sulfate precipitation from sera of rats immunized 25–100 days earlier with AChR and adjuvants or adjuvants only (8). 1 ml vol containing 40–60 mg protein was injected into the exposed jugular vein under ether anesthesia.

Cobra Venom Factor Preparation and Inoculation. Cobra Factor (CoF) was prepared from the venom of *Naja naja siamensis*, assayed in vitro for anti-complementary activity as described elsewhere (13), and treated with α -paradibromo acetophenone to inhibit phospholipase A₂.² A loading dose of CoF (325 U/Kg) was given i.p. in five divided doses over the first 24 h. For subsequent maintenance of C₃ depression, 65 U/Kg was injected every 48 h.

Clinical and Electrophysiological Assessments. Rats were weighed daily and tested for muscle weakness which was graded on a scale ranging from + for weak cry and grip to +++ for moribund as described previously (6). Electromyograms were performed as described previously, and rats lacking spontaneous decrements of muscle action potential with slow repetitive motor nerve stimulation were retested after i.p. injection of 8 μ g curare (14). A consistent decrement \geq 10% (fifth response compared with first) was considered significant.

Histology. Frozen sections (8 μ m) of forelimb extensor muscles were formalin fixed and stained with hematoxylin and eosin after histochemical reaction with α -naphthyl acetate to locate end plate cholinesterase (7).

Quantitation of Muscle AChR and of AChR Complexed in Situ with Antibody. AChR was extracted with 2% Triton X-100 from washed pelleted membranes of homogenized individual rat carcasses (15). An aliquot of each rat extract was incubated overnight with ¹²⁵I- α -bungarotoxin (α -BT) to label AChR which was then precipitated by sequential addition of excess rat anti-AChR antibodies and goat anti-rat Ig. AChR concentration was expressed in terms of moles of ¹²⁵I- α -BT precipitated. The proportion of AChR which was already complexed with antibody when extracted from muscle was determined from the cpm of ¹²⁵I- α -BT directly precipitated by goat anti-rat Ig without addition of anti-AChR antibodies (15).

Antibody Assays. AChR solubilized from normal rat muscle and labeled with ¹²⁵I- α -BT provided antigen for testing sera in an indirect immunoprecipitation assay (6, 15).

C₃ Assays. Serum C₃ was measured by the Mancini immunodiffusion technique. Anti-rat C₃ antiserum was raised by immunizing rabbits i.d. with an incomplete Freund's adjuvant emulsion containing repeatedly washed precipitates of boiled zymosan reacted with fresh rat serum. The Ig fraction of pooled rabbit antisera was absorbed with rat Ig, and showed a single precipitin band when reacted with whole normal rat serum and little or no reaction with serum of rats depleted of C₃ with CoF. The precipitin band was identified as C₃ in immunoelectrophoretic analysis.

Results

C₃ Values. All experiments were completed within 5 days of commencing

² J. O. Shaw, M. F. Roberts, R. J. Ulevitch, and E. A. Dennis. 1978. *Am. J. Pathol.*

TABLE I
Effects of Complement Depletion on the Outcome of Passively Transferred Anti-AChR Antibodies at Low and High Doses

Ex- peri- ment num- ber	Immunoglobulins injected	Treatment	Incidence of			AChR Extracted from muscle	
			Clinical signs	EMG Decre- ment	Inflam- matory cells	Moles ($\times 10^{-11}$) mean \pm SE (n)	% Com- plexed with anti- body mean \pm SE
I	Anti-AChR (2×10^{-11} mol)	Saline	3/7	6/7	6/6	2.95 \pm 0.09 (6)*	19 \pm 0.8
	Anti-AChR (2×10^{-11} mol)	CoF	0/4	0/4	0/4	4.38 \pm 0.16 (4)	18 \pm 1.0
	Nil	Nil	0/5	—	0/3	4.08 \pm 0.21 (5)	0
II	Anti-AChR (20×10^{-11} mol)	Saline	5/5	4/4‡	4/4	2.05 \pm 0.21 (5)*	53 \pm 3.0
	Anti-AChR (20×10^{-11} mol)	CoF	0/5	0/5	0/5	3.56 \pm 0.13 (5)	60 \pm 2.0
	Adjuvant	CoF	0/2	0/2	0/2	3.55, 4.25	0
	Nil	Nil	0/2	—	—	3.00, 3.16	0

* Values for AChR extracted from muscle of antibody recipients treated with saline were significantly lower than controls ($P < 0.001$, Student's t test). Values for antibody recipients treated with CoF did not differ from controls.

