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Region of peptide 125–147 of acetylcholine receptor α subunit is exposed at neuromuscular junction and induces experimental autoimmune myasthenia gravis, T-cell immunity, and modulating autoantibodies

(nicotinic receptor/synaptic transmission/disulfide-looped peptide/synthetic antigenic determinant/delayed hypersensitivity)

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ABSTRACT **A major antigenic region of native nicotinic acetylcholine receptors (AcChoR) has been identified by using a synthetic disulfide-looped peptide corresponding to α-subunit residues 125–147 of *Torpedo* electric organ AcChoR: Lys-Ser-Tyr-Cys-Glu-Ile-Ile-Val-Thr-His-Phe-Pro-Phe-Asp-Gln-Gln-Asn-Cys-Thr-Met-Lys-Leu-Gly. The peptide bound 26–56% of polyclonal antibodies induced in rat, rabbit, and dog by immunization with native AcChoR. Rats inoculated with 50 μg of unconjugated peptide developed helper T-cell responses, delayed hypersensitivity, and antibodies to native AcChoR. Anti-peptide antibodies were more reactive with native than denatured AcChoR and bound to the α subunit. Some reacted exclusively with mammalian muscle AcChoR, some induced modulation of AcChoR on cultured myotubes, but none inhibited binding of α-bungarotoxin to solubilized or membrane-associated AcChoR. Repeated immunization induced experimental autoimmune myasthenia gravis: clinical signs in one rat and electrophysiologic and/or biochemical signs in 10 of 11 rats. Thus, at least part of the corresponding region of the mammalian AcChoR α subunit is extracellular at the neuromuscular junction and a potential target for pathogenic autoantibodies in patients with acquired myasthenia gravis.**

The nicotinic acetylcholine receptor (AcChoR) was the first neurotransmitter receptor and transmembrane ionic channel whose complete amino acid sequence was deduced (1–8). This molecular information is of clinical importance because AcChoR in the neuromuscular junction are the target of antibodies that disrupt synaptic transmission in the autoimmune disease myasthenia gravis (MG) (reviewed in ref. 9). Experimental autoimmune MG (EAMG) can be induced by immunization with purified AcChoR from fish electric organs (10, 11) or mammalian muscle (12, 13). Binding of antibodies to AcChoR in the motor endplate can directly block binding of acetylcholine (AcCho) (14). More commonly, secondary events lead to a loss of AcChoR from the endplate (9): membrane lysis follows activation of complement, and accelerated endocytosis and degradation of AcChoR (i.e., modulation) follow its cross-linking by antibodies. No information is yet available concerning the identity of AcChoR segments that induce pathogenic antibodies or are the target of antibodies in MG. This information is essential to devising antigen-specific strategies for immunotherapy of patients with MG.

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Antibodies raised against synthetic peptides corresponding to defined sequences of the AcChoR have revealed the disposition of several regions of the five polypeptide subunits (α_2, β, γ, and δ) of the 290-kDa AcChoR glycoprotein in the *Torpedo* electric organ membrane. For example, the COOH termini of all subunits (15, 16), α-subunit residues 152–159 (17), β 350–358 (15), and γ 360–377 (18) have been assigned to the cytoplasmic face. There has been no immunologic confirmation of the topography of segments thought to be extracellular. The α subunits in electric organ (19, 20) and human muscle AChoR (13, 20) contain a binding site for AcCho near a disulfide bond. Numa's group (1) proposed that cysteine-128 and cysteine-142 may be associated with this site, and they tested the hypothesis by site-directed mutagenesis of α-subunit DNA clones (21). Mutants with Cys-128 or Cys-142 converted to serine, to prevent disulfide bond formation between those residues, yielded AcChoR proteins that did not bind α-bungarotoxin (α-Bgt) or translocate ions in response to AcCho. The properties of an AcChoR mutant with α-subunit asparagine-141 converted to aspartic acid supported the prediction that Asn-141 was a site of N-glycosylation (21) and therefore likely to be extracellular. McCormick and Atassi (22) reported that a synthetic peptide, *Torpedo* α-subunit-125–147 (Tα125–147), bound AcCho and α-Bgt, provided that Cys-128 and Cys-142 were disulfide linked.

The purpose of our study was to determine immunologically whether the α-subunit region corresponding to synthetic peptide 125–147 is exposed extracellularly in the mammalian motor endplate and therefore could be a target *in vivo* for autoantibodies. In addition, we tested whether or not antibodies raised against the peptide could inhibit binding of ^{125}I-labeled α-Bgt (^{125}I-α-Bgt) to native AcChoR.

MATERIALS AND METHODS

Peptide Synthesis. The disulfide-looped peptide Tα125–147 (Lys- Ser- Tyr- Cys- Glu- Ile- Ile- Val- Thr- His- Phe -Pro-Phe-Asp-Gln-Gln-Asn-Cys-Thr-Nle-Lys-Leu-Gly) was synthesized, in which Met-144 of the *Torpedo* (T)AcChoR α-subunit sequence is replaced by norleucine (22). Lys-Lys-

Abbreviations: α-Bgt, α-bungarotoxin; AcCho, acetylcholine; AcChoR, acetylcholine receptor(s); D.TAcChoR, denatured *Torpedo* AcChoR; DH, delayed hypersensitivity; EAMG, experimental autoimmune myasthenia gravis; ELISA, enzyme-linked immunosorbent assay; MEPP, miniature endplate potential; MG, myasthenia gravis; N.TAcChoR, native *Torpedo* AcChoR; PPD, purified protein derivative; TAcChoR, *Torpedo* AcChoR.

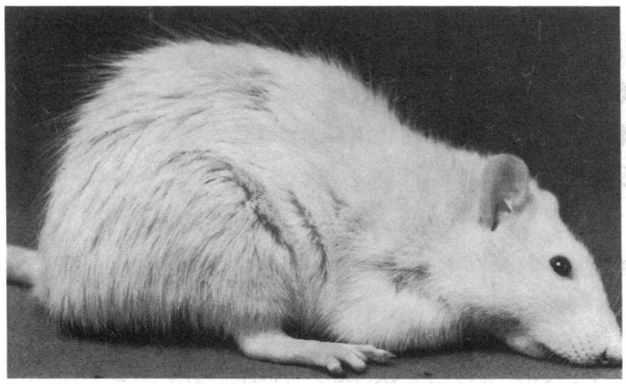

FIG. 1. Chronic EAMG 4 weeks after a second immunization with 50 μg of Tα125–147. Fatigued after mild exercise, the rat was tremulous on attempted ambulation, adopted the hunched posture characteristic of EAMG, and was unable to raise its mandible or forearms from the floor. Difficulty chewing, evidenced by lengthening of the incisor teeth, resulted in 13% weight loss in 2 weeks. MEPP amplitude, 0.18 mV, was 29% of the mean adjuvant control value, and AcChoR content of the tail, 258 fmol/g, was 32% of the control. AcChoR-modlulating antibodies in the serum caused 70% loss of AcChoR from cultured muscle cells. Other serologic tests were negative except for the immunoprecipitation of N.TAcChoR complexed with ^{125}I-α-Bgt, 0.92 nM (see Table 3).

Gly was added to Lys-125 to promote peptide solubility and N-terminal coupling to Sepharose 4B.

Animal Procedures. Female Lewis rats (Charles River), age 10 weeks or more, were anesthetized with diethyl ether for denervating, immunizing, and retroorbital bleeding or with intraperitoneal sodium pentobarbital (≈33 mg/kg of body weight) for amputating tails. Antigens diluted in 0.1 M sodium phosphate buffer, pH 7.0, were emulsified in complete Freund's adjuvant and injected intradermally; *Bordetella pertussis* vaccine was given subcutaneously as additional adjuvant (23).

Microelectrophysiologic Studies. Miniature endplate potential (MEPP) amplitude was measured *in vitro* (24) with intracellular microelectrodes in interosseous muscle from the tail. The mean amplitude of 15 MEPPs, excluding giant potentials, was determined in each of 16–18 fibers per rat.

AcChoR Quantitation, Purification, and Denaturation. AcChoR were extracted in buffers containing 2% Triton X-100 (13, 25). TAcChoR purified by immunoaffinity chromatography (25) bound 5 nmol of ^{125}I-α-Bgt per mg. AcChoR from rat muscle (normal or denervated 10 days) were used as crude extracts. Quantities of AcChoR and bound immunoglobulin were measured as mol of ^{125}I-α-Bgt immunoprecipitated (13). TAcChoR denatured (D.TAcChoR) by reduction and carboxymethylation in 6 M guanidine hydrochloride (26) bound 2 pmol of ^{125}I-α-Bgt per mg.