‡ One rat died before testing. The four survivors had +++/+++ weakness and spontaneous decrements of muscle action potential on repetitive motor nerve stimulation; rats in all other groups were tested after challenge with curare, and none showed a decrement except the saline-treated recipients of the lower dose of antibodies.

CoF and the mean serum C_3 value of rats treated with CoF (48 h earlier) was 11% of that in rats treated with saline.

Effects of Passively Transferred Anti-AChR Antibodies. Two separate experiments were performed by employing syngeneic Ig preparations which differed 10-fold in their content of antibodies to muscle AChR (2 and 20×10^{-11} mol per ml, respectively). This allowed comparison of the effects of antibody doses less than and in excess of the total amount of AChR extractable from an average rat's musculature ($3-5 \times 10^{-11}$ moles). CoF (or saline) was commenced 2 days before Ig injection and rats were killed by ether inhalation 3 days after Ig injection, immediately after electromyogram (EMG) tests.

Rats treated with saline, i.e. having normal C_3 levels, developed clinical and electrophysiological signs of EAMG after injection of anti-AChR antibodies (Table I). The smaller dose of antibodies (2×10^{-11} moles) used in experiment I induced mild clinical signs (+), without a spontaneous EMG decrement, but enhanced curare sensitivity was demonstrated electromyographically. There was a 32% reduction in muscle AChR. In experiment II, rats injected with a larger dose of antibodies (20×10^{-11} moles) had clinically more severe EAMG (+++/+++), with profound weight loss (Fig. 1), and spontaneous EMG decrements; muscle AChR was 41% less than that of control groups which did not receive anti-AChR antibodies. Inflammatory cells, predominantly mononucleated, were found in the motor end plate regions of muscle from recipients of both high and low doses of antibodies. The inflammatory cells were intimately associated with cholinesterase-positive material (i.e. postsynaptic membrane) which, in many instances, was sequestered away from the muscle fiber and in a granular, disintegrated form (Fig. 2).

Injection of anti-AChR antibodies into rats depleted of C_3 by CoF treatment, induced neither weakness, significant weight loss (Fig. 1), a decremating

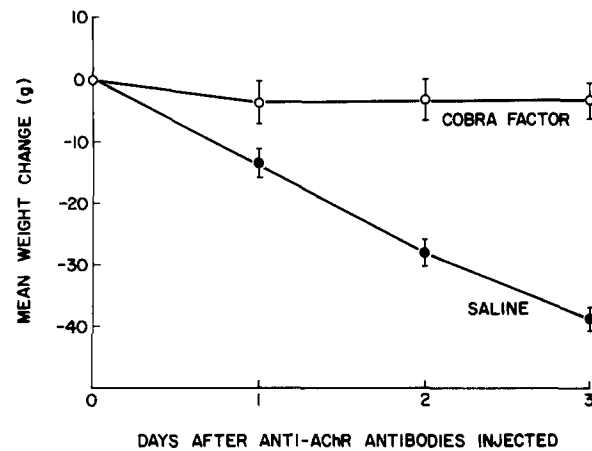


FIG. 1. Mean weight changes (\pm SD) of two groups of five rats injected on day 0 with 20×10^{-11} mol anti-rat muscle AChR antibodies (mean starting weight = 195 ± 12 g). The group treated with CoF had neither clinical signs of weakness, nor enhanced curare sensitivity; the group treated with saline had +++/+++ EAMG and spontaneous decrements of muscle action potential when tested by EMG with repetitive motor nerve stimulation.