Delayed Hypersensitivity (DH). Native TAcChoR (N.TAcChoR) and D.TAcChoR (5 μg), synthetic peptides (1 μg), and purified protein derivative (PPD, 50 μg) of human tuberculin were injected in phosphate-buffered saline (23).

Table 1. Microelectrophysiologic and biochemical evidence of EAMG in tail muscle of rats immunized with Tα125–147, D.TAcChoR, or adjuvants only

Immunogen	No. of rats	MEPP amplitude, mV	AcChoR extracted	
			Total, fmol/g	% complexed with Ig
Adjuvants	7	0.61 ± 0.047	448 ± 32.6	0
Tα125–147	11	0.40 ± 0.054*	310 ± 32.3*	5 (0–30)
D.TAChoR	3	0.25 ± 0.057†	227 ± 36.7†	30 (7–69)

MEPP amplitudes were corrected for nonlinearity of the endplate response to AcCho (assuming an AcCho equilibrium potential of −15 mV) and to a membrane potential of −75 mV. This correction did not bias the data because the mean resting potential (−74 mV) was the same in all groups. Results are mean ± SEM or with the range in parentheses.
*Significantly different from adjuvant controls ($P < 0.02$).
†Significantly different from adjuvant controls ($P < 0.01$).

Radiolabeling of Proteins. Purified immunoglobulins and α-Bgt were labeled with ^{125}I (Amersham) by using chloramine-T (27, 28).

Antibody Assays. (*i*) An enzyme-linked immunosorbent assay (ELISA) (29) used purified N.TAcChoR or D.TAcChoR (2.5 μg/ml). (*ii*) An immunoprecipitation assay used equimolar concentrations of native *Torpedo* or rat muscle AcChoR complexed with ^{125}I-α-Bgt (13). (*iii*) A radiobinding assay used living muscle cells in microculture (24). After syngeneic rat myotubes had been exposed to 25% test serum for 16 hr at 37°C, ^{125}I-α-Bgt-binding sites were measured in the presence and absence of carbamoylcholine (30). Retesting with metabolism inhibited distinguished AcChoR-modulating from AcChoR-blocking antibody activity (11). (*iv*) A modified immunoprecipitation assay (31) allowed detection of antibodies binding sufficiently close to the AcCho-binding region of solubilized AcChoR to impede binding of ^{125}I-α-Bgt. (*v*) Immunoblotting (32) was used to investigate binding of rat antibodies to subunits of TAcChoR separated electrophoretically (13) and transferred to nitrocellulose paper (Hoefer, San Francisco). (*vi*) Binding of ^{125}I-labeled anti-TAcChoR immunoglobulins to synthetic peptides and proteins conjugated to Sepharose 4B was titrated quantitatively by an immunoabsorbent assay (27).

RESULTS

Peptide Tα125–147 proved to be a major antigenic region of the native AcChoR molecule. It produced the immunologic phenomena previously described by this laboratory in rats immunized with purified AcChoR (10, 11, 13, 23). Furthermore, the peptide bound up to 56% of antibodies induced by immunization with N.TAcChoR.

Induction of EAMG. One of 11 rats immunized with Tα125–147 developed clinical signs of EAMG (Fig. 1). This indicates that at least part of the mammalian AcChoR's α

Table 2. DH skin responses in rats 10 days after primary immunization

Immunogen	Dose, μg	No. of rats	Diameter of induration at 48 hr, cm				
			N.TAcChoR	D.TAcChoR	PPD	Tα125–147	Hb-A
None	—	4	0	0	0	0	0
Adjuvants	—	12–15	0	0	2.1 ± 0.65	0	0
N.TAcChoR	7.5	8–14	1.6 ± 0.24	1.9 ± 0.49	2.1 ± 0.56	0	0
D.TAcChoR	7.5–100	12–20	2.2 ± 0.59	2.5 ± 0.43	1.9 ± 0.41	0	0
Tα125–147	50	10–16	1.5 ± 0.31	1.2 ± 0.26	1.8 ± 0.36	1.7 ± 0.44	0

Induration less than 0.5 cm diameter was considered negative. Results are mean ± SD. Hb-A is human hemoglobin peptide α45–54 (33). Skin-tested rats were not included in any other studies.

Table 3. Mean peak titers of serum antibodies to solubilized AcChoR induced by Tα125–147 or D.TAcChoR

Immunogen	No. of rats	Reactivity in ELISA*		Binding to native AcChoR complexed with ^{125}I-α-Bgt, nmol/liter of serum		
		N.TAcChoR	D.TAcChoR	*Torpedo*	Rat muscle: Innervated	Rat muscle: Denervated
Adjuvants	6	<5	<5	0.01 ± 0.00	0.01 ± 0.00	0.00
Tα125–147	11	40 (20–80)	8 (5–20)	2.8 ± 1.65	0.68 ± 0.43†	0.72 ± 0.41
D.TAcChoR	3	10,240 (10,240)	15,360 (10,240–20,480)	1185 ± 250	5.7 ± 4.26	18.2 ± 12.32

Results are mean ± SEM or with the range in parentheses.

*Mean reciprocal titer (and range). An absorbance at 405 nm of 1.5 times the value for adjuvant control sera at 1:5 dilution was the endpoint in titrations. Reactivities with N.TAcChoR and D.TAcChoR, respectively, were not found in four and five rats immunized with Tα125–147. They were excluded in calculating the means.

†By immunoprecipitation, antibodies reactive with native *Torpedo* and denervated muscle AcChoR were detected in all rats immunized with Tα125–147, but five rats lacked demonstrable reactivity with innervated muscle AcChoR, possibly due to absorption *in vivo*. They were excluded in calculating the mean.

subunit corresponding to residues 125–147 is extracellular in innervated muscle. Because no other rat became weak, electrophysiologic and biochemical evidence of EAMG was sought in amputated tails after antibodies reactive with muscle AcChoR were found in two consecutive bleedings, or after four immunizations.

MEPP amplitudes in 11 rats immunized with Tα125–147 were significantly lower than in 7 age-matched adjuvant control rats ($P < 0.02$, Student's t test; Table 1). Amounts of AcChoR in tails of the peptide group were significantly less than in the controls ($P < 0.02$) and 5% AcChoR was complexed *in situ* with immunoglobulin in the former group while none was complexed in the adjuvant group. Altogether, 10 of 11 rats immunized with Tα125–147 had electrophysiologic and/or biochemical evidence of EAMG. AcChoR content in 7 rats was less than the range of adjuvant controls (320–550 fmol/g); the mean was 55% of the control. The mean (± SEM) MEPP amplitude in those 7 rats was 0.36 ± 0.061 mV, i.e., 59% of the control value ($P < 0.01$).

Induction of DH to AcChoR by Tα125–147. Rats skin tested with TAcChoR after primary immunization with Tα125–147 had typical DH responses, as did rats immunized with TAcChoR (Table 2). Only rats immunized with Tα125–147 responded to the peptide itself; none responded to a hemo-