FIG. 2. Motor end plate region of digit extensor muscle from a rat 72 h after being treated with saline and injected i.v. with anti-AChR antibodies. Numerous inflammatory cells surround three areas of darkly stained cholinesterase reaction products (arrows) which are sequestered from muscle fibers. Granular appearance is particularly evident in the area on the right (Fig. 3). (hematoxylin and eosin, $\times 400$).

response to repetitive nerve stimulation, nor an increased curare sensitivity (Table I). The amount of AChR solubilized from muscle did not differ significantly from that of control rats not injected with anti-AChR antibodies. However, AChR extracted from rats treated with CoF was found to have

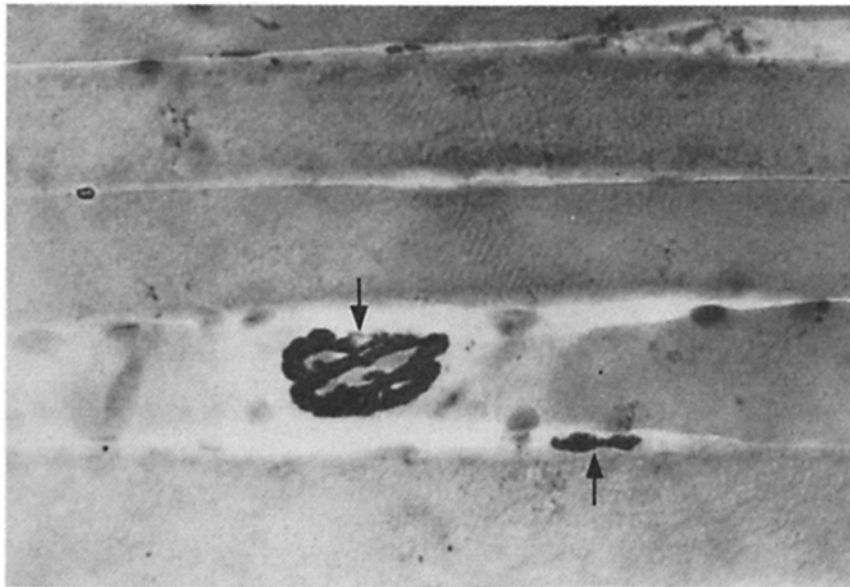


FIG. 3. Motor end plate region of digit extensor muscle from a rat 72 h after being deplemented with CoF and injected i.v. with anti-AChR antibodies. Two areas of darkly stained cholinesterase reaction products (arrows) are seen from above and from the side. They are compact, have a normal appearance, and can be demonstrated to be closely applied to the muscle fibers. No inflammatory cells are seen. (hematoxylin and eosin, $\times 400$).

antibody bound to it, and, indeed, the absolute amount of AChR complexed with antibody was greatest in C_3 -depleted rats injected with the larger dose of antibodies (Table I). Motor end plate regions (Fig. 3) lacked inflammatory cells, and the cholinesterase reaction products resembled those of normal rats and rats injected with adjuvant Ig alone.

Evidence for Complement's Role in Destruction of the Postsynaptic Membrane in the Acute Phase of EAMG Induced by Immunization with AChR. C_3 has been reported to play a role in the induction of immune responses to T-dependent antigens (16). Thus, to ascertain whether our protocol for CoF administration might inhibit induction of T-cell responses in Lewis rats, a preliminary experiment was performed in another model disease—hyperacute experimental autoimmune encephalomyelitis (HEAE). HEAE induced in Lewis rats by guinea pig myelin basic protein has the same time-course as acute EAMG (17) but immunopathologic lesions appear to be mediated entirely by effector T cells. Injection of CoF from the 7th day after inoculation with 30 μ g guinea pig myelin basic protein plus CFA and *B. pertussis* caused depletion of serum C_3 , but did not delay the onset or inhibit the severity of HEAE.