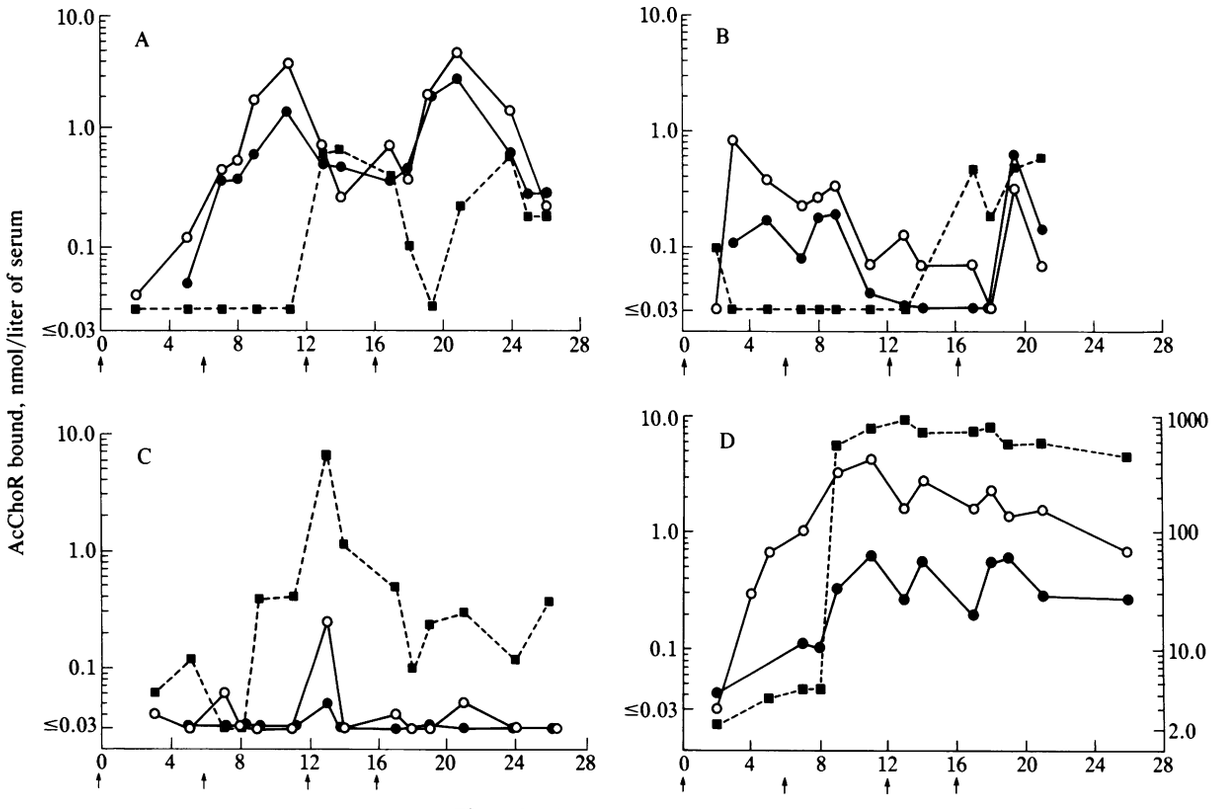

FIG. 2. Antibodies to native AcChoR of *Torpedo* (■), denervated rat muscle (○), and innervated rat muscle (●) after serial immunizations (arrows) with 50 μg of Tα125–147 in rats A, B, and C, and 50 μg of D.TAcChoR in rat D, all with adjuvants. Scales are the same for all antibodies except for anti-TAcChoR in rat D, on the right. Rats A, B, and D had EAMG evidenced by MEPP amplitudes of 0.31, 0.19, and 0.32 mV and tail AcChoR contents of 120, 220, and 190 fmol/g, all significantly below the ranges for adjuvant controls (0.41–0.79 mV and 320–550 fmol/g). In rat C, MEPP amplitude (0.45 mV) and AcChoR content (395 fmol/g) were normal.

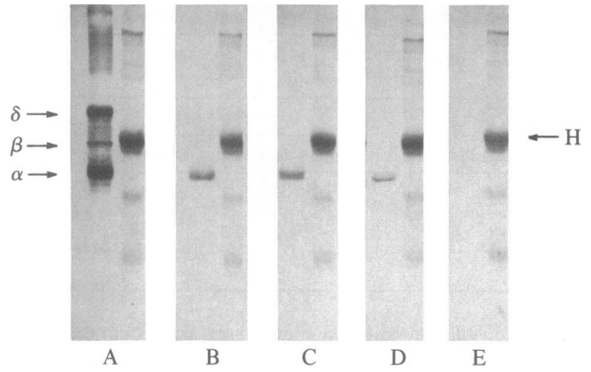

FIG. 3. Immunoblots of 7 μg of TAcChoR (left lanes) and immunoglobulin fraction of rat serum precipitated in 50% saturated ammonium sulfate (right lanes). The blots were incubated with the following: *A*, rat anti-N.TAcChoR antiserum (1:200 dilution); *B*, rat monoclonal anti-AcChoR antibody McAb4 (24) (300 nmol/liter); *C* and *D*, sera from rats C and A (Fig. 2), immunized with Tα125–147 (1:25 dilutions); *E*, adjuvant control rat serum (1:25 dilution). Peroxidase coupled to anti-rat IgG (Dako–Accurate Chemicals, Westbury, NY) with substrate revealed the following: right lanes, a broad band of immunoglobulin heavy chain (H), a molecular weight marker ($M_r \approx 53{,}000$); left lanes, *A*, TAcChoR subunits α ($M_r \approx 44{,}000$), β ($M_r \approx 52{,}000$), and δ ($M_r \approx 66{,}000$); *B*, *C*, and *D*, TAcChoR α subunits; *E*, no reaction.

globin peptide or buffer. PPD elicited DH responses in all adjuvant-inoculated rats.

Specificities of Antibodies Induced by Tα125–147. Antibodies to native AcChoR were detected in the primary response of 10 of 11 rats immunized with 50 μg of Tα125–147, and in all after a second immunization (Table 1). Means for the highest serum titers are summarized in Table 3. Unlike the response of rats immunized with D.TAcChoR, the duration of antibody peaks in peptide-immunized rats was transitory (Fig. 2). Reactivities with TAcChoR and rat muscle AcChoR were often dissociated. Reactivity with innervated muscle AcChoR (detected in only 6 rats) was usually lower than with denervated muscle AcChoR (detected in 11 rats).

Antibodies induced by Tα125–147 bound exclusively to the α subunit of TAcChoR in immunoblot analyses (Fig. 3). Surprisingly, antibodies were not detected by ELISA until after a third immunization, and those were more reactive with N.TAcChoR than with D.TAcChoR (Table 3). In contrast, antibodies induced by D.TAcChoR were detected earlier by ELISA than by immunoprecipitation assay, and reactivity was always less with N.TAcChoR than with D.TAcChoR.

Antibodies reactive with muscle AcChoR were detected in some sera only by their modulating activity (see Fig. 1 legend). Sera from 8 of 11 rats immunized with Tα125–147 caused loss of binding sites for ^{125}I-α-Bgt on living myotubes (Table 4), but they had no effect when metabolism was inhibited and did not block ^{125}I-α-Bgt binding to solubilized AcChoR. Control antisera to N.- and D.TAcChoR also caused modulation of myotube AcChoR and did not directly block ^{125}I-α-Bgt binding to muscle AcChoR, but antisera to N.TAcChoR blocked 96% of ^{125}I-α-Bgt binding to solubilized TAcChoR.

Table 5. Binding of anti-N.TAcChoR antibodies to immobilized peptide Tα125–147 and control proteins and peptides

Sepharose conjugate	^{125}I-labeled antibodies bound, cpm		
	Rabbit	Rat	Dog
N.TAcChoR	93,900	85,400	50,200
D.TAcChoR	32,500	26,000	28,500
Tα125–147	37,700	22,200	28,200
Bovine albumin	2,900	3,500	2,800
Hemoglobin A	2,000	4,700	NT
β1–15*	2,100	3,000	3,300
α121–135*	NT	4,500	3,700

IgG was purified from immune sera (27); the dog preparation contained IgG, IgM, and IgA. ^{125}I-labeled antibodies ($275–315 \times 10^3$ cpm) were added to serial volumes (25–200 μl) of packed Sepharose conjugates. The mean plateau values of binding in triplicate analyses varied ± 1.6% or less and were not corrected for nonspecific binding. NT, not tested.
*Synthetic hemoglobin peptides β1–15 and α121–135 (34, 35).

Binding of Anti-Native AcChoR Antibodies to Tα125–147. The binding of rabbit, rat, and dog anti-native TAcChoR antibodies (^{125}I-labeled) to Sepharose-linked Tα125–147 ranged from 26% to 56% of the net plateau binding to N.TAcChoR (Table 5). Binding to Tα125–147 and to D.TAcChoR (30–57%) was quantitatively similar; nonspecific binding to control proteins and peptides ranged from 2% to 7%. Binding of ^{125}I-labeled rabbit anti-hemoglobin IgG to Sepharose-linked AcChoR and peptide Tα125–147 was less than 4% of its binding to hemoglobin (data not shown).