To investigate whether complement may have an effector role in the acute phase of EAMG induced by active immunization, CoF treatment was commenced on the 7th day after inoculation with Torpedo AChR. Of 18 rats inoculated with AChR and adjuvants, 6 received CoF, and 12 received saline. When sacrificed on the 11th postinoculation day (the expected time of peak incidence of acute EAMG), serum titers of antibody to Torpedo AChR in the

TABLE II
Effect of CoF on the Acute Phase of EAMG Induced by Active Immunization with Torpedo AChR

Immu- nogen	Treatment	Incidence of			AChR Extracted from muscle		
		Clini- cal signs	EMG Dec- re- ment	In- flam- matory cells	Range (moles \times 10^{-11})	% Complexed with antibody	
					Mean	Range	
AChR	Saline	8/12	8/11*	7/10	3.2 \rightarrow 8.5‡	3.2	(1-6)
AChR	CoF	1/6	0/6	0/4	5.1 \rightarrow 5.4	1.0	(0-2)
Adjuvant	CoF	0/5	0/5	0/3	5.0 \rightarrow 5.6	0	—

* Six rats had spontaneous decrements of muscle action potential on repetitive motor nerve stimulation, and two more in this group, but none in the other groups, showed a decrement after challenge with curare.

‡ A wide range of values for muscle AChR is characteristic of acute EAMG (see text).

group treated with CoF (mean \pm SD = $1.99 \pm 0.85 \times 10^{-7}$ M, $n = 4$) were in the lower range of titers of the group treated with saline (3.65 ± 1.38 , $n = 11$). This difference ($P = 0.05$, Wilcoxon's rank test) suggests that injection of CoF slightly depressed the humoral immune response to AChR, which requires participation of helper T cells (2, 4). One rat treated with CoF developed mild weakness on day 9 but none had EMG evidence (curare test) of impaired neuromuscular transmission. 8 of 12 rats treated with saline developed weakness ranging from + to +++ severity, and EMG evidence of impaired neuromuscular transmission (Table II). Three of the eight rats with clinical and electrophysiological signs of acute EAMG had antibody titers in the same range as rats treated with CoF.

The amount of AChR extracted from the musculature of rats inoculated with AChR and treated with CoF ($5.1-5.4 \times 10^{-11}$ mol) did not differ significantly from that of controls inoculated with adjuvants ($5.0-5.6 \times 10^{-11}$ mol), whereas values in rats inoculated with AChR and treated with saline varied over a wide range ($3.2-8.5 \times 10^{-11}$ mol). A wide range of muscle AChR content is characteristic of acute EAMG (15), and presumably reflects variable loss and resynthesis of AChR. In both saline and CoF-treated groups, only a small amount of the extracted AChR had antibody bound to it. This also is characteristic of the acute phase of EAMG in normal rats (15).

In 7 of 10 muscle biopsies available from the saline-treated group, mononuclear cells were abundant and in intimate association with granular cholinesterase-positive material. The cholinesterase-positive material (i.e. postsynaptic membrane) was frequently detached from muscle fibers. The motor end plate regions of four rats examined after CoF treatment (including the one with mild weakness) resembled those of control rats inoculated with adjuvants only: the cholinesterase-positive material was compact and closely applied to muscle fibers with no evidence of inflammatory cells.

Discussion

The present study has established that complement plays a critical role in vivo in mediating impairment of neuromuscular transmission in the acute

phase of EAMG induced either by passive transfer of anti-AChR antibodies or by active immunization with AChR. Injection of syngeneic anti-AChR antibodies in excess of the muscle's content of AChR was without any measurable effect in rats treated with CoF. There was no weakness or impairment of neuromuscular transmission as judged electromyographically by curare sensitivity, and the absolute amount of AChR was uniformly within the normal range despite complexing of 60% of AChR with antibody.

Had binding of antibodies to the postsynaptic membrane induced redistribution (4, 12) and accelerated degradation of surface AChR (18-21), as have been reported *in vitro* from studies with both rat and human anti-AChR antibodies on extrajunctional AChR of cultured muscle cells, one might expect an increase in curare sensitivity and also some alteration (either a decrease, or increase, by 72 h) in the amount of AChR extractable from muscle. Thus if antibody-induced modulation of AChR does occur in the intact adult neuromuscular junction, that mechanism does not appear to contribute significantly to the reduction of ACh sensitivity of the postsynaptic membrane induced *in vivo* by passive transfer of anti-AChR antibodies.

Impairment of the ionophore function of AChR by antibodies has also been suggested from *in vitro* studies as a mechanism by which antibodies might impair neuromuscular transmission. Again one might anticipate an increase in curare sensitivity, if this mechanism were a significant pathogenic factor. However, since neuromuscular transmission in the rat has such a large "safety factor", these apparently minor mechanisms of impairing neuromuscular transmission could be of importance in membranes whose content of AChR has been greatly reduced by complement-dependent mechanisms.

Observations by Toyka et al. (22) in mice injected with IgG from patients with MG, suggested that components of the complement system activated early in the cascade were important mediators of neuromuscular transmission impairment by anti-AChR antibodies *in vivo*. They found that recipient mice treated with CoF developed less severe signs of MG (reduction of miniature end plate potential [MEPP] amplitude and decreased α -BT binding sites in muscle) than did normal recipient mice. In contrast, recipient mice genetically deficient in C₃ were affected as severely by MG immunoglobulins as normals. This implied that lytic components of complement were not involved in impairment of neuromuscular transmission by antibodies and complement.

The present study has provided evidence for a complement-dependent mechanism of membrane destruction which need not require the terminal components of complement. Activation of complement by antibodies binding to AChR in the postsynaptic membrane appears to provide a signal that is directly or indirectly chemotactic for mononuclear inflammatory cells. Inflammatory cells did not accumulate in the motor end plates of rats depleted of complement either before passive transfer of anti-AChR antibodies or 7 days after active immunization with AChR. A destructive mononuclear cellular response, which is similarly both transferable by antibodies and complement-dependent, has been described in a guinea pig model of experimental autoimmunity to renal tubular basement membranes (23). Electron microscopy has revealed that destruction of the postsynaptic membrane in acute EAMG is effected principally by cells resembling macrophages (7). Nonimmune lymphoid cells which attack

the postsynaptic membrane presumably bear receptors for C_3 and the Fc piece of IgG complexed with antigen. We previously reported that anti-AChR antibodies detectable in the serum of rats with EAMG (day 10) were predominantly 7S (2). It has been demonstrated in the rabbit that homologous anti-AChR antibodies are cytophilic for macrophages (24). C_3 receptors on lymphoid cells have been proposed to play a role *in vivo* in promoting antibody-dependent cell-mediated cytotoxicity (ADCC), which is inhibited *in vitro* by concentrations of IgG which exist *in vivo* (25). Furthermore, Fc receptors on lymphoid cells have been reported to bind inefficiently to particle bound Fc (16). Thus IgG and C_3 probably act synergistically in opsonization and phagocytosis of particulate antigens. The binding of a macrophage's C_3 receptors to the activated form of C_3 on the postsynaptic membrane would promote interaction between its Fc receptors and IgG on the postsynaptic membrane, thus triggering phagocytosis.