DISCUSSION

Previous studies from this laboratory established that the AcChoR molecule has more than one antigenic site that can induce EAMG (24). This study has identified a myasthenogenic region of AcChoR at the biochemical level. The occurrence of EAMG in rats after immunization with a synthetic peptide comprising residues 125–147 of the TAcChoR α subunit demonstrated unequivocally that at least part of this region of the AcChoR is extracellular in the mature mammalian neuromuscular junction. Unlike native AcChoR

Table 4. Assays for antibodies reactive with the AcCho-binding region of solubilized and membrane-bound AcChoR in rats immunized with Tα125–147 or N.- or D.TAcChoR

Immunogen	No. of rats	% inhibition of ^{125}I-α-Bgt binding				
		Solubilized AcChoR			Intact membranes of cultured rat muscle	
			Rat muscle			
		Torpedo	Innervated	Denervated	37°C	22°C/DNP*
Adjuvants	3	0	2 ± 1.1	8 ± 3.3	8 ± 4.1	0
Tα125–147	11	0	3 ± 0.8	0	52 ± 7.3	0
D.TAcChoR	3	21 ± 1.2	10 ± 5.8	2 ± 4.0	39 ± 2.9	0
N.TAcChoR	3	96 ± 3.3	0	0	73 ± 11.3	11 ± 5.8

Results are mean ± SEM.
*Indirect immunofluorescence established that antibodies and AcChoR to which they bind disappear from myotube membranes in 6 hr at 37°C, presumably as a result of endocytosis (9, 11). This AcChoR loss is prevented by inhibiting metabolism with 0.5 mM 2,4-dinitrophenol and lowering the temperature (11).

(21, 36), the synthetic peptide was not glycosylated at Asn-141. Thus, the carbohydrate moiety is not essential for myasthenogenicity. The disulfide-looped peptide Tα125–147 appears to encompass a major antigenic region of the AcChoR's α subunit because it bound 26–56% of the polyclonal antibodies induced in three different species by immunization with N.TAcChoR. By comparison, we found that only 15–17% of these antibodies bound to peptide Tα159–169 (27), a region reported to be cytoplasmic in electric organ membranes (17).

Peptide Tα125–147 was a potent inducer of T-cell responses to native AcChoR. It induced DH and a thymus-dependent antibody response (23) without conjugation to a carrier. The oligoclonal antibodies bound to the α subunit of TAcChoR. Plümer *et al.* (37) reported that antibodies raised against peptide Tα127–132 bound to the α subunit of TAcChoR and to membrane fragments of the electric organ, but extracellular exposure was not proven. Although antibodies induced by Tα125–147 caused modulation of AcChoR on living myotubes, they did not inhibit binding of ^{125}I-α-Bgt to either solubilized or membrane-bound AcChoR (Table 4). If a binding site for AcCho resides in the native AcChoR molecule's α subunit region 125–147, failure of the anti-peptide antibodies to inhibit binding of α-Bgt may reflect a higher affinity of native AcChoR for α-Bgt than for the antibodies. Alternatively, antibodies that block α-Bgt's binding to AcChoR may be directed at the carbohydrate associated with Asn-141. This suggestion is consistent with a report that blocking antibodies from serum of a patient with MG did not inhibit binding of α-Bgt to AcChoR with added *N*-acetylglucosamine (38). A third possibility is that additional residues or α-subunit segments outside the region 125–147 may be involved in binding α-Bgt in the native AcChoR. This suggestion is supported by the affinity labeling data of Kao *et al.* (39), which implicated Cys-192 and possibly Cys-193 in binding AcCho.

The greater reactivity of anti-peptide antibodies with N.TAcChoR than with D.TAcChoR, and with membrane-associated than with solubilized muscle AcChoR, suggests that the antigenic site(s) in Tα125–147 is (are) to a considerable extent conformation dependent. The potential AcChoR-like conformational states of the loop peptide were presumably maximized by using unconjugated immunogen. This would account in part for the different antigen-binding profiles of antibodies at different times in an individual rat's sera (Fig. 2). Both affinity and specificity differences would contribute to these profiles. For example, adsorption *in vivo* to motor endplates would selectively remove high-affinity antibodies reactive with innervated muscle AcChoR. The transience of antibody peaks, presumably reflecting the influence of regulatory lymphocyte subsets, was quite striking in the response induced by a 23 amino acid segment of TAcChoR, and it contrasted with the persistence of antibodies in the polyclonal response induced by the multideterminant D.TAcChoR.

Our demonstration that a synthetic peptide can induce an autoimmune response to the AcChoR gives promise that modified synthetic autoantigens might find therapeutic application in antigen-specific immunotherapy for MG.

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