If this picture of acute EAMG is correct, then the absence of inflammatory cells at the motor end plate in chronic EAMG must be explained. First, it is possible that after initial destruction of the postsynaptic membrane by complement-dependent mechanisms, immune complexes (AChR, IgG, and C_3) on the remaining membrane are no longer of sufficient number or in optimal array for effective interaction with lymphoid cells bearing C_3 and Fc receptors, and no longer give rise to an adequate chemotactic signal for inflammatory cells. This hypothesis would most readily explain the abrupt termination of the acute phase of EAMG. Pronounced clinical recovery from acute EAMG, which is often observed over the course of 8 h (V. Lennon, unpublished observation), probably reflects return of the nerve terminals into proximity to remnants of the postsynaptic membrane after disappearance of inflammatory cells. A second possible explanation for termination of acute EAMG is that Fc and C_3 receptors on effector lymphoid cells might become occupied by soluble complexes of antigen and antibody appearing in the circulation with progression of the immune response induced by inoculation with AChR and adjuvants. Because the ratio of antigen and antibody is critical for effecting ADCC *in vitro* (26), a third possibility is that the transient increase in muscle AChR which follows separation of nerve terminals from postsynaptic membranes by inflammatory cells (15), might inhibit lymphoid cells from binding to postsynaptic immune complexes. In MG, a possible explanation for rarity of inflammatory cells at neuromuscular junctions could be that the Fc piece of the IgG subclass(es) of human anti-AChR antibodies may not be cytophilic for lymphoid cells.

Deposition of homologous C_3 has been demonstrated by indirect immunofluorescence on the membranes of cultured muscle cells exposed to anti-AChR antibodies from rats with acute or chronic EAMG (4). Although cultured myotubes were not lysed after binding homologous antibody and complement (4), complement-mediated lysis may occur *in vivo* in the highly specialized postsynaptic membrane. Engel and co-workers have demonstrated focal lysis of the tips of postsynaptic membrane folds electron microscopically in MG (27), and in chronic EAMG, and also early in acute EAMG in the absence of inflammatory cells (7). Immunoelectron microscopic studies of the neuromuscular junction in both chronic EAMG (28) and MG (27) suggest that lysis of the postsynaptic membrane is complement-mediated—both IgG and C_3 were demonstrated on degenerating membrane fragments in the synaptic cleft. C_3 was

demonstrated also on the postsynaptic membrane in the same distribution as bound IgG (27) and residual AChR (i.e. α -BT binding sites) (9). In mild cases of MG, where ultrastructural abnormality of the postsynaptic membrane and reduction of AChR were minimal, a maximal area of membrane was covered by IgG and C₃, but reduction in MEPP amplitude was at most only 40% (27). In more clinically severe cases of MG, MEPP amplitude reduction was paralleled by reduction in AChR, IgG, and C₃ on the postsynaptic membrane. It could be concluded from those studies that in severe cases of MG the mere presence of IgG and C₃ on the postsynaptic membrane was not the major cause of the marked reduction in MEPP amplitude; membrane destruction and accompanying loss of AChR (29) were more important.

The present study in deplemented rats has provided direct evidence that binding of antibody to AChR at the neuromuscular junction does not significantly compromise neuromuscular transmission unless the amount of AChR also is reduced. Complement appears to play a central role *in vivo*, at least in the acute phase of EAMG in rats, in mediating both destruction of the postsynaptic membrane and loss of AChR which is initiated by the binding of anti-AChR antibodies.

Summary

An acute phase of experimental autoimmune myasthenia gravis (EAMG) occurs transiently early in the immune response of Lewis rats to nicotinic acetylcholine receptors (AChR) when *Bordetella pertussis* is used as adjuvant. It is characterized by a destructive cellular attack directed at the postsynaptic membrane of muscle. Acute EAMG can be passively transferred to normal rats by IgG from serum of rats with chronic EAMG. In the present study, acute EAMG, induced either by passive transfer of syngeneic antibodies or by active immunization, was inhibited in rats depleted of complement by treatment with cobra venom factor (CoF). Furthermore, passive transfer of antibodies in excess of the muscle's content of AChR was without any measurable effect in rats treated with CoF. Although 60% of the muscle's AChR was complexed with antibody, there was no reduction in the muscle's content of AChR, and neuromuscular transmission was not compromised as judged electromyographically by curare sensitivity. These data imply that redistribution, accelerated degradation, and impairment of the ionophore function of AChR, effects of antibodies described *in vitro* on extrajunctional AChR, do not play a significant role *in vivo* in impairing neuromuscular transmission in an intact neuromuscular junction. Complement appears to be a critical mediator of anti-AChR antibodies' pathogenicity *in vivo*.

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